STUDIES ON THE MICROTUBULES
IN HELIOZOA

II. The Effect of Low Temperature on These Structures
in the Formation and Maintenance of the Axopodia

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ABSTRACT

When specimens of Actinosphaerium nucleofilum are placed at 4°C, the axopodia retract and the
birefringent core (axoneme) of each axopodium disappears. In fixed specimens, it has been
shown that this structure consists of a highly patterned bundle of microtubules, each 220 A
in diameter; during cold treatment these microtubules disappear and do not reform until
the organisms are removed to room temperature. Within a few minutes after returning the
specimens to room temperature, the axonemes reappear and the axopodia begin to reform
reaching normal length 30–45 min later. In thin sections of cells fixed during the early stages
of this recovery period, microtubules, organized in the pattern of the untreated specimens,
are found in each reforming axopodium. Reforming axopodia without birefringent axonemes
(and thus without microtubules) are never encountered. From these observations we conclude
that the microtubules may be instrumental not only in the maintenance of the axopodia but
also in their growth. Thus, if the microtubules are destroyed, the axopodia should retract
and not reform until these tubular units are reassembled. During the cold treatment short
segments of a 340-A tubule appeared; when the organisms were removed from the cold,
these tubular segments disappeared. It seems probable that they are one of the disintegration
products of the microtubules. A model is presented of our interpretation of how a 220-A
microtubule transforms into a 340-A tubule and what this means in terms of the substructure
of the untreated microtubules.

INTRODUCTION

The disposition of microtubules in cells and cell
processes has led several investigators to suggest
that these elements perform a skeletal role (Byers
and Porter, 1964; Porter et al., 1964; Fawcett and
Witebsky, 1964; Behnke, 1965; Tilney and Porter,
1965; Maser and Philpott, 1964; de-The, 1964;
Pitelka, 1963; Roth, 1956) in the production and
maintenance of linear cell extensions. Examples of
these are found in cilia and flagella (Gibbons,
1961), in neurons (Gonatas and Robbins, 1965;
Porter et al., 1964), in numerous structures in the
protozoa (Tilney and Porter, 1964; Pitelka, 1963;
Rudzinska, 1965), in cells in culture (Taylor,
1966), in certain types of sperm (Gordon, 1966,
personal communication; Moses, 1966), or even in
cells undergoing elongation such as during mitosis
(de-The, 1964; Robbins and Gonatas, 1964; Roth
et al., 1966) or in embryonic differentiation
(Byers and Porter, 1964; Arnold, 1966).

It has further been noted that these elements are
present in regions of the cytoplasm which can be
characterized as gelled or regions of so called
could elucidate the role of the microtubules in the before-mentioned functions. By virtue of this association, the microtubules might then be instrumental in the regulation of cytoplasmic movements within specific regions of the cytoplasm adjacent to these gelled areas. Such regions in which cytoplasmic flow occurs could then be defined as regions of plasmagel.

In order to investigate more rigorously the relationship of the microtubules to the production and maintenance of cell asymmetry, on the one hand, and to the regulation of motility, on the other hand, we undertook a group of experiments designed to cause the breakdown of the microtubules. We felt that through careful comparisons of the experimentally treated specimens with controls we could elucidate the role of the microtubules in the before-mentioned functions.

Toward this end, we have treated the protozoan, *Actinosphaerium nucleofilum*, with cold temperature (Tilney, 1965 a and b). It was reported a number of years ago (Inoué, 1952) that the birefringence of the mitotic spindle disappeared when dividing cells were placed in the cold. More recently, a number of investigators (Roth and Daniels, 1962; Ledbetter and Porter, 1963; Robbins and Gonatas, 1964; de-Thé, 1964; Roth, 1964; Manton, 1964; Harris, 1962; to mention only a few) have shown that each spindle fiber as resolved by the light microscope is, in reality, composed of a number of microtubules. Since each microtubule in the spindle appears morphologically similar to the microtubules in the cytoplasm of interphase cells, and since a reduction in birefringence of the isolated mitotic spindle results in a concomitant loss in the number of microtubules (Kane and Forer, 1965), it seemed reasonable to expect that cold temperature might bring about a destruction of cytoplasmic microtubules and that, in situations other than the mitotic spindle, temperature control might thus be used as an analytical tool to provide more information on the characteristics and function of these cytoplasmic elements.

