STUDIES ON THE MICROTUBULES IN HELIOZOA

III. A Pressure Analysis of the Role of These Structures in the Formation and Maintenance of the Axopodia of *Actinosphaerium nucleofilum* (Barrett)

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ABSTRACT

Electron microscope preparations were made of specimens of *Actinosphaerium nucleofilum* fixed in glutaraldehyde before, during, and after exposure to high pressures (4,000 to 8,000 psi). A study of this material showed that, although other organelles were relatively stable, the microtubular elements of the axopodia and cytosome became unstable under pressure. Their rapid disintegration under pressure was correlated with beading and retraction of the axopodia. Moreover, after the release of pressure, microtubules reappeared as soon as, or sooner than the reextension of the axopodia. The rate of disintegration increased as the pressure was raised. At 4,000 psi, few if any tubules remained after 10 min, whereas at 6,000 and 8,000 psi the disintegration was much more rapid. Some adaptational reorganization of the microtubules and axopodia occurred while relatively low pressures were maintained. This was accompanied by an actual elongation of the axopodia in specimens maintained for 20 min at 4,000 psi, but was confined to knoblike axopodial remnants in animals kept at 6,000 psi. No regeneration of tubules or axopodia occurred at 8,000 psi. The presence of fibers and a finely fibrillar material in pressurized animals suggests that these may be derivatives of microtubular disintegration. This evidence, though purely morphological, is consistent with the hypothesis that microtubules play an important role not only in maintaining the form-stability of the axopodia, but also in the active process by which the axopodia reextend themselves after retraction.

Kitching recently (1964) has shown that the birefringent core, or axoneme, which constitutes the axis of the heliozoan axopodium, displays a unique fine structure. This consists of numerous precisely arranged "hollow-appearing filaments," now generally designated as microtubules. Microtubules have also been demonstrated in a wide variety of other forms: in the mitotic spindle (Harris, 1962; Kane, 1962; deThé, 1964; Krishan and Buck, 1965); in cilia and flagella (Burgos and Fawcett, 1956; Fawcett and Porter, 1954; Gibbons, 1961); in many protozoan structures (Roth, 1964; Rudzinska, 1965; Sommer, 1965); in the cytoplasm of various animal and plant cells (Ledbetter and Porter, 1963; Byers and Porter, 1964; Porter et al., 1964); and in many other cases (See review of Slautterback, 1963).

As to the functional significance of microtubules,

Tilney and Porter (1965), in a detailed analysis of the form and general occurrence of these elements, have proposed that microtubules may be important not only in the development of movements by cells generally but also in the stabilization of cellular form. Such movements would include: movements of the cell as a whole; changes in cell form (Byers and Porter, 1964); various kinds of protoplasmic streaming, as in plant cells, various amoeboid forms, axopodia, suctorian tentacles, etc.; and finally the movements of chromosomes and other intracellular entities. Moreover, owing to the tensional forces that exist at cell surfaces generally, some sort of form-stabilizing agencies must be present in all cells, except those that naturally possess a spherical shape.

Hydrostatic pressure has provided an excellent analytical tool in the present experiments, since pressure is known to have marked effects upon motion and upon the form stability of cells. A wide variety of cells tend to become rounded and inert when exposed to moderate pressure intensities, *i.e.* 4,000 to 8,000 psi. These cells include: various free-living amoebae (Marsland and Brown, 1936; Landau, Zimmerman, and Marsland, 1954); human fibroblasts in tissue culture (Landau, 1961); various ciliates (Auclair and Marsland, 1958; Asterita and Marsland, 1961); *Euglena* and other flagellates (Byrne and Marsland, 1965); suctorians (Kitching and Pease, 1939); heliozoa (Kitching, 1957); and egg cells in process of division (Marsland, 1956). Moreover, microtubular elements have been demonstrated in many of these cells (see p. 77 for references).

Actinosphaerium provided ideal experimental material. The needlelike axopodia, which radiate out from the cell surface, display an intrinsically unstable form; and since there is a well defined system of microtubules in the axopodial axis, opportunity is afforded for investigating the hypothesis that these elements play an essential role in the formation and maintenance of the axopodia. The axopodia become unstable at moderate intensities of pressure (Kitching, 1957). Each undergoes a rapid process of beading, followed by a slower process of withdrawal. Moreover, after decompression, the axopodia soon begin to reform and gradually they attain full length and normal appearance.

A method of fixing cells during the period in which they are exposed to high pressure was devised by Landau and Thibodeau (1962). This provided a means for preparing the pressurized Actinosphaerium for electron microscopic study, with particular reference to changes in fine structure of the microtubular elements. If the proposed hypothesis is valid, the microtubules should undergo disorganization as fast as, or faster than, the whole axopodium; and during reorganization, a reappearance of the microtubules should not lag behind the total regeneration of the axopodium.

MATERIALS AND METHODS

THE ORGANISMS: Originally a culture of *Actinosphaerium nucleofilum* (Barrett, 1958) was obtained from the Carolina Biological Supply Co.[1] and serially subcultured in a wheat medium (Looper, 1928). Small amounts of a rich culture of mixed ciliates were added from time to time as food for the Actinosphaerium. Subcultures which were some 3 to 5 wk old provided a good supply of animals in excellent condition.

IN VIVO OBSERVATION OF THE PRESSURIZED CELLS: The pressure equipment, which permitted direct observation of the pressurized cells at magnification up to 300, has been described by Marsland (1950). During the pressure period the organisms were kept under continuous observation except for momentary intervals during which photomicrographs were taken. The pump permitted a pressure build-up at the rate of about 4,000 pounds/in.^{2} (psi) per sec. Decompressions, by means of a needle valve, were instantaneous.

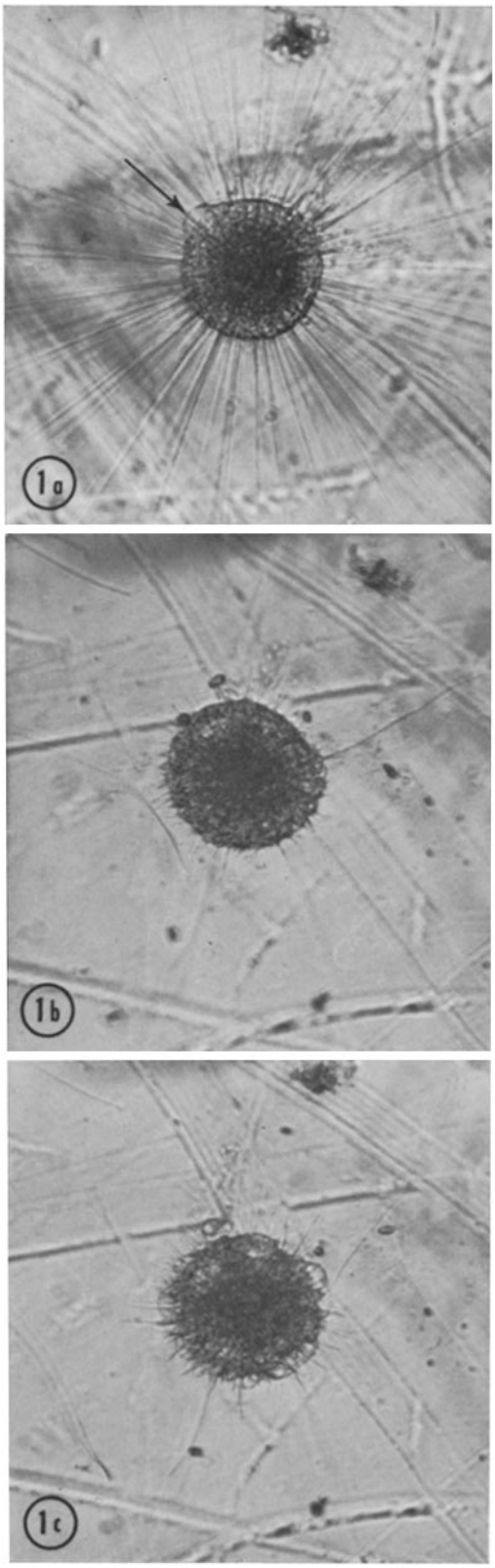
FIXATION DURING COMPRESSION: The apparatus used in this technique has been described by Landau and Thibodeau (1962).

