

# THE EFFECTS OF HIGH HYDROSTATIC PRESSURE ON THE MICROTUBULES OF *TETRAHYMENA PYRIFORMIS*

JOHN R. KENNEDY and ARTHUR M. ZIMMERMAN

From the Department of Zoology, University of Tennessee, Knoxville, Tennessee 37916, and the Department of Zoology, University of Toronto, Toronto, Canada

## ABSTRACT

Exposure of *Tetrahymena pyriformis* to 7,500 or 10,000 psi of hydrostatic pressure for 2, 5, or 10 min intervals results in a change in cell shape and ciliary activity. Shape changes occur concurrently with a degradation of longitudinal microtubules in a posterior to anterior direction. High pressure also causes a disruption of ciliary activity. Fine structural analysis reveals a breakdown (presumably microtubule depolymerization) of the central ciliary microtubules. The depolymerization begins at the junction of the central ciliary microtubules with the axosome and progresses distally along the ciliary shaft for a distance of about 0.5  $\mu$ .

## INTRODUCTION

The cortex of *Tetrahymena pyriformis* contains a system of highly organized microtubules. This series of tubules has recently been described in detail by Allen (1967). Associated with each ciliary row is a group of longitudinal tubules which extends in an anterior to posterior direction in the cell. Arising from the anterior portion of each basal body, transverse tubules extend to the adjacent ciliary row. A posterior group of microtubules extends upward to the immediate longitudinal microtubules. It has been proposed that this system of microtubules controls coordination of ciliary movement or is responsible for form maintenance in ciliates (Jahn and Bovee, 1967).

Recent studies have demonstrated that the axopodial microtubules are responsible for form maintenance in *Actinosphaerium*. Exposure of the cell to high hydrostatic pressure (Tilney et al., 1966) or to other agents capable of microtubule degradation (Tilney and Porter, 1967; Tilney, 1968; Tilney and Byers, 1969) causes breakdown of microtubules and retraction of the axopodia. These investigators ob-

served that the pressure effects are reversible, and that upon release of pressure the microtubules reformed (repolymerization) and axopodia reappeared.

Shape changes have also been observed in ciliated protozoa upon exposure to high pressure (Auclair and Marsland, 1958; Kitching, 1957). Generally the cells have been described as attaining a roundish shape. Asterita and Marsland (1961) attributed this shape change in part to damage to the pellicle of the cell, and in part to alterations of the cortical gel. In view of this and the extensive studies on the effects of high pressure on macromolecular synthesis (see Zimmerman, 1969, 1970), we decided to investigate the effects of high pressure on cell fine structure, particularly on cortical and ciliary microtubules.

## MATERIALS AND METHODS

The organism used in this study was *Tetrahymena pyriformis* strain GL. Cells were grown in 2% proteose peptone supplemented with 0.1% liver ex-

tract. The temperature control housing, pressure pump, and microscope-pressure chamber were similar to that described by Marsland (1950). A pressure-fixation chamber identical with that designed by Landau and Thibodeau (1962) was employed for fixation of cells under hydrostatic pressure. Cells in logarithmic growth (at 28°C) were subjected to pressures of 7,500 or 10,000 psi for intervals of 2, 5, and 10 min.

At the above intervals, cells were fixed with 3% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2). Fixation was carried out under pressure for 5 mins. Upon decompression, cells and fixative were transferred to test tubes for an additional 25 min fixation. Cells were washed 3 times over a period of 30 min and stored for 1–3 days in cacodylate-buffered sucrose (10%) prior to postfixation. Samples were postfixated for 45 min in 1% OsO<sub>4</sub> in cacodylate buffer (pH 7.2), dehydrated, and embedded in Epon 812 (Shell Chemical Co., New York) (Luft, 1961). Sections were cut on a Porter-Blum MT-1 microtome with diamond knives and stained with uranyl acetate and lead citrate (Reynolds, 1963). Material was examined in an RCA-EMU-3 F microscope.

For light microscopy, samples were taken from Epon-infiltrated (Shell Chemical Co., New York) material prior to polymerization, mounted on slides, and examined with a Zernike phase-contrast system.