*Actinosphaerium nucleofilum* seemed ideally suited for experiments of this kind. As is well known, its needle-like pseudopodia (axopodia), which can be up to 400 µ in length, contain within them a well defined system of microtubules, the so called axoneme (Kitching, 1964; Tilney and Porter, 1965). It was reasoned that, if the microtubules are instrumental in the maintenance of these slender protoplasmic extensions, then low temperature, which, as previously stated, should cause the breakdown of the microtubules, ought secondarily to cause retraction of the axopodia. Likewise, if the microtubules are important in the production of these linear cell extensions, then re-extension of the axopodia, when the *Actinosphaerium* is brought back to room temperature, should not occur in the absence of oriented microtubules.

It also proved worthwhile to examine the products of the microtubule disassembly since this cryopathology gave us some additional information about the composition of the microtubule through the behavior of these breakdown products.

**MATERIALS AND METHODS**

**THE ORGANISMS:*** Cultures of Actinosphaerium were obtained from the Carolina Biological Supply Co. and serially subcultured in a wheat medium (Looper, 1928). At intervals, ciliates were added as a food source.

**LIGHT MICROSCOPIC PROCEDURES:** For observation, cells were put either on a glass slide and covered by a cover slip supported on its edges, or in a shallow depression slide. Each preparation was allowed to equilibrate for a short period prior to cold treatment. Such preparations were viewed with an ordinary light microscope or with a Zeiss polarizing microscope equipped with a mercury arc lamp. With the latter, the 16x objective and a compensator setting of 3° proved optimal.

Living organisms were studied during the cold treatment by placing the microscope in a 4° cold room. Once the polarizing microscope had reached the temperature of the cold room, it could be used to study slides prepared as already described. To watch regrowth, we removed the microscope from the cold room and allowed it to equilibrate at room temperature. The slide was then taken from the cold room to room temperature and viewed immediately.

Photomicrographs were taken at intervals during and following cold treatment on 35-mm film. We subsequently developed the film in Diafine to increase the effective film speed.

**ELECTRON MICROSCOPIC PROCEDURES:** Specimens were fixed after periods varying from 1 to 24 hr following the onset of cold treatment. The fixative was cold (4°C) 3% glutaraldehyde in 0.05 m phosphate buffer at pH 7.0 which contained 0.0015 m CaCl₂. The specimens were washed in 0.1 m phosphate buffer with 0.0015 m CaCl₂, postfixed in 1% OsO₄ in 0.1 m phosphate buffer and 0.0015 m CaCl₂, dehydrated rapidly, and embedded in Epon 812 (Laft, 1961).

Other specimens which had been cold treated for

1 Burlington, North Carolina.

2 Baumann Photo-chemical Corporation, Chicago.
several hours were brought out of the cold room in small petri dishes which contained about 5 ml of fluid. These were examined with a dissecting microscope. When the axopodia first began to reform, about 10 min after removal from the cold, they were fixed in the identical fixative as that used on the cells in the cold; the only difference being that the fixative was at room temperature. The remaining steps were carried out as already described.

Specimens 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 the Siemens Elmiskop I electron microscope.

OBSERVATIONS

Normal Structure

The axoneme or birefringent core of each axopodium (in rare instances, a second axoneme parallel to the first is present within a single axopodium) extends without interruption from the tip of the axopodium to the medullary region of the cell body. The birefringence, which can be readily demonstrated in living specimens (Fig. 1 a), is positive, uniaxial, and has been shown to be form birefringence (McKinnon, 1909). The limiting membrane, ingested material such as rotifers (Fig. 1 a), and twinkling medullary particles of unknown nature are also birefringent.