DIFFERENTIAL FIXATION OF THE AXOPODIA AND CYTOSOME: Unfortunately, optimal fixation of both axopodia and cytosome could not be effected simultaneously, since best preservation of the fine structure of these parts of the cell was attained only when two different concentrations of glutaraldehyde were employed.

For the axopodia, 1% glutaraldehyde (Sabatini et al., 1962) with 0.05 M phosphate buffer and 0.0015 M $CaCl_2$, at pH 7.0 and 265 mOsmol (Powell et al., 1964), was employed for 30 min. Then the organisms were washed briefly in 0.1 M phosphate buffer containing 0.0015 M $CaCl_2$ and postfixed for 20 min in 1% osmium tetroxide in the same concentration of phosphate buffer and $CaCl_2$ at pH 7.0. Finally, the specimens were embedded in Epon 812 (Luft, 1961) after rapid dehydration in acetone.

For the cytosome, the procedures were the same, except that 3% glutaraldehyde with 0.05 M phosphate buffer containing 0.0015 M $CaCl_2$ at pH 7.0

[1] Elon College, North Carolina.



(520 mOsmol) was employed, and the fixation period was approximately 1 hr.

OTHER PROCEDURES: Thin sections were cut with a diamond knife on a Servall MT2, Porter-Blum ultramicrotome, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and examined with a Siemens-Elmiskop I. At least three specimens from each of the experiments were studied electron microscopically.

RESULTS

Light Microscope Observations

For orientational purposes, the familiar normal appearance of a nonpressurized control specimen is shown in Fig. 1 *a*.

CHANGES OBSERVED AT 4,000 PSI, PRESSURE SUSTAINED: Little or no change was discernible for the first 3 min. Then a slight beading occurred at the distal extremities of some of the axopodia. Gradually, all of the axopodia developed terminal beading and started to grow noticeably shorter and more slender. Shortening continued for 10 min, by which time the stiff, slender, generally straight axopodia had shrunk to about $\frac{1}{10}$ of their original length (Fig. 1 *b*). Further shortening was not observed. Instead, a slow extension was observed, so that by the end of 20 min most of the axopodia had elongated to almost twice the minimal length (Figs. 1 *c* and 2).

By 20 min, the only change in the cytosome was a slight enlargement of the cortical vacuoles which showed a tendency to protrude from the cell surface. A contractile vacuole was observed to function during the 20-min compression period.

Electron Microscope Observations

NORMAL DISTRIBUTION OF MICROTUBULES IN ACTINOSPHAERIUM: This very

FIGURE 1 Light micrographs taken of the same living specimen at a magnification of × 100.

a. Specimen in the pressure chamber prior to compression. Needlelike processes, or axopodia, extend from the cell body or cytosome into the culture medium. The cytosome is divided into an outer cortical region, in which a single contractile vacuole is present (see arrow), and an inner denser medullary region. The axopodial axis, or axoneme, continues into the latter.

b. Micrograph taken after 10 min of sustained pressure at 4,000 psi. Only short stubs of the axopodia remain.

c. Micrograph taken after 20 min of continuous pressure at 4,000 psi. The axopodia have become twice as long as those shown in the previous photograph.

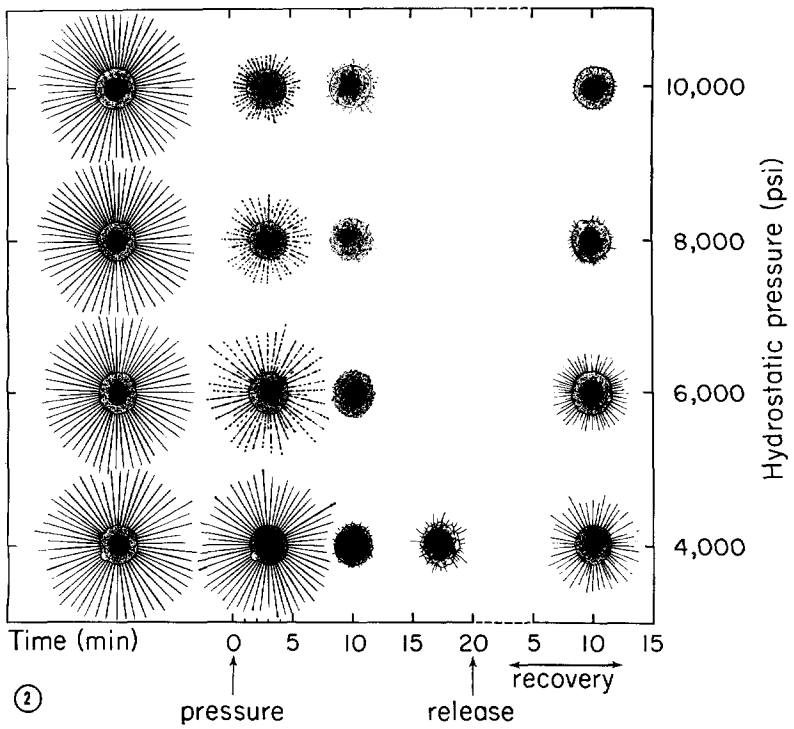


FIGURE 2 Drawing summarizing the changes observed in living Actinosphaerium before, during, and after application of hydrostatic pressure. The row on the far left illustrates the form of Actinosphaerium prior to compression. The row on the far right illustrates Actinosphaerium 10 min after the release of pressure.

brief account, derived from the detailed study of Tilney and Porter (1965), is designed to provide a basis of comparison between the normal and the experimentally altered microtubular systems.

The axoneme, or birefringent core of the axopodium, is constituted essentially of a large number of microtubules running parallel with one another and arranged, in cross-sectional view, in the form of an interlocking double coil (Fig. 3). The diameter of each microtubule is about 220 A, and the space between the coiling rows is about 350 A. An amorphous material is found between the rows of the double coil.

A maximum number of tubules, about 500, which give rise to 5 to 6 turns of the double coil, is found near the axopodial base. A minimum of 5 or less are present at the tip. Apparently, the microtubules centrally situated in the axoneme extend all the way from base to tip, whereas the peripheral units terminate at a relatively short distance out from the base, and the intermediate tubules run a series of intermediate distances out into the axopod. The tubules terminate distally in a dense material immediately subjacent to the plasma membrane of the axopodium. Generally, two small groups of tubules terminate in the same cross-sectional plane, on opposite sides of the axopodial circumference, and it may be assumed that each of these terminal clusters is derived from one or the other of the two rows of tubules present in the double coil.

The axonemes extend inward from the axopodia through the cortex and into the medulla of the cytosome where they frequently indent, but do not penetrate the nuclear envelope. The end of each microtubule is surrounded by small masses of a dense material.

Cytoplasmic streaming, which carries mitochondria and other cytoplasmic elements back and forth between the axopodia and the cytosome, is prominent in Actinosphaerium, as in other heliozoan forms.

FINE STRUCTURAL CHANGES AT 4,000 PSI: (1% glutaraldehyde fixation.) In the first group of experiments, fixation (under pressure) was executed after 10 min of compression, at

which time the axopodia had shrunk to minimum length (Figs. 1*b* and 2).

The few short residual axopodia were found, for the most part, to be devoid of recognizable microtubular elements. Instead, in the position normally occupied by the microtubules was a mass of finely fibrillar material (Fig. 4). Peripheral to this material, mitochondria and electron-opaque granules were often seen just inside the limiting plasma membrane.

Cytoplasmic preservation of the cytosome varied considerably. In some cases, it seemed excellent even though the fixative employed was 1% (rather than 3%) glutaraldehyde. No definitive microtubules were found in the cytosome (Fig. 5). Fibers (similiar to those shown in Fig. 10) ranging from 100 to 200 A in diameter and up to 1 μ in length were sparsely distributed in the cortical cytoplasm. Similar structures were almost never present in the control specimens. The finely fibrillar material was also present in the cortical cytoplasm, but unlike that in the axopodia, this material appeared in partially membrane-limited masses 0.5 μ in diameter (Fig. 5). The plasma membrane, on the other hand, was intact. It is probable that the fibers and the finely fibrillar material represent disintegration products of the microtubules. These matters will be considered more carefully in the Discussion.