## RESULTS

Certain changes in cell shape and behavior are observed as the duration of exposure and levels of pressure are increased. After only 2 min exposure to a pressure of 7,500 psi, the posterior portion of the cell begins to round (Fig. 2). Control *Tetrahymena* at atmospheric pressure are shown in Fig. 1. Following 10 min at 7,500 psi, all of the cells continue to exhibit forward translational movement, although cellular velocity is approximately one-half that of the controls. Immediately upon decompression all cells exhibit a rapid increase in movement, and within 1 min the speed is almost comparable to that of nonpressurized controls. After exposure to 10,000 psi for 2 min, cell movement sharply decreases. As the duration of pressure is increased (up to 10 min), all effective forward movement stops and the cells show considerable rounding (Figs. 3 and 4). Although swimming movement has essentially stopped, ciliary activity continues for the full 10 min of pressure treatment,

and the cells can be seen to vibrate. For technical reasons it was not possible to quantitate the reduction in either rate of beat or ciliary coordination. Within 1 min after decompression, 95% of the cell population shows signs of recovering (increased activity). At 5 min after decompression most cells are seen to move slowly; they retain their roundish shape and display prominent cytoplasmic vacuoles. The recovery of cell movement is slow, and during the 20 min following decompression the cells never attain the control value, although progressively more cells become elongate. 24 hr later the cells appear normal as observed under the light microscope.

## Cortical Microtubules

Major structural changes evident in cells exposed to high hydrostatic pressure are associated with cortical microtubules. Most sensitive among these are the longitudinal microtubules. These tubules are aligned in rows adjacent to each ciliary row in the cell cortex. The number of microtubules at a specific level varies (compare Figs. 5 and 6), but the number seems constant from row to row at a given level in the cell. In the more posterior and anterior portions of the cell the number of microtubules in each row is fewer than at the midregion, where as many as 20 microtubules per row have been observed (Elliott and Kennedy, in press).

While longitudinal microtubules have been suggested to terminate randomly along the length of the cell (Pitelka, 1961), our data indicate that they reach their greatest number in the midregion and decrease to as few as four per row as they reach the poles of the cell. Underlying each bundle of longitudinal tubules is a thin granular layer of cytoplasm (Fig. 5). At 7,500 psi for 2 min, breakdown of longitudinal tubules begins. This breakdown is seen as an increase in the thickness of the underlying granular layer extending into the region of microtubules above (Fig. 6). The increase of the granular layer is not observed in tangential sections of longitudinal tubules of control animals, and thus is clearly not due to the plane of the sections. Examination of a similar area (Fig. 7) shows that some microtubules at this time retain their integrity, while others are already losing structure. After 10 min of exposure to either 7,500 or 10,000 psi, the structure and organization of the longitudinal microtubules is lost in most of the cell, and generally only areas of granular material persist. This can be observed in some regions of the cell as

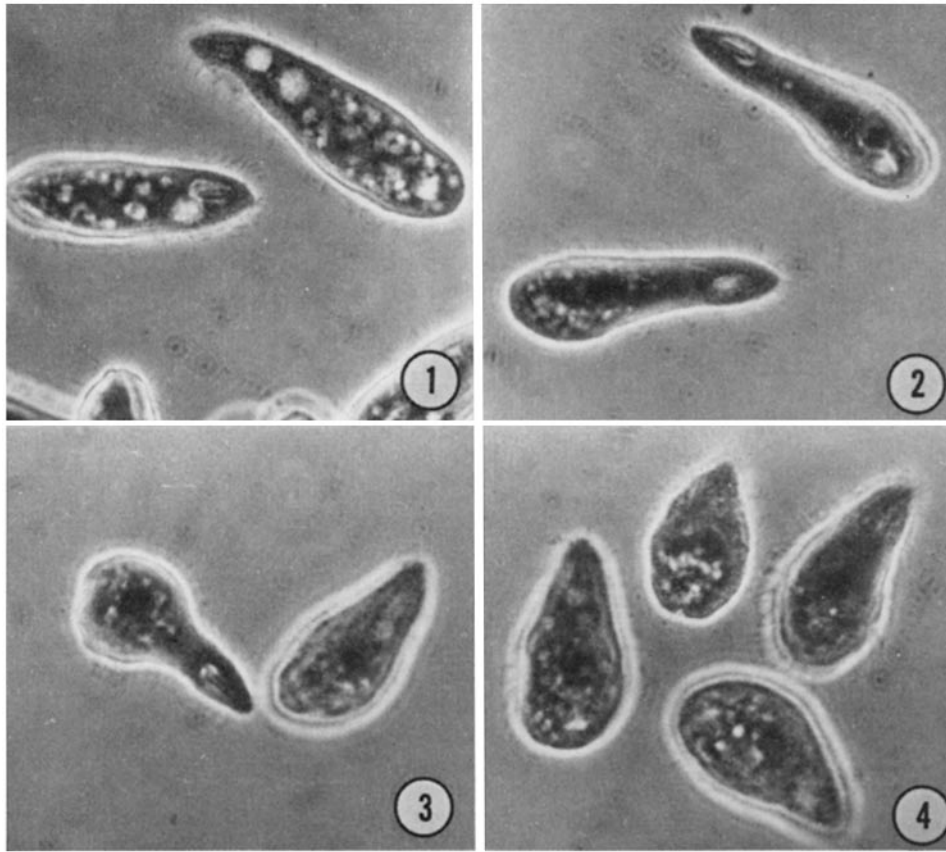


FIGURE 1 Untreated control cells of *Tetrahymena pyriformis*.  $\times 600$ .