When an axoneme is examined with the electron microscope, it is found to exist as a bundle of microtubules arranged parallel to one another and, in cross-sectional view, organized into an interlocking double coil. (For a more detailed description of the organization of the microtubules in the

FIGURE 1. These micrographs and the micrographs of Fig. 4 are all taken of the same organism with a Zeiss polarizing microscope. The large birefringent spot in the center of the organism is an ingested rotifer. X 170.

a, 3 min at 4°C. Note the birefringent axonemes which extend from the medulla to the tip of each axopodium. b, 55 min at 4°C. The axopodia are reduced in length and have become beaded. Their short birefringent axonemes extend from the corticomedullary junction out into the axopodia. c, 24 hr at 4°C. Note the almost complete absence of axopodia. In four, possibly five, instances thin axonemes extend from the corticomedullary boundary out into short axopodia. In other instances, the birefringence of the axonemes has totally disappeared. A number of beads, presumably the short axopodia seen in Fig. 1 b, are seen surrounding the cell body. It is possible that they are connected together and to the cell body.
FIGURE 2. Micrograph taken through a portion of the cell body of an organism fixed after 2½ hr at 4°C. Mitochondria (M), short segments of rough-surfaced endoplasmic reticulum and profiles of tubules (see arrows) are apparent in this micrograph. The ground substance which we refer to in the text as "matrix substance" is very prominent in these cold-treated cells. X 43,000.

FIGURE 3. Micrographs taken of portions of cold-treated cells. a, Most prominent in this micrograph are tubular profiles measuring approximately 340 Å in diameter. In some, a small dot can be seen in the center. X 82,000. b, Many of the 340-Å tubules are present in longitudinal section in this micrograph. In the tubule designated by the arrows, one can make out diagonal striations across the wall of the tubule. These striations make approximately a 45° angle with the long axis of the tubule. X 110,000.
axoneme, see Tilney and Porter, 1965). Figs. 6 and 7, taken of recovering axopodia, depict this structure. Within each axopodium, dense granules, believed to function in food capture, and mitochondria are situated between the axoneme and the limiting plasma membrane. These cytoplasmic particles, in living organisms, move up and down the axopodia in the periaxonemal space.

Cold Treatment

1. Light Microscopic Observation of Living Animals: A series of small beads appears along the length of most of the axopodia within a few minutes after preparations containing the organisms are placed at 4°C (Fig. 1 a). Several minutes later, the axopodia begin to shorten. In all but the final stage of axopodial retraction, the cytoplasm which once made up each axopodium withdraws into the cortical region of the cell body.

Alterations in the axoneme occur during the cold treatment as well. These can be studied most profitably in living organisms with the polarizing microscope. With this instrument, quantitative as well as qualitative measurements on the amount of birefringence in a single axoneme or in a portion of a single axoneme can be made with respect to the length of time the organisms are exposed to the cold. The accompanying Figs. 1 a–c, photomicrographs of the same organism, were taken after periods of 3 min, 1 hr, and 2 1/4 hr of cold treatment. As can be readily seen, the birefringence of the axoneme gradually disappears; each axoneme does not merely shorten in length but rather, while each decreases in length, the over-all birefringence of the whole axoneme diminishes. Thus, if we compare the birefringence of a specific region of the axoneme, for example that portion near the surface of the cell body, before axoneme shortening (Fig. 1 a) and during axoneme shortening (Fig. 1 b), we find that roughly the birefringence diminishes in this region in proportion to the amount of axoneme shortening.

After longer periods in the cold (Fig. 1 c) few axopodia remain, but in those that do persist axonemes can still be seen. A number of small beads lie just outside the cell body and persist in this position over long periods of cooling. Since these beads cannot account for all the cytoplasm in the axopodia, it would seem that some of the cytoplasm from the retracting axopodium has migrated into the cell body. It is probable that these beads are connected to each other and to the cell body by a plasma membrane and that during axopodial re-extension (Fig. 4) they again become a part of the axopodal cytoplasm.

With increased time in the cold, up to 1 1/4 days, further changes in the axopodia and axonemes do not occur. An occasional short axopodium with a faintly birefringent axoneme similar to that depicted in Fig. 1 c remains. Redevelopment of straight rigid axopodia does not occur in the cold.

In contrast to the diminution in axoneme birefringence during cold treatment, the birefringence of the ingested material, the limiting membranes (this includes the plasma membrane on the surface of the cell body as well as the membranes which limit all the vacuoles), and twinkling medullary particles is unaltered.

Although no further changes take place in the axonemes and remaining axopodia with prolonged cold treatment, changes do take place in the size of the cortical vacuoles and the over-all density of the organisms. The cortical vacuoles increase in size and the optical density diminishes. We presume that these changes are related to the effect of low temperature on the contractile vacuole. This structure either completely fails to function or empties at a rate insufficient to counteract water inflow. Each organism gradually increases in diameter and eventually ruptures.