In a second group of experiments, fixation was carried out at the end of a 20-min period of pressurization. By this time, considerable reorganization had occurred, since the axopodia had reextended to about twice their minimal length (Fig. 1 *c*). As demonstrated in Fig. 6, microtubules arranged in the double coiled configuration are present in the reextending axopodia; more than 1 to 1½ turns of each row were never seen (Fig. 6). Double coiled configurations of microtubules also were found in the cytosome, despite the fact that 3% glutaraldehyde was not employed as the fixative (Fig. 7). As summarized in Table I, the fibers were absent from the axopodia, although present in the cytosome. The finely fibrillar material was found in both the axopodia and the cytosome; in the former, it was seen only when the microtubules were absent or sparse and poorly oriented. The plasma membrane was always intact, although a few discontinuities were observed in some of the vacuolar membranes.

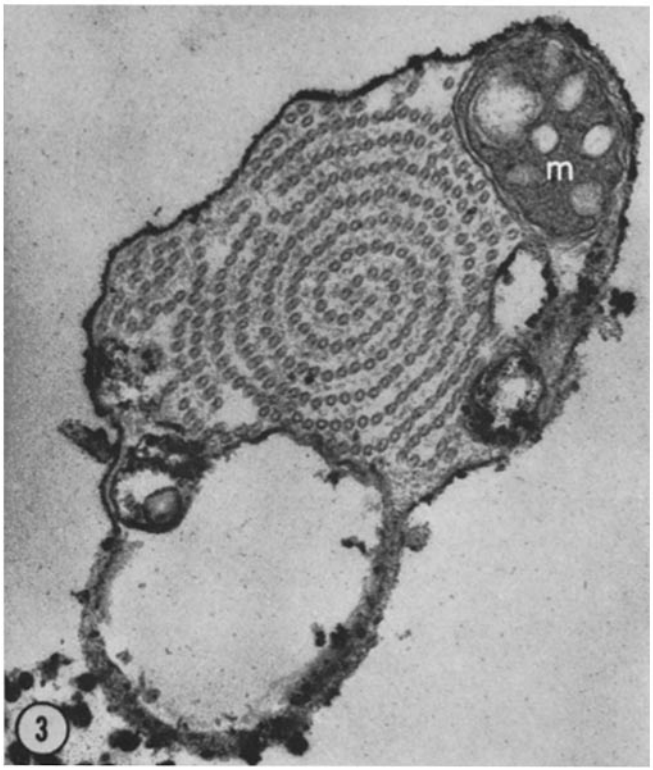


FIGURE 3 Cross-section through an axopodium of an unpressurized control specimen. The microtubules, which form the axoneme, run parallel to each other and are arranged in an interlocking double coil. An amorphous material is present between the rows of the double coil. A mitochondrion (*m*) is present peripheral to the axoneme but beneath the plasma membrane which is a unit membrane. Fixation, 1% glutaraldehyde. × 75,000.

Light Microscope Observations at 6,000 psi

Beading of the axopodia became apparent during the first minute of pressurization (Figs. 2 and 8 *a*). Moreover, such beading was not confined to the tips, but extended throughout the whole length of the axopodia, leaving only delicate strands of connection between the beads. Gradually thereafter, the axopodia shrank, carrying the beads towards the cell surface. In 5 min, the average length had decreased by 50% and in 10 min no true axopodia remained. There were, however, numerous regularly disposed knoblike protrusions which preempted the entire cell surface (Fig. 8 *b*).

Fine Structural Observations at 6000 psi

3 MIN AT 6,000 PSI (1% glutaraldehyde fixation): The beaded portions as well as the unbeaded portions of the axopodia are almost devoid of microtubules (Fig. 9 *a*). Instead, axopodia were filled with the finely fibrillar material already described. The microtubules, when present, were drastically reduced in number and considerably disoriented (Fig. 9 *b*) so that rarely could the coiled arrangement be recognized. There were never more than 1½ turns per coil. Fibers were not found in the axopodia.

The cytosome of these specimens contained no definitive microtubules but randomly arranged cortical fibers were common (Fig. 10 *a*). The fine structure of these fibers was variable; some appeared with less dense centers as if hollow, others solid throughout. The diameters did not seem constant, varying from 100 to 200 A (Fig. 10 *b*). Also, many of the fibers displayed a periodicity along their length. The finely fibrillar material was generally encountered in small masses incompletely limited by a unit membrane. Areas either devoid of cytoplasmic constituents or with markedly reduced numbers of organelles and inclusions were also present. With longer compressions at 6,000 psi (see below), these "empty areas" became more conspicuous, at least in the material fixed with 1% glutaraldehyde.

10 MIN AT 6,000 PSI: Fixation made at the end of a 10-min compression period yielded a quite different fine structural picture. These micrographs indicated that a considerable degree of adaptational reorganization had occurred while the pressure was sustained. This was particularly apparent in the group of specimens fixed in 3% glutaraldehyde.

Virtually all the short knoblike axopodial remnants observed by light microscopy contained microtubules arranged in the double-coiled pattern; up to 3 full turns were present in some of the coils (Fig. 11). But what is most unusual is that many of these knoblike processes displayed not one but several (up to 8) of these coils (Fig. 11). There were some interruptions in the pattern of the coils and thus some scattering of the individual microtubules. A few of these processes, especially those in which the axonemes were not well developed, contained some of the finely fibrillar material previously described. The axonemes in most instances penetrate well into the medullary region as in the nonpressurized controls. Correspondingly, fibers were very rarely present.

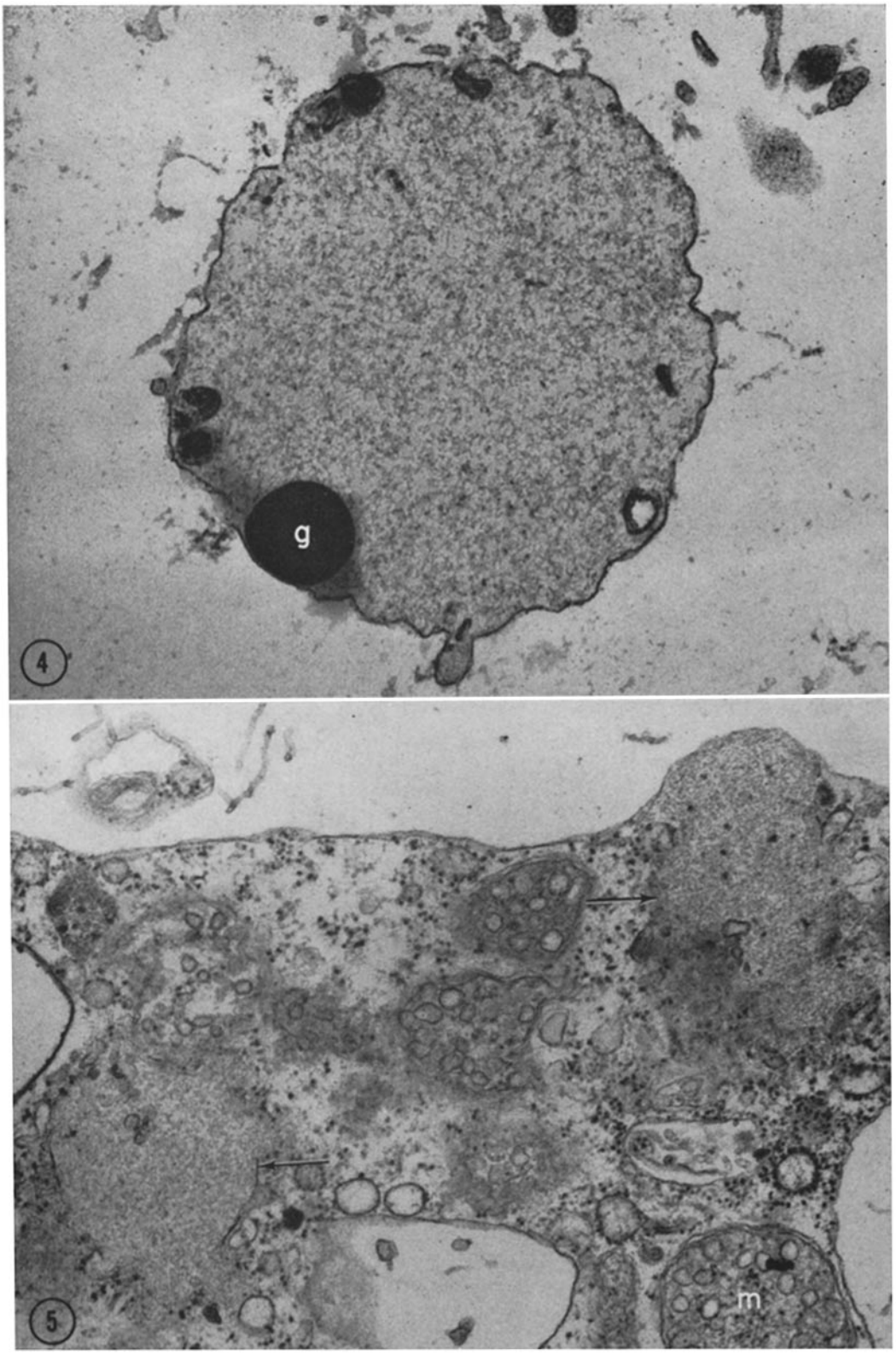
In material fixed with 1% glutaraldehyde, the cytosome was not well preserved. In these cases, axonemes were not seen in the cytosome, but fibers and large areas devoid of cytoplasmic constituents were common. Most of the knoblike axopodial protrusions did show microtubules arranged in the usual double-coiled pattern. Often there were several such coils in each protrusion.