FIGURE 2 Cells exposed to 7,500 psi for 2 min.  $\times 600$ .

FIGURE 3-4 Cells exposed to 10,000 psi for 10 min.  $\times 600$ .

early as 2 min at 10,000 psi (Fig. 8), but more commonly after 10 min at 7,500 psi (Fig. 9) or 10,000 psi. In Fig. 8 the raised area to the left of the cilium, which normally contains longitudinal microtubules, is entirely granular. At this time no change in ciliary microtubules is observed. The increase of granular material in this area seems to be greatest after 10 min exposure (Fig. 9). The breakdown of longitudinal microtubules seems to occur first in the posterior portion of the cell, progressing in an anterior direction in much the same manner as the gross morphological alterations evident with the light microscope.

It should be emphasized that disappearance of longitudinal microtubules can be observed in some parts of the cell as early as 2 min after exposure to

either 7,500 or 10,000 psi (Fig. 8) of pressure. As pressure is increased, or as time of exposure to a specific pressure is extended throughout the ranges employed in this study, further degradation of the longitudinal microtubules occurs. However, even after exposure to pressures of 10,000 psi for 10 min, some persistence of longitudinal microtubules is observed (Fig. 12). This is particularly true for longitudinal microtubules in the anterior portion of the cell, as demonstrated by their presence in the region of the oral apparatus.

Disorganization of longitudinal microtubules may be through breakdown of the tubules into their granular subunits, since no filamentous material has been observed in cells exposed to as much as 10,000 psi for 10 min. Just what effect

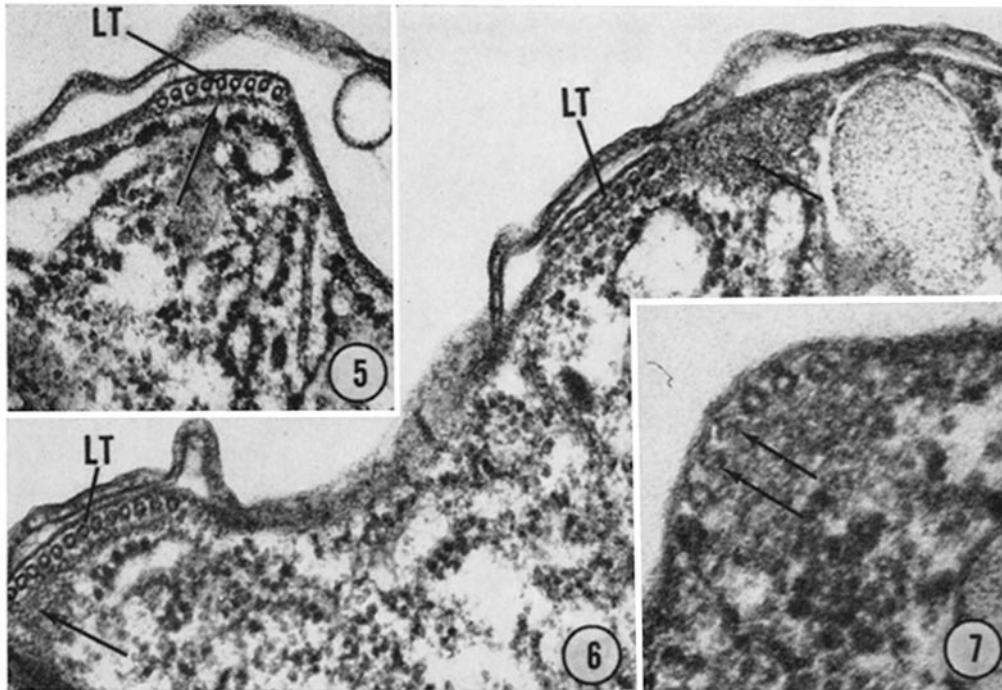


FIGURE 5 Longitudinal microtubules (*LT*) cut in cross-section are shown in this portion of an untreated control cell. Beneath is a granular layer (arrow).  $\times 72,000$ .

FIGURE 6 Cells exposed to pressure show increased amounts of granular material (arrows) in regions normally containing longitudinal microtubules (*LT*). The cell was exposed to 7,500 psi for 2 min.  $\times 72,000$ .