2. Electron Microscope Observations

a. Cells Fixed after 2 1/4 hr at 4°C: Since few axopodia remain after 2 1/4 hr of cold treatment, it is not surprising that few were encountered in thin sections cut from several organisms. Present within some of these axopodia we found small numbers of microtubules (never more than 20); in other axopodia, which appeared larger in diameter, we found mitochondria, dense granules, small numbers of structures presumed to be excretion bodies (Tilney and Porter, 1965), and short segments of a randomly oriented, tubular component measuring 310–360 A in diameter. The tubular component was embedded in an amorphous material (Figs. 2 and 5). In no instance were both the 220- and the 340-A tubular profiles present in the same axopodium.

Since axopodia were rare, our attention focused on the cytoplasm of the cell body. As might be expected by the absence of birefringent axonemes (Fig. 1 c), organized arrays of microtubules were no longer present; in fact, the 220-A microtubules had completely disappeared from the cell body.
Short segments of the larger, 310–360-A tubules, already described in some of the remaining axopodia, were commonly encountered scattered throughout the cell body. But in most instances, these units bore no specific orientation either to each other or to the free surface. A closer examination of their fine structure, in particular in sections cut parallel to their long axes, revealed that some of these tubular units possessed an irregular transverse striation. The pitch of the striae was not constant, but, in some preparations favorable for observation, it approximated a 45° angle relative to the central axis of the tubule (Fig. 3b). The wall of each of the tubules was approximately 50 A thick, comparable to the wall thickness of the 220-A microtubules (Fig. 3a). In the center of some of these elements there was a small electron-opaque dot about 75 A in diameter.

Certain alterations in the nature of the material which occupies the area between the formed elements of the cytoplasm also seem to occur in cold-treated specimens. This material in untreated cells is very inconspicuous and possesses a fluffy appearance. In cold-treated cells, on the other hand, the equivalent space is packed with a finely fibrous cotton-like material which is illustrated in Figs. 2a, 2b.

**Figure 4** These micrographs are taken of the same organism illustrated in Fig. 1. In this series, the organism has been removed from the cold room and placed at room temperature; so these three micrographs depict recovery. As in Fig. 1, the highly birefringent spot in the center of the cell is due to an ingested rotifer. X 170. a, 3 min at room temperature. Even at this short time at room temperature, a few short axopodia had reformed; within each is a birefringent axoneme. Axonemes are beginning to form in the cell cortex (see arrow). b, 15 min at room temperature. Short axonemes now extend from the cell body out into the surrounding pond water. Within each is a strongly birefringent axoneme which extends from the tip of the axopodium into the medullary region. Through a comparison of Figs. 4b and 1b in which the axopodia are approximately the same length, it can be easily seen that not only are the reforming axopodia more rigid than the retracting ones but also the amount of birefringence is greater. c, 45 min at room temperature. By this time, the axopodia are of normal length. A comparison of Fig. 1c with this figure reveals that axopodia, in both instances, seem to appear in approximately the same locations and in the same numbers.
It is interesting to compare Fig. 5, a retracting axopodium, with Fig. 6, a reforming axopodium.

**Figure 5**  Section of an axopodium which remained after $3 \frac{1}{4}$ hr of cold treatment. Beneath the limiting membrane are mitochondria ($M$), electron-opaque granules ($G$), excretion bodies ($E$), and a randomly arranged tubular component enmeshed in a dense matrix substance. Each tubule measures about 340-A in diameter. $X 48,000$.

3 b, 5, and 8. In the absence of a better name, we will refer to this material as “matrix substance.” It is probable that it is derived in part from the disassembly of the axoneme and thus is related to the microtubules. The segments of 310-360-A tubules are commonly found in this matrix substance (Fig. 2).

b. CELLS FIXED AFTER 24 HR AT 4°C: The morphology of cells fixed after 24 hr at 4°C is comparable to that of cells fixed after shorter periods at 4°C. The frequency of the 310-360-A tubule segments has certainly not diminished by this stage. In certain micrographs these tubular units are oriented parallel to each other (similar to Fig. 3 a), but in most micrographs the tubules are randomly arranged within the matrix substance (Fig. 2). In one instance a short axopodium was found. This contained within it a few 220-A microtubules, but in no other instance were any 220-A microtubules seen at this stage. The matrix substance appears in the same quantity as is present after $3 \frac{1}{4}$ hr of cold treatment.