Light and Electron Microscope Observations at 8000 psi

Within 3 min, the entire length of each axopodium became beaded. By 6 min, all of the axopodia in most of the specimens had been fully retracted.

FIGURE 4 Cross-section through one of the short axopodia present after 10 min at 4,000 psi. Note the finely fibrillar material in the central region normally occupied by the axoneme. Peripheral to this material, beneath the unbroken plasma membrane, are several electron-opaque granules (*g*). Fixation, 1% glutaraldehyde. × 35,000.

FIGURE 5 Portion of the cortical region of same specimen (Fig. 4). Note that there are two rounded masses of finely fibrillar material (arrows) and that one of these is partially delimited by a membrane. The mitochondria (*m*) are slightly swollen in this preparation. × 35,000.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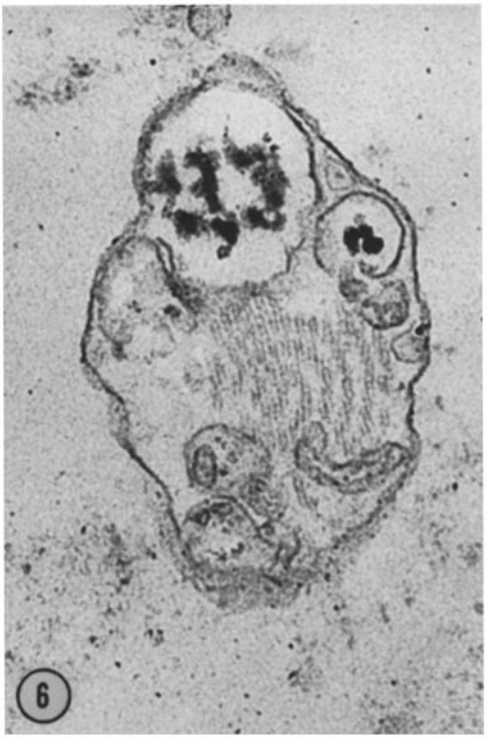
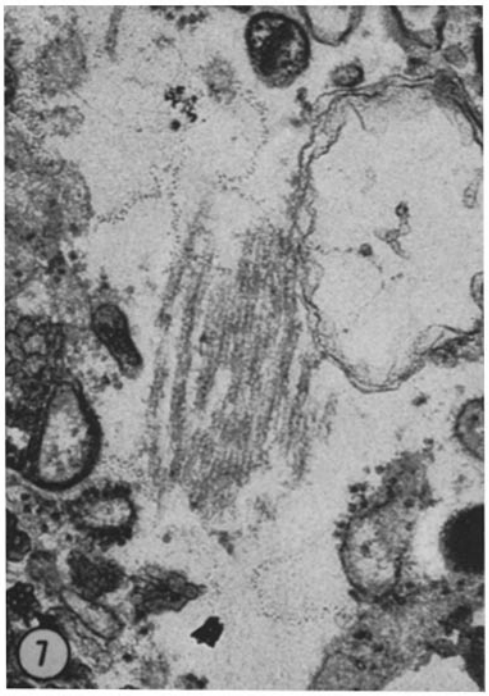




Nevertheless, knoblike protrusions still persisted over the cell surface, as was the case in the 6,000 psi experiments. By 10 min, however, the knoblike protrusions had also disappeared in most, if not all, of the specimens, permitting the smooth surface contours of the cells to be clearly seen (Fig. 2).

As demonstrated in Table I, fixation with 3% glutaraldehyde after 10 min showed a complete absence of microtubules, few fibers, and no finely fibrillar material. The cytoplasm, instead of containing numerous, possibly interconnected vacuoles, appeared to take the form of a reticulum, with many areas which seemed to be in direct contact with the outside medium. The density of organelles and inclusions in this cytoplasmic reticulum differed markedly from region to region. The mitochondria were uniformly swollen to about twice their normal diameter (Fig. 14). The pattern of their vesicular cristae were unaltered, however.

Light Microscope Observation at 10,000 psi

Beading and retraction of the axopodia occurred even more rapidly than at 8,000 psi (Fig. 2). A fine structural analysis was not carried out.

Axopodial Recovery Following Pressure Release

Recovery of normal form and activity was observed following release of each magnitude of compression (Fig. 2). Axopodia first reappeared within 2 min following release of 4,000 and 6,000 psi pressure. It took 4 min for reappearance following release of 8,000 psi pressure, and 6 min following release of 10,000 psi pressure. Attainment of full axopodial length required 15, 20, 25, and 50 min after exposures to pressures of 4,000, 6,000, 8,000 and 10,000 psi, respectively. Recovery was complete and organisms observed 24 hr later were completely normal.

One group of organisms was fixed in 1% glutar-

FIGURE 6 Slightly oblique section through an axopodium of specimen fixed in 1% glutaraldehyde after 20 min at 4,000 psi. The axoneme consists of microtubules arranged in the form of a small, imperfect double coil. Peripheral to this are several poorly preserved electron-opaque granules. × 49,000.

FIGURE 7 Oblique section through a portion of the cortex of the cytosome of specimen from the same experiment as above. Note axoneme.

TABLE I

Relationship between Hydrostatic Pressures and the Presence of Microtubules, Fibers, and Fibrils in Both the Axopodia and the Cytosome

Description	Time under pressure	Glutaraldehyde	Axopodia			Cytosome		
			Microtubule	Fibers	Fibrils	Microtubule	Fibers	Fibrils
	min	%						
Control		1	+++++	0	0			
		3				+++++	0	0
4,000 psi	10	1	+	0	++	0	++	+++
	20	1	++	0	++	+	++	+++
6,000 psi	3	1	+	0	++	0	++++	+++
	10	1	+	0	+	+	+++	+++
	10	3	+++	0	+	+++	+	+
8,000 psi	10	3	No axopodia			0	+	0
Recovery following 6,000 psi)		1	+++	0	0	++	+	+

aldehyde during recovery following 6,000 psi compression for 10 min; at the time of fixation, their axopodia had reextended to half their normal length. Microtubules within each axopodium were found to be arranged in the double-coiled configuration (Figs. 13 *a* and *b*), but sometimes this pattern was distorted or partially broken. The amorphous material found between the rows of microtubules in nonpressurized control organisms (Fig. 3) was not well defined in the regenerating axonemes (Fig. 13 *a* and *b*). Furthermore, the number of turns of the double coil appeared proportional to the axopod length which, in this case, was half that present in the controls. Mitochondria, electron-opaque granules (Fig. 12), and "excretion granules" (Fig. 13 *a* and *b*) were encountered in the regenerating axopodia just peripheral to the axoneme, beneath the unbroken limiting plasma membrane. A single axopodium contained occasionally more than one (up to 3) axoneme.

The cytosome, although fixed in only 1% glutaraldehyde, appeared well preserved, and axonemes could be followed into the medullary region. As in the axopodia, the turns of the double coil in the cytosomal axonemes were reduced to about half their normal number. In fact, not more than 3 turns were ever present at the widest point. Randomly arranged fibers, identical to those depicted previously, were common, but the finely fibrillar material was rare.