FIGURE 7 In this group of longitudinal microtubules, both normal and disrupted (arrows) microtubules are evident. (7,500 psi for 2 min).  $\times 144,000$ .

pressure has on the transverse and posterior microtubules and the basal microtubule is difficult to assess. These tubules are rather sparsely distributed through the cell, and, since they twist through the cytoplasm often for only short distances, it is difficult to determine whether they are actually degraded or simply omitted in a given section.

### *Ciliary Microtubules*

Since ciliary structure in *Tetrahymena* has recently been described in detail (Allen, 1969; Elliott and Kennedy, in press), only the general features common to most cilia will be mentioned. The nine pairs of peripheral tubules extend inward to make up the wall of the basal body (Fig. 10). The central pair of tubules terminates at the axosomal granule. Spokelike cross-connections extend from the peripheral to the central tubules.

The basal body contains a centrally placed granular matrix of unknown function and composition (Fig. 11).

One of the first changes evident in ciliary structure after exposure to pressure is the breakdown of the central ciliary tubules at their site of junction with the axosome. This appears as a separation of the central tubules from the axosome (Fig. 11). Little or no change is seen in the structure of the granular matrix of the basal body. Continued pressure results in further degradation of the central ciliary tubules (Fig. 12), with little or no effect on the peripheral tubules or the spokelike fibrils from the peripheral to the central tubules. Continued exposure (10,000 psi for 10 min) results in further degradation of both the central ciliary microtubules and the spokelike filaments to about  $0.5 \mu$  above the axosome (Fig. 13). Furthermore, at this

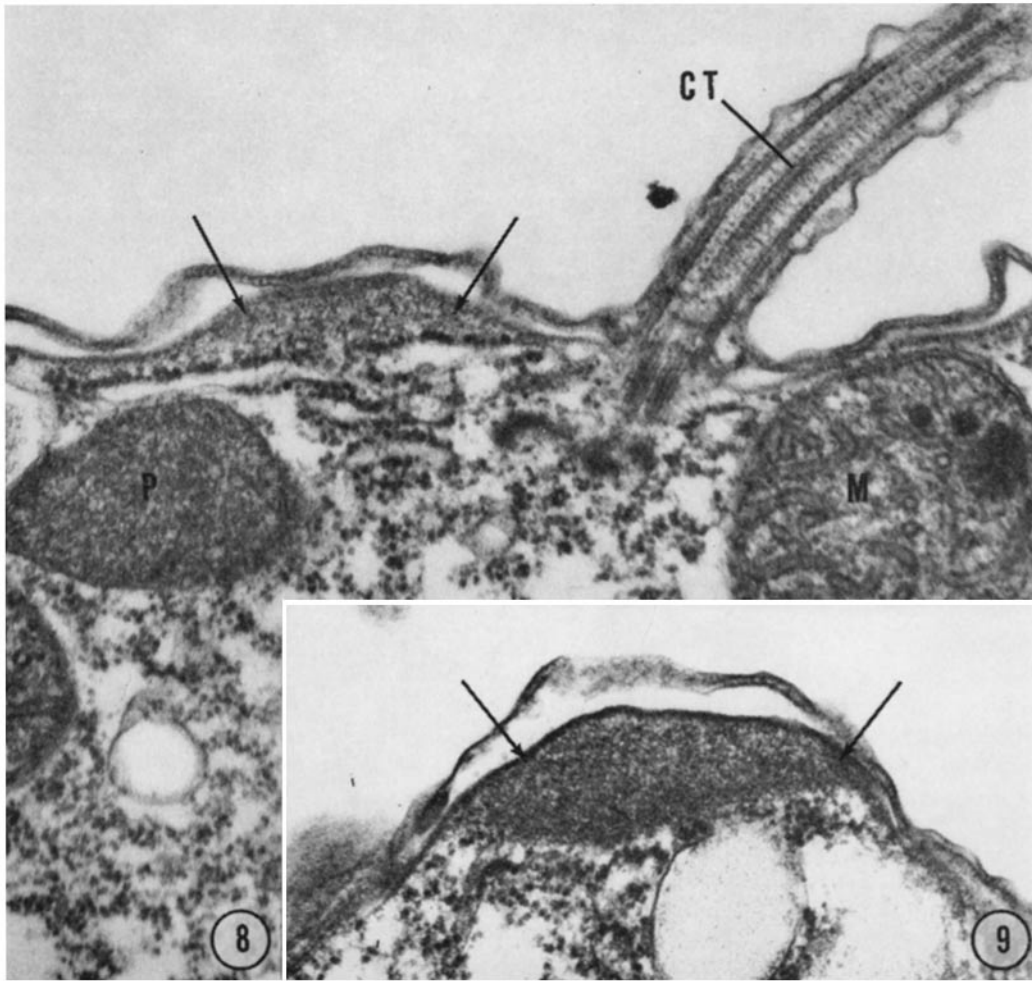


FIGURE 8 This section is from a cell exposed to 10,000 psi for 2 min. The central tubules (CT) of the cilium appear unaltered. To the left, the raised area in which longitudinal microtubules are normally found is granular (arrows). A mitochondrion (M) and a peroxisome (P) are indicated.  $\times 52,000$ .