3. Recovery Following Low Temperature

a. LIGHT MICROSCOPIC OBSERVATIONS OF LIVING ORGANISMS: Within 3-4 min after the preparations were removed from the cold, axopodia begin to reform; the few short axopodia which had remained during the cold treatment begin to elongate. Within each of these, the polarizing microscope reveals the presence of a birefringent axoneme (Figs. 4 a and b).

Birefringence attributable to an axoneme is first visible in the cortical region of the cell. These regions are indicated by the arrows in Fig. 4 a and appear to give rise to the axoneme depicted in Fig. 4 b. These reforming axonemes initially ex-
tend in both directions. Once the basal end has made contact with the medullary region, it ceases to elongate basally; its only growth now will be in width. The apical end, on the other hand, continues to elongate as well as to grow in width. After making contact with the limiting membrane, it extends out into the surrounding medium. It is covered by the plasma membrane which becomes the axopodial membrane. As this reforming axopodium extends, presumably through the action of the axonemes, small granules move out into it and begin their characteristic movements. (For a more detailed description of these movements, see Tilney and Porter, 1965.) We have never observed a reforming axopodium which lacks a birefringent axoneme. It also appears that the residual beads present after full axopodial retraction (Figs. 1 c and 4 a) are incorporated into the reforming axopodia. (Compare Fig. 1 c with Figs. 4 a and b. Figures in the series 1 and 4 are from the same organism.)

It is interesting to compare the birefringence of reforming axopodia (Fig. 4 b) with the birefringence of retracting axopodia (Fig. 1 b). It is apparent by the comparison of these two figures (Figs. 1 b and 4 b) in which these cell extensions are approximately the same length, that the axonemes of the reforming axopodia are more strongly birefringent than the axonemes of the retracting axopodia. Of even greater significance is the fact that the surfaces of the reforming axopodia are smooth and relatively even while those of the retracting axopodia are beaded and clearly very irregular (Fig. 1 b).

By 35–40 min, the average length of the axopodium is within the normal range. Furthermore, organism movement has begun; a mechanism for
this movement, related to the axopodia, has been suggested (Tilney and Porter, 1965).

Through a careful comparison of Fig. 1 a with Fig. 4 c (micrographs of the same organism, which had been slightly compressed under a cover slip so that it could not change position), it is possible to show that the reforming axopodia appear in the same number and in approximately the same positions as before the cold treatment. These comparisons are technically difficult because a slight change in the focus will bring axopodia in a lower plane of focus into focus and those that were formerly in focus out of focus. When this is compensated for, one can match an axopodium before cold treatment to the same axopodium following cold treatment with reasonable certainty.

b. ELECTRON MICROSCOPIC OBSERVATIONS: Cells were fixed during the initial stages of axopodial reformation (about 10–15 min following removal from the cold). By this time, the axonemes were well developed and consisted of a bundle of 220-A microtubules (Figs. 6 and 7). These elements in cross-section were organized into their characteristic double-coiled configuration (Fig. 6) with an amorphous material present between the interlocking coils. (This material is present in untreated specimens as well.) In some cases irregularities or gaps in the double coil were seen, but, in all instances, microtubules were found in the reforming axopodia; never did we find a reforming axopodium without microtubules. Furthermore, these elements were invariably arranged parallel to the long axis of the process (Fig. 7). The diameter of the axoneme or the greatest number of microtubules present at the widest point, the axopodial base, was, in specimens fixed in the initial stage of recovery, seldom more than \( 1/2 \) that present in untreated specimens. (Absolute numbers of microtubules would be meaningless here, for there is considerable variation not only in the maximum number of microtubules in the axoneme in untreated organisms but also in the speed of recovery.) When recovery was completed, the number of microtubules was equivalent to that found in untreated specimens.

Figure 7: Longitudinal section through a recovering axopodium. Dense granules are present just peripheral to the axoneme. The latter is made up of microtubules arranged parallel to each other and to the long axis of the axopodium. \( \times 56,000 \).
FIGURE 8  Portion of the cortex of the cell body from an organism in the early stages of recovery. Of particular interest here is the "matrix substance" in which ribosomes, mitochondria (M), and an occasional dense granule are embedded. This dense material is prominent in cells which have been fixed during cold treatment or in the early stages of recovery. We presume that it is derived from the breakdown of the axoneme. X 43,000.