DISCUSSION

Microtubules in Relation to the Formation and Maintenance of the Axopodia

The results reported here are altogether consistent with the hypothesis that microtubules play an important role not only in maintaining the form-stability of axopodia, but also in the process of axopodial reextension. Whenever the axopodia became unstable and underwent beading and retraction, whether slowly as at 4,000 psi or more rapidly as at 6,000 psi, the microtubules were shown to have undergone more or less complete disintegration. Also, whenever the axopodia began to reextend, it was shown that there had been a regeneration of a well organized system of microtubules concomitant with axopodial extension. And finally, with higher pressures, namely 8,000 psi, there were no signs of axopodial regeneration while the pressure was maintained, and likewise no persisting microtubular elements were found. In short, the evidence tends to support the hypothesis that microtubules are important in the formation and maintenance of the axopodia.

Reorganization under Pressure

Adaptation to sustained pressures has not been reported previously except, perhaps, in the case of *Euglena* (Byrne and Marsland, 1965). With Ac-

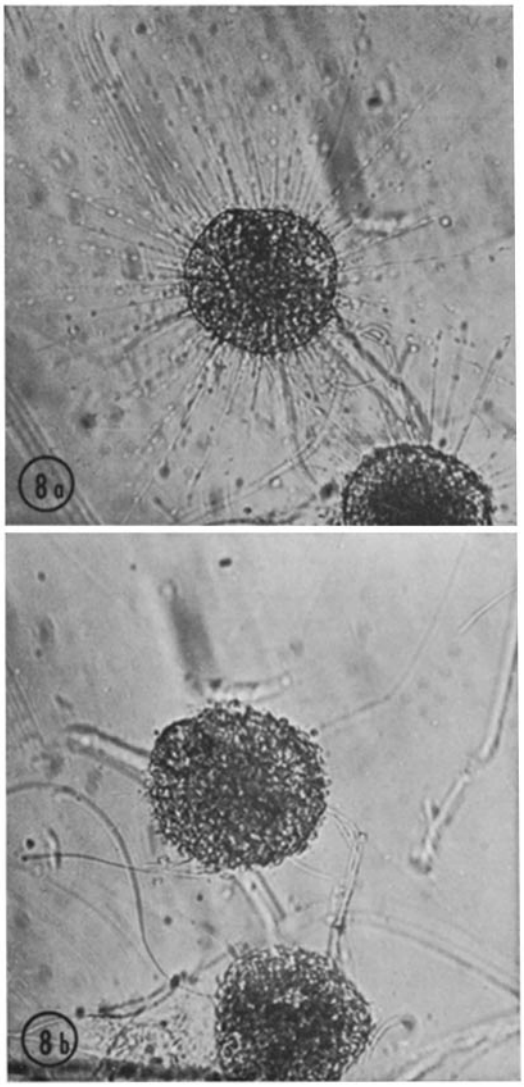


FIGURE 8 Light micrographs of the same living specimen during compression at 6,000 psi. × 100.

a. After 3 min all the axopodia are beaded.

b. After 10 min the axopodia have retracted, leaving only short knoblike protrusions at the cell surface.

tinosphaerium, at 4,000 psi, reextension of the axopodia began to be visible after 10 min, and this was correlated with the reappearance of an organized system of microtubules in the axopodia. At 6,000 psi, disorganization of the microtubules was virtually complete within 3 min, but by the end of 10 min a considerable reorganization was found in the persistent knoblike axopodial protrusions. In many of the knobs, several axonemal complexes were present, indicating that more than one axopodium could be formed from a single knob. Furthermore, the number of knobs was far less than the number of axopodia formed subsequent to decompression, when only rarely, were axopodia found containing more than one axoneme.

Apparently, disorganization of the microtubules and retraction of the axopodia at 4,000 psi is relatively slow. Thus, a maximum disorganization and retraction was observed at 10 min, after which reorganization and reextension began to occur. At 6,000 psi, in contrast, retraction reached a maximum in about 5 min, and by 10 min considerable reorganization was found, especially at the prospective sites of axopodial regeneration. At 8,000 psi, however, no signs of adaptational reorganization could be recognized.

Stability of the Cytosomal vs the Axopodial Tubules

Generally speaking, the cytosomal and axopodial microtubules appear to display approximately the same degree of susceptibility to pressure-induced disintegration. However, optimal fixation of both these elements was not obtained using the same concentration of glutaraldehyde. Until a method of fixation is found that can be used in common for both these elements, the evidence will remain somewhat equivocal. Meanwhile, further experiments should be undertaken in which both 1% and 3% glutaraldehyde fixations are systematically employed under all the different sets of experimental conditions.

Transitional Stages in Microtubular Disintegration

Concurrent with the pressure-induced disappearance of microtubules is the appearance of two components not present in well fixed control organisms. These are fibers, 100 to 200 A in diameter, and the finely fibrillar material. Whereas the fibers have been seen only in the cytosome and never in the resorbing axopodia, the finely fibrillar material appears not only in the axopodia in the position of the former axoneme but also in the cytosome in partially membrane-limited rounded masses.

There are four pieces of evidence which suggest a relationship between the fibers and the microtubules: (1) Microtubules disappear at approxi-

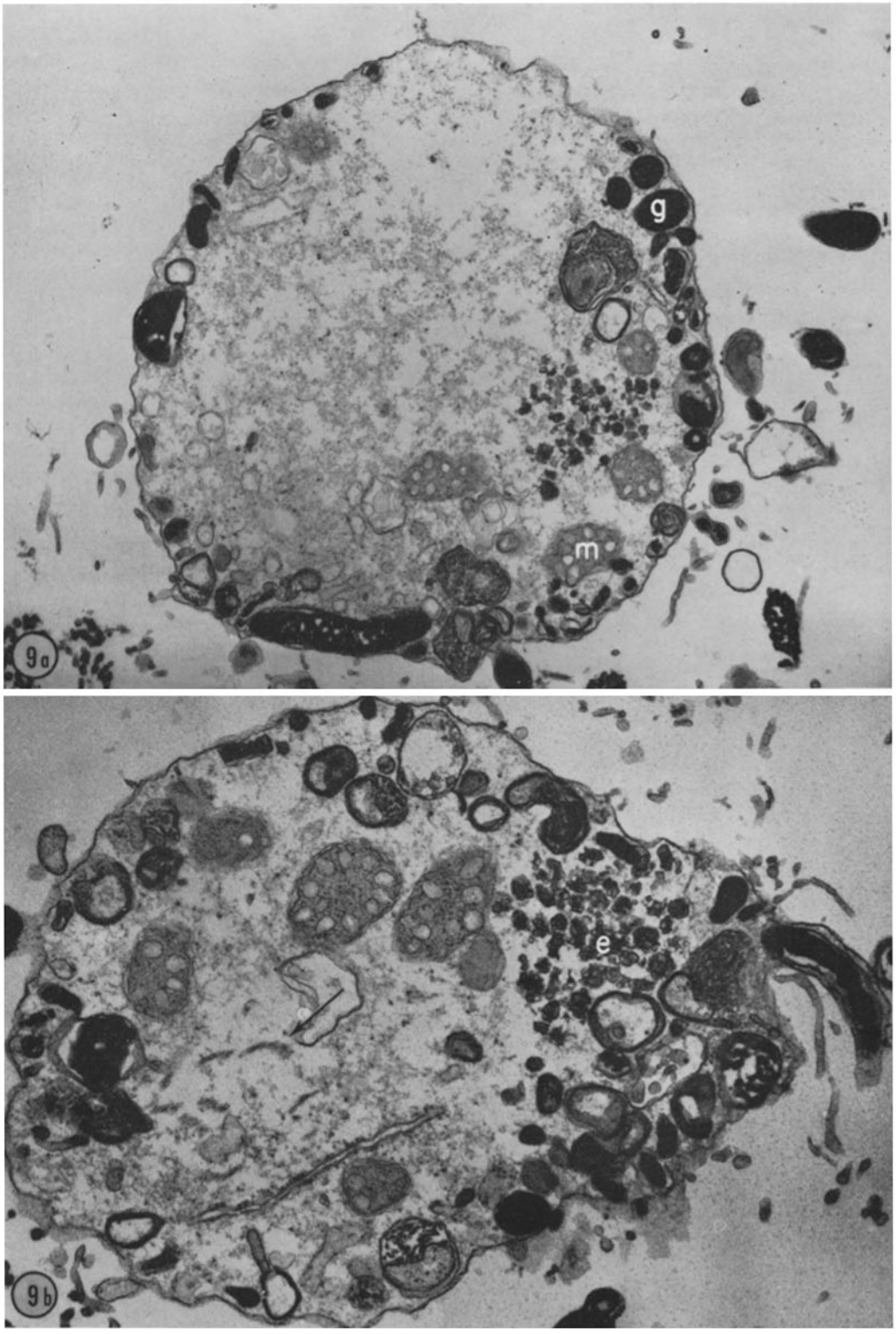


FIGURE 9 Nearly transverse sections through two axopodial beads similar to those depicted in Fig. 8 *b*. Fixation with 1% glutaraldehyde was performed after 3 min at 6,000 psi. Note that no definitive microtubules are present in *a*; whereas several may be seen in *b* (arrow). Also note, in both micrographs, the centrally situated finely fibrillar material and the more peripheral mitochondria (*m*), electron-opaque granules (*g*), and "excretion bodies" (*e*). *a*, × 50,000. *b*, × 75,000.