FIGURE 9 This section through an area which would normally contain longitudinal microtubules (arrows) is completely granular in composition. 7,500 psi for 10 min.  $\times 52,000$ .

time, the granular material in the basal bodies may be either displaced to one side or broken down (Fig. 13). The septum and axosome persist. This tubular degradation progresses in a distal direction and may result in swelling of the basal body and ciliary shaft (Fig. 14). No alteration is seen in the peripheral ciliary microtubule structure (Fig. 14, insert). No evidence of an accumulation of granular or fibrous material has been found, suggesting that these microtubules break down to a molecular

level not preserved by the fixation methods employed.

Although there is a marked reduction in cell movement by the time the first changes in ciliary structure are observed (10,000 psi for 5 min; Fig. 11) ciliary activity is not completely blocked. Thus, apparently neither the central ciliary tubules nor the granular material of the basal body are totally necessary for continued ciliary activity. However, whether these structures are functional with regard

to the rate, direction, or coordination of ciliary beat is as yet undetermined.

## DISCUSSION

Numerous functions have been attributed to the cellular microtubules (Kennedy, 1969). However, only recently has any substantial evidence been presented to support the hypothesis that microtubules are form maintenance organelles. Experimental studies on *Actinosphaerium* have clearly shown that when cells are subjected to various antimitotic agents, i.e. colchicine (Tilney, 1968), low temperature (Tilney and Porter, 1967), or pressure (Tilney *et al.*, 1966), axopodial microtubules are broken down and axopodial form disappears. Thus it appears that these microtubules in *Actinosphaerium* are responsible for form maintenance. A similar situation may exist with regard to the longitudinal microtubules of *Tetrahymena*. The pressure degradation of these tubules seems to correspond with the loss of normal cell form, appearing first in the posterior region of the cell and progressing anteriorly. Furthermore, as suggested by Tilney and Porter (1967), the cell membrane alone seems incapable of maintaining cellular form.

Unlike the situation in *Actinosphaerium*, in which pressure (Tilney *et al.*, 1966) degrades microtubules to a filamentous material, possibly by separating the microtubules into the longitudinal subfilaments, 8–13 in number, (see Kennedy, 1969 for review of microtubule substructure data), in *Tetrahymena* the longitudinal and central tubules seem to be broken down into the granular subunits. These subunits may be comparable to the 40 Å microtubule subunit described by Shelanski and Taylor (1968). Thus, it is possible that, in *Tetrahymena*, subunit bonds in a given row of granules are no stronger than bonds between adjacent rows. Nevertheless, it may be that degradation is more complete for the longitudinal microtubules of *Tetrahymena* than for the axopodial microtubules of *Actinosphaerium*.