Besides the microtubules which penetrate the medullary region of the cell body at the basal end of the axoneme, small numbers of microtubules, unassociated with one another, were also encountered in this zone of the cytoplasm. The 310-360-A tubular elements, on the other hand, were no longer present; the matrix substance was still abundant and found throughout the cytoplasm (Fig. 8).

DISCUSSION
The Role of the Microtubules in the Formation and Maintenance of the Axopodia

The observations presented above demonstrate that during the cold treatment the axonemes disappear and the axopodia withdraw so that after 21/4 hr at 4°C few axopodia and thus few axonemes remain. Thin sections of cells fixed at this time or after longer periods in the cold show that the microtubules which make up the axoneme and thus appear to relate to the birefringence of the axoneme in living material, disassemble and do not reassemble until the specimens are returned to room temperature.

Within a few minutes after returning the cells to 22°C (room temperature), the axonemes begin to reappear and the axopodia to reform. 30-45 min later, axopodia of normal length are encountered. In thin sections of axopodia from cells fixed during the early stages of this recovery period, microtubules are found in each reforming axopodium; they are organized into the semicrystalline array characteristic of untreated specimens and extend from the axopodial tip to the medullary region of the cell body. Reforming axopodia without axonemes are never encountered.

From these observations and the fact that the...
limiting membrane alone is not known to support rigidly such long protoplasmic extensions in any other cell type, we conclude that the microtubules are intimately involved not only with the maintenance of the axopodia but also with their growth. Thus, if the microtubules are destroyed, these linear cell extensions ought to retract and not reform until these tubular units are reassembled. In work presented elsewhere, two other agents also antimitotic in nature, namely hydrostatic pressure (Tilney et al., 1966) and colchicine (Tilney, 1965 a, b), give similar results. In higher organisms as well, the microtubules appear to play a similar role (Tilney and Gibbins, 1966). It is, therefore, not unreasonable to suggest that these tubular elements may not only supply their elongation the force necessary for axopodium regrowth but also may give form and rigidity to each newly extended axopodium.

The Relationship of the Microtubules to the Plasmagel

The work of Marsland and coworkers over the last 30 yr has demonstrated that protoplasmic gel structures undergo solational weakening if exposed to hydrostatic pressure or low temperature. In a recent study, Tilney et al. (1966) reported that the microtubular component which makes up the axonemes of Actinosphaerium disassembled when the cells were subjected to hydrostatic pressure and from this study suggested that the microtubules may be an important factor in the development of the plasmagel.

Porter (1966) has suggested that the microtubules may also act to shape and orient the plasmagel with which they are coexistent. Thus by organizing the surrounding matrix, the microtubules may define or enclose regions of plasmagel, regions in which cytoplasmic movement may occur. As pointed out in an earlier paper in this series (Tilney and Porter, 1965), the cytoplasmic movement of particles proceeds in linear paths or tracks similar to the tracks described in the movement of pigment granules in chromatophores (Bikle et al., 1966) or by the chromosomes in the mitotic spindle. One of the first reactions of the cells to cold temperature is a beading of the axopodia or a conversion of regions of plasmagel into nearly spherical solated volumes. One effect of this is that the tracts or paths through which the particles normally move no longer exist owing to the loss of the gelated region represented by the axoneme. This means, in our interpretations, that now the streaming particles, disintegration products of the microtubules, and material associated with the microtubules accumulate into local regions which we recognize in the light microscope as "beads."

Microtubules and Other Fibrous Proteins: Similarities in the Assembly and Disassembly