mately the same time that fibers appear. For example, in the 6,000 psi/10-min compression experiments, few microtubules but many fibers are found after fixation with 1% glutaraldehyde; whereas with 3% glutaraldehyde fixation, many microtubules but only a few fibers were found. (2) When specimens are placed at 4°C, the axopodia rapidly withdraw and the microtubules break down (Tilney, 1965 *a*). In these cells, fibers are present as well, although not in such large numbers as seen at 6,000 psi. (3) Some of the fibers, although usually of smaller diameter and length, as compared with microtubules, have a less dense center, as if hollow. (4) Under conditions of poor fixation, *i.e.*, with 0.5% glutaraldehyde, microtubules are not well preserved but fibers are present in the cytosome, even in nonpressurized specimens. (5) Similar-appearing fibers have been found in amoebae (Wolpert et al., 1964; Marshall and Nachmias, 1965). That amoebae may contain poorly preserved microtubules was suggested by the work of Marshall and Nachmias (1965), when they demonstrated microtubule-like elements in negatively stained preparations of the amoeba cytoplasm.

The foregoing evidence suggests, therefore, that the fibers may represent a stage in disintegration of microtubules or a stage of partial reaggregation of smaller microtubular subunits. It is curious that they are never seen in the retracting axopodia. This may merely indicate, however, a very rapid disassembly of the axopodial microtubules into units that are much smaller than the fibers.

The finely fibrillar material may also be related to the microtubules. It may be a disintegration product or possibly a remnant of the amorphous material normally present between the coils of microtubules. Or the finely fibrillar material may represent a secondary product derived from an aggregation of the disassociated subunits of the microtubular structures. It cannot, however, be a product of pressure-induced protein coagulation since such coagulation requires the application of much greater (about 75,000 psi) pressures (Bridgeman, 1914).

Fate of the Membranous Materials of a Retracting Axopodium

It is interesting to note that myelin figures or areas containing other identifiable forms of lipoidal material were not found in the peripheral cytoplasm during and subsequent to a generalized retraction of the axopodia. In view of the elongate needlelike form of the axopodia, the quantity of membrane material drawn back into the cell must be *very* great as compared to the material in the membrane of the cytosome proper. (We have calculated that the axopodial surface is at least 10 times the surface area of the cytosome, assuming a completely smooth spherical shape.) Usually no part of an axopodium is pinched off from the cell when retraction occurs, and thus it may be concluded either that the membrane material assumes a finely particulate, readily dispersible form as it is incorporated into the peripheral cytoplasm or that there is a dramatic increase in cytosomal vacuoles. The latter possibility, although difficult to document, does not seem to take place.

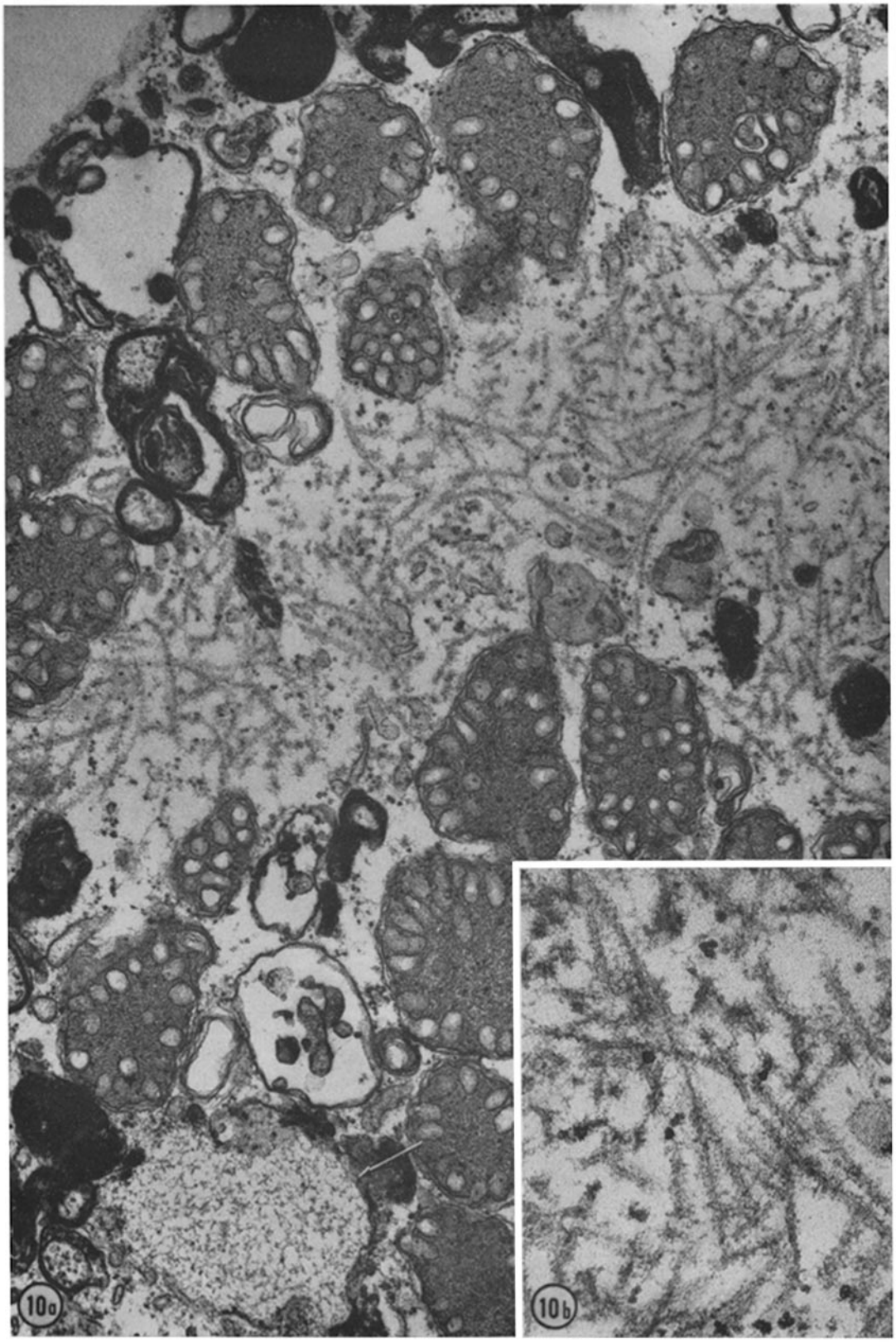
Microtubules in Relation to Gel Structures Generally

The extensive work of Marsland and coworkers (*see* Marsland, 1956) has shown that protoplasmic gel structures generally are susceptible to solational weakening upon exposure to high pressures (and low temperatures) and that motile activities associated with such structures are inhibited quantitatively in proportion to the pressure effects on the physical properties of the gel systems. The formation of protoplasmic gel structures represents an endergonic process which involves a definitive increase in the volume of the gelling system, and these volume and energy factors account for

FIGURE 10 Portions of the cortex of the cytosome fixed in 1% glutaraldehyde after 3 min at 6,000 psi.

a. Note the usual complement of organelles and inclusions, as well as the numerous fibers. Some of these fibers have a less dense central cavity as if hollow; others are solid. Some display a periodicity. The arrow points to a mass of finely fibrillar material, partially membrane delimited. × 30,000.

b. Micrograph depicting the fibers at a higher magnification. × 47,000.