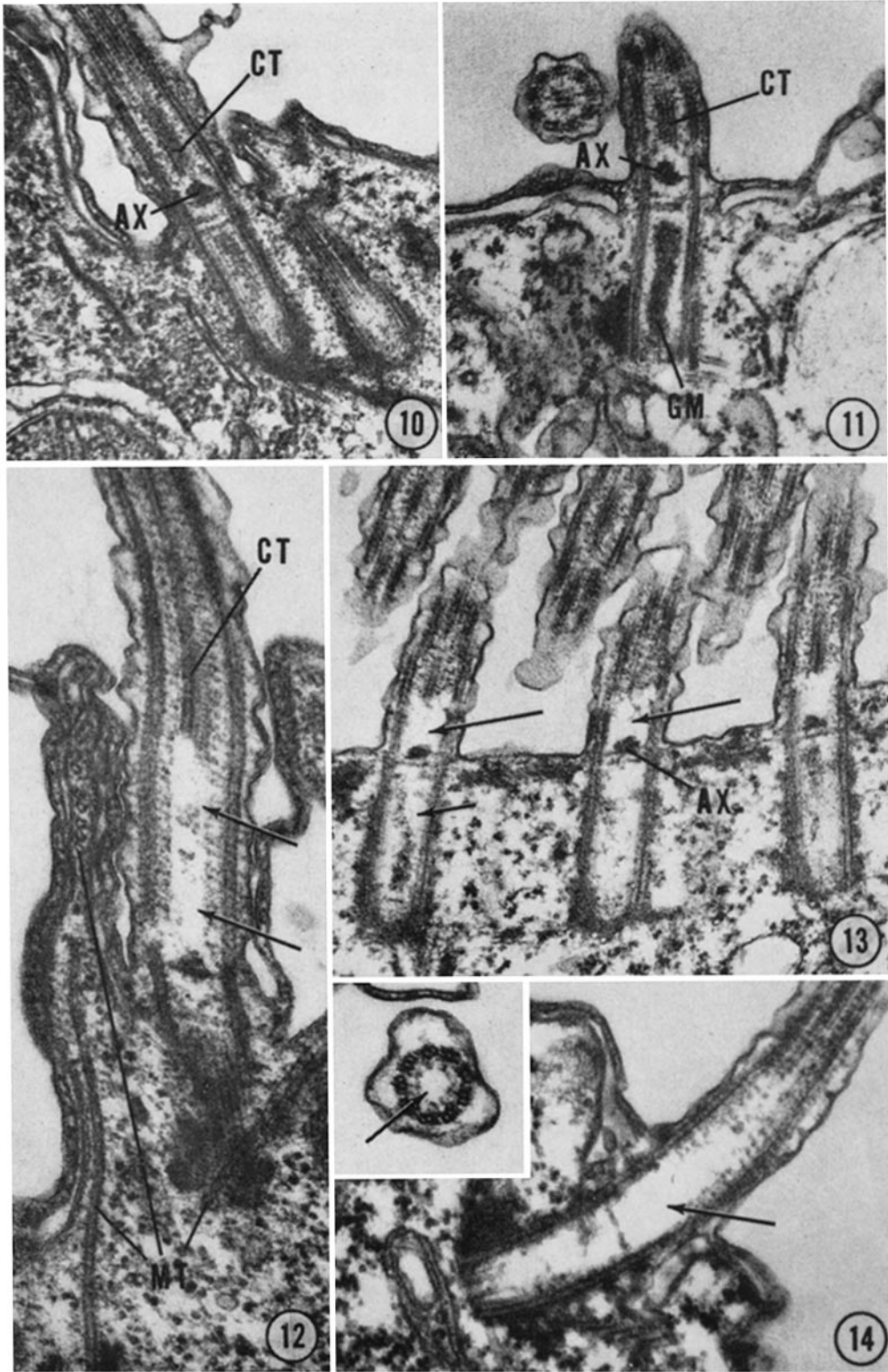
The central ciliary microtubules are also affected by high hydrostatic pressure. Degradation occurs from the axosome distally for as much as 0.5  $\mu$  without any apparent damage to peripheral microtubular structure in either the cilium or the adjoining basal body. The granular matrix of the basal body, however, is disrupted or displaced. These apparent structural alterations do not completely block ciliary activity in *Tetrahymena*, but

may be responsible for reduced ciliary activity or loss of coordination. Kitching (1957) observed that *Tetrahymena* exhibited some movement up to 11,000 psi. At higher pressures the cells were stationary, and some individuals became bulbously deformed and finally spherical. Following decompression (from 11,000 psi), there was an immediate resumption of slow movement, but in most individuals it took up to 20 min to recover moderate swimming activity. When *Stentor* was subjected to high hydrostatic pressure, Kitching (1957) reported that there was a decrease in beat frequency in the adoral membranelle, and ultimately the metachronal rhythm was lost. In *Spirostomum*, he observed that coordination was lost before the capacity to beat. Thus in large organisms such as *Stentor*, where more careful analysis of pressure effects are possible, rate of ciliary beat and coordination are both lost. Just how this relates to central tubule breakdown requires further examination.

Unlike the situation in *Tetrahymena*, embryos of *Arbacia*, when exposed to 6,500 psi for 1 hr, show no changes in either ciliary activity or structure (Tilney and Gibbins, 1968). Furthermore, no alteration occurs in basal body structure. Though ciliary activity persists, directed movement seems to be lost. These investigators were not certain whether this was due to alteration in rate of beat or loss of coordination. Cytoplasmic microtubules are degraded and development is arrested.

Young *et al.* (1970) also studied pressure effects on the cilia of *Strongylocentrotus purpuratus* embryos, and reported that, following 10,000 psi for 10 min, or 7,500 psi for 20–30 min, almost all of the blastulae lost over 90% of their cilia. At 6,500 psi for durations up to 50 min, the results were variable. Whereas some cells retained most of their cilia, others were mostly devoid of cilia. Unlike Tilney and Gibbins (1968), these investigators found that pressurizing embryos of *Arbacia punctulata* at 2,000 psi (for 20 min) caused most of the cilia to be removed; in several experiments, 5,000 and 10,000 psi were found to remove all cilia within 10 min. Sperm flagella are more resistant to pressure than cilia. Hinsch and Marsland (Marsland, 1970, p. 289) found that the microtubules present in the flagella of a variety of sperm cells retain a high degree of structural integrity and normal arrangement following treatment of 10,000 psi for 10 min.