In this study, we have demonstrated that the polymerization of microtubules is favored by increasing the temperature, depolymerization by reducing the temperature. Inoué (1952; 1964) made quantitative measurements on the birefringence of the mitotic apparatus and found that an almost linear relationship existed between temperature and birefringence and that increasing the temperature resulted in an increase in birefringence. It is now well known that each spindle fiber contains a number of microtubules (de-Thé, 1964; Robbins and Gonatas, 1964; Roth et al., 1966; Harris, 1962); and recently Kane and Forer (1965) have demonstrated that a correlation exists between birefringence and microtubule number, both in isolated spindles (Kane and Forer, 1965) and after treatment with heavy water (Kane, 1966, personal communication). Thus, it appears that, at least in Actinosphaerium and in the mitotic apparatus, increasing the temperature favors polymerization of the microtubules. Furthermore, polymerization of the microtubules appears to involve an increase in volume (Tilney et al., 1966). Even though electron microscopy on mitotic spindles has not been carried out on cells fixed during the application of hydrostatic pressure, light microscope studies (Pease, 1941; Zimmerman and Marsland, 1964) have demonstrated that the mitotic apparatus solates under pressure so that it is probable that the microtubules in the mitotic spindle act as those in Actinosphaerium and depolymerize when the pressure is increased. Similar results have been observed on globular to filamentous transformation in F actin (Ikkai et al., 1966), tobacco mosaic virus protein polymerization (Lauffer, 1962), and in aggregation of protein and crystal formation in sickle cell anemia (Murayama, 1966). In all these cases as well as microtubule formation, polymerization appears to be an endothermic reaction involving an increase in volume.

In studies on the polymerization of the tobacco mosaic virus protein (Lauffer, 1962) and forma-
ion of the mitotic spindle (Inoué, 1964), it has been demonstrated that polymerization involves an increase in enthalpy and a decrease in free energy. In view of the endothermic nature of polymerization, one arrives at the rather unexpected conclusion that the entropy must increase during polymerization. This conclusion is opposed to one's intuitive understanding of thermodynamics, and it indicated to Lauffer that its explanation must be in the release of "something" during polymerization which is bound to the subunits of the polymer. In an elegant set of experiments he was able to show that this "something" was water.

Since the proteins of tobacco mosaic virus, of sickle cells, of the mitotic spindle, and of the axoneme are similar both in certain structural proportions and in behavioral characteristics (they all assemble into microtubules), it is not unreasonable to suggest that the mechanisms for polymerization of these proteins may be similar, that is, involving the release of water. Therefore, any change in the characteristics of water should affect polymerization or depolymerization, and one is not surprised that heavy water (D₂O) stabilizes the mitotic spindle (Marsland and Zimmerman, 1965) and appears to increase not only the birefringence (Inoué, 1964) but also the number of microtubules in the mitotic apparatus (Kane, 1966, personal communication). An increase in axoneme birefringence and number of microtubules in the axopodia was likewise observed when Actinosphaerium was subjected to heavy water (unpublished observations).

It may be useful to carry this analysis one step further and to suggest that each microtubule polymerizes by the addition at its end (or ends) of subunits, i.e., micelles which assemble one unit after another in the single position always available in a continuous coil. In the case of the tobacco mosaic virus (Casper, 1963), there appear to be 16½ subunits per turn while in the microtubules of plants and animals, where it is more difficult to work out the polymerization steps, the best evidence suggests that there are only 13 micelles per turn (Ledbetter and Porter, 1964; Gall, 1965). Further work on the polymerization of microtubules following in vitro isolation of the subunits should obviously be carried out.

Other proteins form a tubular component as well and possess some of the characteristics already mentioned. For example, the assembly of flagellin to form the bacterial flagella (Köfllier, 1966; Kerridge et al., 1962) appears to be a similar case. (See Lauffer's reviews (1962, 1964) for other examples.)

The Significance of the 340-A Cold-Produced Tubules

The appearance of two types of structures in the cytoplasm during cold treatment, namely the 310-360-A tubular units and the amorphous material designated as matrix substance, and the simultaneous disappearance of the 220-A microtubules lead us to conclude that these new components are related to the breakdown of the axoneme. (This conclusion is not unreasonable since in untreated cells the microtubules make up a sizable proportion of the cytoplasm in the axo-podium. We have calculated that the axonemes of Actinosphaerium comprise roughly 10% of the total bulk of the cytoplasm. These are, in turn, composed of microtubules and an amorphous material around the microtubules. Furthermore, in cold-treated cells changes in other cytoplasmic constituents do not occur.) One immediately wonders if the two types of tubules are related. Does the 220-A tubule transform into the 340-A tubular unit?

This question cannot be definitively answered until such a time as microtubules have been isolated in a pure fraction and made to disassemble and repolymerize in vitro. Nevertheless, all the facts, to be enumerated presently, point to the interpretation that the 220-A microtubules transform into the 340-A tubular units. A mechanism for this transformation is suggested, although it is recognized that other interpretations are possible.