10a
10b

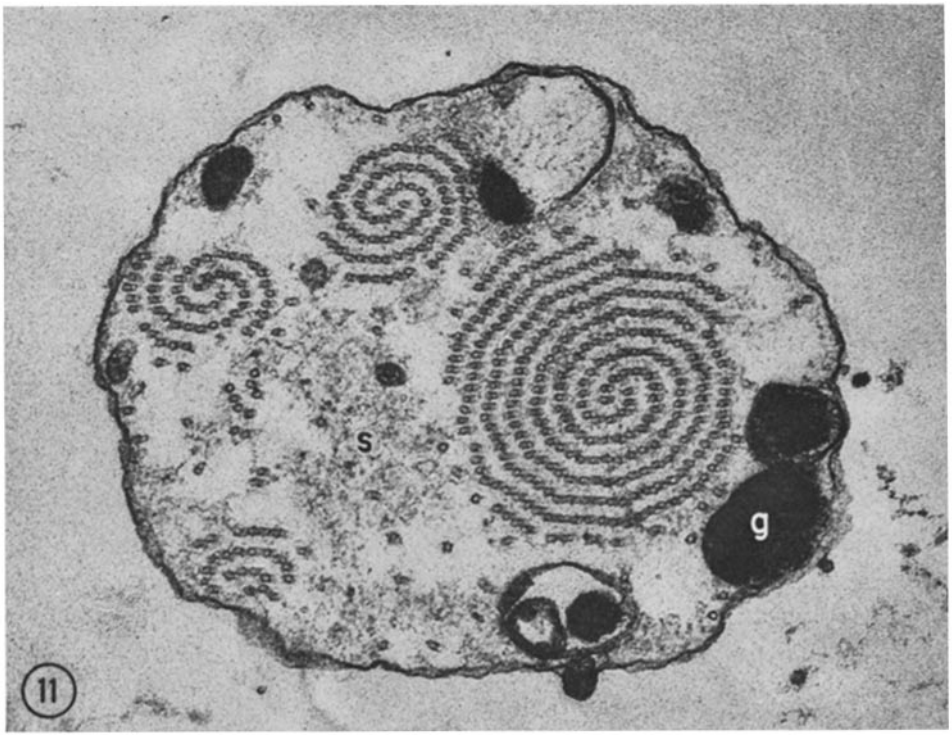


FIGURE 11 Specimen at 6,000 psi for 10 min and then fixed in 3% glutaraldehyde. This cross-section of a knoblike axopodial remnant shows three (possibly four) axonemes composed of interlocking double coils of microtubules. The direction of coiling of the rows is the same in all these cases. Several electron-opaque granules (*g*) are present peripheral to the axonemes, beneath the unbroken plasma membrane. The amorphous material is not obvious between the rows of the coils. Near the center of the axopodium a mass of the finely fibrillar material (*s*) can be identified. × 52,000.

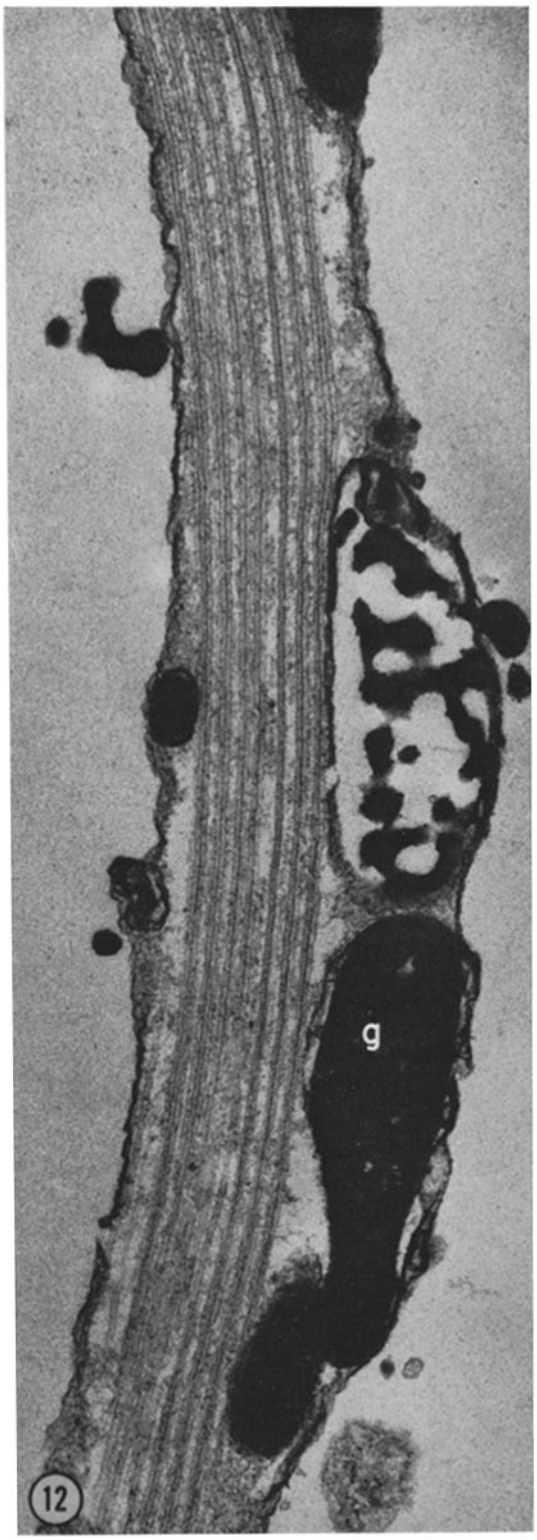
the pressure-temperature sensitivities of such systems (Marsland, 1956).

More recently, it has been recognized that microtubular elements constitute an intrinsic part of many protoplasmic structures, e.g., mitotic spindles (Harris, 1962; Kane, 1962), suctorian tentacles (Rudzinska, 1965), the cortical cytoplasm of many plant cells (Ledbetter and Porter, 1963), the processes which extend out from unicellular melanophores (Bikle et al., 1966; Green, 1965) and, of course, heliozoan axopodia (Kitching, 1964). In the formation of such microtubular structures, the processes of polymerization which underlie gelational reactions generally (Marsland and Zimmerman, 1965; Lauffer, 1962) must be guided into a very precisely determined spatial pattern. However, the nature of such guidance has remained obscure.

It seems probable that microtubules may represent an important factor in the development of gel strength, at least in the protoplasmic structures which have just been mentioned. Furthermore, in other structures in which microtubules have not been found, owing perhaps to their extreme lability and difficulty in preservation, gel strength may depend upon microtubules. As already mentioned, for example, the fibers in the plasmagel of the amoeba may represent improperly preserved microtubules (Marshall and Nachmias, 1965). Also, the sensitivity of protoplasmic gel structures generally to low temperatures and the corresponding sensitivity of microtubules to temperature, as in the mitotic spindle (Inoué, 1964) (birefringence evidence) and axopodia (Tilney, 1965 *b*) (electron microscopic evidence), further suggest a close relationship between gel strength and microtubular structure.

Pressure Effects upon Other Cytoplasmic Structures

Aside from the rather drastic disintegration of the microtubule system, the general effects of



pressure upon cytoplasmic organelles and inclusions appear quite mild. Mitochondrial swelling was not observed at levels below 8,000 psi, and below this level in 3% glutaraldehyde fixation, at least, cytoplasmic areas devoid of the usual assortment of structural elements were seldom encountered. Such stability of the cytoplasmic fine structure constitutes an important observation in itself, especially in view of the fact that only one pressure–electron microscope study has been carried out previously (Landau and Thibodeau, 1962). Moreover, it seems pertinent to suggest that there is a relationship between the pressure-stability of the cytoplasmic fine structure and the continuance of at least certain aspects of metabolism in pressurized cells. For example, Landau and Peabody (1963) have reported that ATP production does not cease, and Zimmerman (1963) has shown that DNA synthesis continues while cells are exposed to pressures in the range employed in our experiments.