The apparent sensitivity of central ciliary microtubules is not unique to pressurized *Tetrahymena*. Central tubules from chemically disrupted cilia ap-



pear to be more sensitive to dialysis than the peripheral tubules (Gibbons, 1965). Furthermore, Shelanski and Taylor (1967) observed that colchicine was bound to the central ciliary microtubules of sea urchin sperm, which are also more sensitive to dialysis than the peripheral microtubules.

Other degradative methods which have been employed to study microtubule structure and stability show somewhat variable results (Behnke and Forer, 1967). In crane fly spermatids, brief pepsin digestion has no apparent effect on either the central tubules or the cytoplasmic tubules, while subfibril A of the peripheral tubules is lost. Only after prolonged pepsin digestion are the central and cytoplasmic tubules broken down. Colchicine and low temperature (0°C) affected only the cytoplasmic tubules. High temperatures (50°C) caused disappearance first of the cytoplasmic tubules, and later of the central, and finally the peripheral, tubules. On this basis, Behnke and Forer classed the cytoplasmic and central ciliary microtubules into two separate groups. Our data indicate that longitudinal cytoplasmic tubules and at least a part of the central ciliary tubule in *Tetrahymena* respond similarly to high hydrostatic pressure. Microtubular sensitivity to various agents may represent the degree of macromolecular bonding of the subunits. Thus peripheral ciliary tubules may be more stable than central or cytoplasmic microtubules, since the former are doublets sharing a common wall and also contain side arms of adenosine triphosphatase (ATPase) (Gibbons, 1965).

The fact that microtubules of the old mouth in

*Tetrahymena* are generally unaffected by high pressure, while oral microtubules of developing anlagen are broken down (Kennedy and Zimmerman, unpublished data), suggests that newly polymerized microtubules are the least stable. If longitudinal microtubule elongation (polymerization) occurs at the posterior end of the cell, the first effects of high pressure would be most evident in this area. This seems to be the case in *Tetrahymena*, with degradation progressing anteriorly with time.

Central ciliary microtubules show alteration first at their junction with the basal body, and extend distally about 0.5  $\mu$ . A similar sensitivity was observed in cilia of *Paramecium* treated with chloral hydrate (Kennedy and Brittingham, 1968), where first alteration of both central and peripheral microtubules occurred at the ciliary-basal body junction. However, in the case of *Paramecium*, complete breakdown of all ciliary microtubules was observed. Certainly, further studies are needed to establish the relationship between the various types of microtubules and their formation.

This research was supported in part by United States Public Health Service Grants GM 13386 and AI 09656 and by the National Research Council of Canada.

Received for publication 26 November 1969, and in revised form 13 July 1970.

#### REFERENCES

- ALLEN, R. D. 1967. Fine structure, reconstruction and possible functions of components of the cortex of *Tetrahymena pyriformis*. *J. Protozool.* 14:553.

---

FIGURE 10 This cilium from an untreated control shows normal structure. Labeled are the axosomal granule (AX) and central ciliary microtubules (CT).  $\times 52,000$ .

FIGURE 11 Initial breakdown of the central ciliary microtubules (CT) just above the axosomal granule (AX) is shown in this cell exposed to 10,000 psi for 5 min. The granular mass (GM) of the basal body appears unaffected.  $\times 52,000$ .

FIGURE 12 Central ciliary microtubule (CT) degradation progresses distally (arrows) from the axosomal granule. Some longitudinal and transverse microtubules (MT) persist even at these pressures. Treatment, 7,500 psi for 10 min.  $\times 72,000$ .

FIGURE 13 Further ciliary degradation (long arrows) above the axosomal granule (AX) is shown here in cells exposed to 10,000 psi for 10 min. The granular mass of the basal bodies is absent or displaced (short arrow).  $\times 52,000$ .

FIGURE 14 Further ciliary degradation results in what appears as swelling of the ciliary shaft at the basal body junction (arrow). The insert shows a similar cilium in cross section with the central tubules missing (arrow). 10,000 psi for 10 min.  $\times 52,000$ .