It seems to us that there are five pertinent facts:
1) the 340-A tubules appear during the period of disappearance of the 220-A microtubules; 2) the 340-A tubule is 50% larger in diameter than the 220-A microtubule, yet is still a tubule; 3) no intermediate-sized tubular profiles are present, only the 220- and 340-A; 4) the wall thicknesses of both the 220- and the 340-A tubules are similar, about 50 Å; 5) high resolution microscopy of the 340-A tubule reveals that there is a diagonal striation on the wall of the tubule. This banding makes an angle of approximately 45° with the long axis of the tubule and resembles the red stripes on a barber pole.

Before developing our interpretation of the above facts, we should mention several bits of information extracted from the literature which per-
tain to our interpretation. First, Pease (1963) and André and Thiéry (1963) have shown that the microtubules in sperm tails which had been dried on a grid and negatively stained tend to fray out at their tips; this reveals that the wall of each tubule is, in reality, composed of a series of 11 or more filaments about 40–50 Å in diameter. Furthermore, the wall of microtubules in plant cells (Ledbetter and Porter, 1964), in neurons (Gall, 1965) and in dividing cells (Barnicot, 1966) has now been shown to be made of 9–13 filaments. (The number of filaments, although appearing to be variable, may be and probably is constant in the latter three cases. Two procedures were used here: thin sections and negatively stained preparations. In the latter, because the microtubules are dried down on the grid, it is difficult to reconstruct in three dimensions the number of subunits for, upon flattening of the tubule, superposition of the filaments will occur. We, therefore, put more con-

![Figure 9](image-url)  
**Figure 9** Plasticine model showing our conception of the transformation of the 220-A microtubule into the 340-A tubule produced during the cold treatment. The wall of each microtubule appears to be composed of filamentous units.

![Figure 10](image-url)  
**Figure 10** This drawing depicts our conception of how the 340-A tubule is formed. We suggest that the globular subunits which make up each filament which, in turn, aggregate to form the wall of the microtubule have discrete bonding sites along their surfaces. This is depicted in this drawing by the Black connecting bars. When the microtubules begin to transform into the 340-A tubular units, these bonds break and the filaments begin to twist and at the same time to slide past each other. Once the filaments have slid past one another by approximately 50 Å, then the bonding sites on adjacent globular subunits along the length of the filaments are in register again so bonding can occur. It does. When this happens, the filaments lie at a 45° angle to the long axis of the tubular unit. Thus, each globular subunit has slid only one subunit past its neighbor on an adjacent filament.

...
filaments which make up the wall of the tubule slide relative to each other; at the same time, the diameter of the tubule, owing to the twisting motion, increases by 50%. It can, in fact, be demonstrated with models that if each of the filaments were to slide 50 Å relative to the subunit on its left, then a 340-Å tubule would be formed having a 50-Å wall thickness and a banding at 45° to the long axis of the tubule. The 220- and 340-Å tubules, as we envisage them, are illustrated in the clay model seen in Fig. 9.

There is one other fact so far unaccounted for by our interpretation, and that is that tubules intermediate in size between 220 and 340 Å were not found. The explanation for this in terms of the model is illustrated in Fig. 10. We assume that there are discrete lateral bonding sites on the subunits such that there is only one position per subunit in which lateral bonding with an adjacent subunit can succeed. Thus, when the filaments slide past one another, the only place at which they can bond again is where the subunits on the filaments come in register. In this position, the filaments would lie at 45° to the long axis of the tubule and the diameter will have increased by exactly 50%.

Another possible interpretation which will fit all the data mentioned above is that the 220-Å microtubule may break down in the cold into its component filaments, or into its subunits, and then reassemble as a 340-Å tubule. We do not wish to give the impression that the 340-Å tubular elements present in a cold-treated cell could account for the total 220-Å microtubule population present prior to cold treatment. Rather, this tubular component may account for only a small number of the microtubules in an untreated or a fully recovered cell. The matrix substance may account for the remainder.

No 340-Å tubular units were present in cells fixed during the initial stages of axopodial reformation. This indicates that these units either break down or reform into the 220-Å microtubules or both during the early stages of recovery.

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