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FIGURE 12 Longitudinal section through an axopodium of a specimen fixed in 1% glutaraldehyde after a 10-min recovery period, following 10 min at 6,000 psi. The microtubules appear well preserved and relatively straight; in fact, one of these can be followed throughout most of the length of the micrograph. Electron-opaque granules (*g*) are situated peripheral to the axoneme. × 50,000.

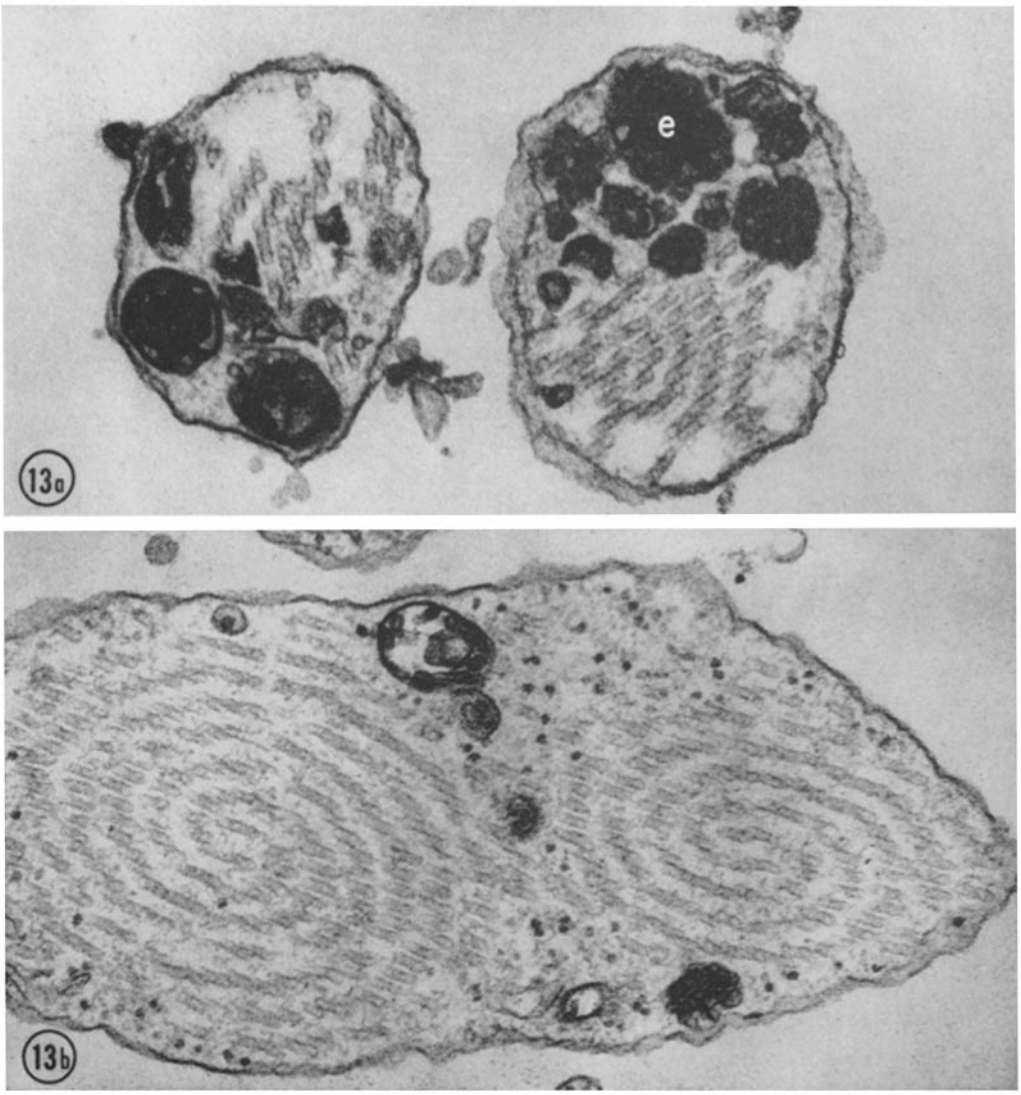

FIGURE 13 Recovery following a 10-min compression at 6,000 psi. Fixation, 1% glutaraldehyde.

a. Transverse section through the tips of two axopodia. Microtubules are found in both; in one instance, at least, they are arranged as a double-coiled configuration. "Excretion bodies" (*e*) are present peripherally. × 75,000.

b. Section through the cortex showing two axonemes, probably those depicted in *a*. Note that the direction of coiling of these axonemes is the same. × 65,000.

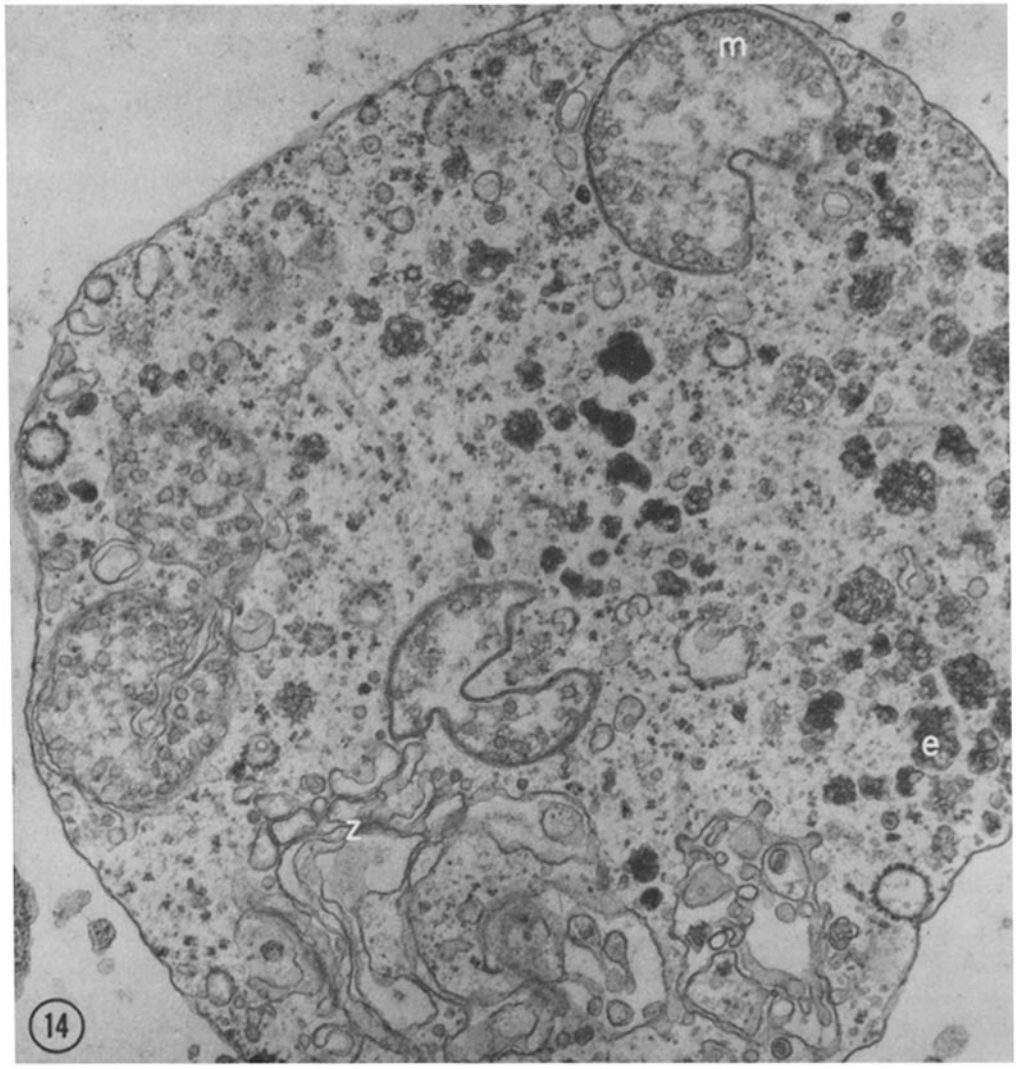

FIGURE 14 Fixation of this organism was carried out in 3% glutaraldehyde after 10 min at 8,000 psi. A portion of the Golgi zone (*Z*) is depicted here. The mitochondria (*m*) are twice the normal size and the electron opacity of the mitochondrial matrix appears to be abnormally low. "Excretion bodies" (*e*), ribosomes, and a few fibers may be identified. × 40,000.

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