- ALLEN, R. D. 1969. The morphogenesis of basal bodies and accessory structures of the cortex of the ciliated protozoan *Tetrahymena pyriformis*. *J. Cell Biol.* **40**:716.
- ASTERITA, H., and D. MARSLAND. 1961. The pellicle as a factor in stabilization of cellular form and integrity: Effects of externally applied enzymes on the resistance of *Blepharisma* and *Paramecium* to pressure induced cytolysis. *J. Cell. Comp. Physiol.* **58**:49.
- AUCLAIR, W., and D. MARSLAND. 1958. Form stability of ciliates in relation to pressure and temperature. *Biol. Bull.* **115**:384.
- BEHNKE, O., and A. FORER. 1967. Evidence for four classes of microtubules in individual cells. *J. Cell. Sci.* **2**:169.
- ELLIOTT, A. M., and J. R. KENNEDY. 1970. The morphology of *Tetrahymena*. In *The Biology of Tetrahymena*. A. M. Elliott, editor. Appleton-Century-Crofts, New York. In press.
- GIBBONS, I. R. 1965. Chemical dissection of cilia. *Arch. Biol. (Leige)*. **76**:317.
- JAHN, T. L., and E. C. BOVEE. 1967. Motile behavior of protozoa. In *Research in Protozoology* Vol. 1. T. T. Chen, editor. Pergamon Press Inc., New York. 1:41.
- KENNEDY, J. R. 1969. The role of microtubules in the cell cycle. In *The Cell Cycle. Gene-enzyme Interactions*. G. M. Padilla, G. L. Whitson and I. L. Cameron, editors. Academic Press Inc., New York. 227.
- KENNEDY, J. R., and E. BRITTINGHAM. 1968. Fine structure changes during chloral hydrate deciliation of *Paramecium caudatum*. *J. Ultrastruct. Res.* **22**:530.
- KITCHING, J. A. 1957. Effects of high hydrostatic pressures on the activity of flagellates and ciliates. *J. Exp. Biol.* **34**:494.
- LANDAU, J. B., and L. THIBODEAU. 1962. The micro-morphology of *Amoeba proteus* during pressure-induced changes in the sol-gel cycle. *Exp. Cell Res.* **27**:591.
- LUFT, J. H. 1961. Improvements in epoxy resin embedding procedures. *J. Biophys. Biochem. Cytol.* **9**:409.
- MARSLAND, D. A. 1950. The mechanisms of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata*. *J. Cell. Comp. Physiol.* **36**:205.
- MARSLAND, D. 1970. Pressure-temperature studies on the mechanisms of cell division. In *High Pressure Effects on Cellular Processes*. A. M. Zimmerman, editor. Academic Press Inc., New York. 259.
- PITELKA, D. R. 1961. Fine structure of the silverline and fibrillar systems of three tetrahymenid ciliates. *J. Protozool.* **8**:75.
- REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
- SHELANSKI, M., and E. W. TAYLOR. 1967. Isolation of a protein subunit from microtubules. *J. Cell Biol.* **34**:549.
- SHELANSKI, M. L., and E. W. TAYLOR. 1968. Properties of the protein subunit of central-pair and outer-doublet microtubules of sea urchin flagella. *J. Cell Biol.* **38**:304.
- TILNEY, L. G. 1968. Studies on the microtubules in heliozoa. IV. The effect of colchicine on the formation and maintenance of the axopodia and the re-development of pattern in *Actinosphaerium nucleofilum* (Barrett). *J. Cell Sci.* **3**:549.
- TILNEY, L. G., and B. BYERS. 1969. Studies on the microtubules in Heliozoa. V. Factors controlling the organization of microtubules in the axonemal pattern in *Echinospaerium (Actinosphaerium) nucleofilum*. *J. Cell Biol.* **43**:148.
- TILNEY, L. G., and J. R. GIBBINS. 1968. Differential effects of antimitotic agents on the stability and behavior of cytoplasmic and ciliary microtubules. *Protoplasma.* **65**:167.
- TILNEY, L. G., Y. HIRAMOTO, and D. MARSLAND. 1966. Studies on the microtubules in heliozoa. III. A pressure analysis of the role of these structures in the formation and maintenance of the axopodia in *Actinosphaerium nucleofilum* (Barrett). *J. Cell Biol.* **29**:77.
- TILNEY, L. G., and K. R. PORTER. 1967. Studies on the microtubules in heliozoa. II. The effect of low temperature on these structures in the formation and maintenance of the axopodia. *J. Cell Biol.* **34**:327.
- YOUNG, P. G., A. D. YOUNG, and A. M. ZIMMERMAN. 1970. Effect of Hydrostatic pressure on Sea Urchin Cilia. Canadian Federation of Biological Societies, Montreal, Quebec. (Abstr.)
- ZIMMERMAN, A. M. 1969. Effects of high pressure on macromolecular synthesis in synchronized *Tetrahymena*. In *The Cell Cycle. Gene-enzyme Interactions*. G. M. Padilla, G. L. Whitson and I. L. Cameron, editors. Academic Press Inc., New York. 203.
- ZIMMERMAN, A. M. 1970. High Pressure Effects on Cellular Processes. Academic Press Inc., New York.