STUDIES ON THE MICROTUBULES IN HELIOZOA

IV. THE EFFECT OF COLCHICINE ON THE FORMATION AND MAINTENANCE OF THE AXOPODIA AND THE REDEVELOPMENT OF PATTERN IN ACTINOSPHAERIUM NUCLEOFILUM (BARRETT)

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SUMMARY

When specimens of Actinosphaerium are placed in a solution of colchicine, the axopodia retract and that portion of the birefringent core (axoneme) present in each axopodium disappears. In fixed specimens, it has been shown that the axoneme consists of a highly patterned bundle of microtubules each 220 Å in diameter. During colchicine treatment the microtubules, present in the axopodial portion of the axoneme, break down and do not reform until the cells are washed free of the colchicine and allowed to recover. In the basal portion of the axoneme or that portion confined to the cell body, most of the microtubules do not break down during colchicine treatment, although the spacing of the microtubules within the remaining axonemes is altered; in longitudinal section these tubules no longer appear straight but present an undulatory profile. When the specimens are washed free of the colchicine and allowed to recover, axopodia reform and within each numerous microtubules are present. From these observations we conclude that the microtubules are instrumental in the maintenance of the axopodia as well as in supplying the force necessary for their regrowth. Observations are also presented on the disintegration products of microtubules, on microtubules and cytoplasmic motility, and on the redevelopment of pattern. The latter is discussed at some length, for it appears that the inter-axonemal organization is somewhat modified by the colchicine treatment.

INTRODUCTION

In the first paper in this series (Tilney & Porter, 1965) we reported on the fine structure of Actinosphaerium and suggested that the microtubules which make up the central core or axoneme present within each axopodium played a cytoskeletal role, being in fact responsible for the formation and maintenance of these long protoplasmic extensions. In order to test the validity of this hypothesis, we undertook a series of experiments, reported in the second and third papers in this series in which we applied to Actinosphaerium two antimitotic agents, namely low temperature (Tilney & Porter, 1967) and hydrostatic pressure (Tilney, Hiramoto & Marsland, 1966). From the literature, we knew that both these agents affected the integrity of the mitotic apparatus. Since large numbers of microtubules are found in positions occupied by both the spindle fibres and the axonemes, and since in both places the

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microtubules appeared morphologically identical, we reasoned that these agents might be helpful in determining the function of the microtubules. If indeed the microtubules are involved in the formation and maintenance of these linear cell extensions, then the axopodia ought to retract when agents are applied which cause the breakdown or disassembly of the microtubules and not reform without the simultaneous appearance of microtubules in the reforming processes. That is indeed what was observed.

It is rather surprising that other experiments of this kind have not been carried out on cells in the light of the extensive literature now available on the distribution of microtubules. Most of the published reports are concerned either with merely reporting the presence of these elements, or suggesting that they may function in motility (either cell movement or cytoplasmic streaming), or as skeletal elements, but none, to our knowledge, have tried to prove their contentions more rigorously.

In this paper we report on the action of yet another antimitotic agent on the microtubules and the axopodia of Actinosphaerium, in hopes of further substantiating a causal connexion between the microtubules and the formation and maintenance of these cell extensions. As before we have used as a model system Actinosphaerium, for its needle-like pseudopodia provide a particularly well-suited object for such studies. This agent, colchicine, is perhaps better known than low temperature and hydrostatic pressure, for it has been used for many years to block mitosis. Pernice (see Eigsti, Dustin & Gay-Winn, 1949, and Eigsti & Dustin’s (1955) book for the detailed history) first noted its effect on cells in metaphase in 1889, and since then numerous investigators have reported on its effect on the spindle fibres (Inoué, 1952). Included also in this report are observations on what is believed to be the breakdown products of the microtubules as well as observations on the reorganization of pattern following the colchicine treatment. The latter is particularly interesting in relation to the recent studies of Stubblefield & Brinkley (1966), where treatment of Chinese hamster fibroblasts with colcemid, a derivative of colchicine, appears to induce ciliogenesis.

MATERIALS AND METHODS

Organisms

Living specimens of A. nucleofilum (Barrett) were obtained from Carolina Biological Supply Company (Burlington, North Carolina) and were grown in a wheat culture medium (Looper, 1928) to which ciliates were added as a food source.

Treatment with colchicine

Since protozoa are quite impermeable to colchicine (Eigsti & Dustin, 1955), it is not surprising to discover that large doses of colchicine must be used to achieve a demonstrable effect, 0.1% being the minimum dose. Since this amount was not always effective, we generally used 1% colchicine dissolved in pond water. To this, specimens were added bringing the final concentration down to about 0.5%. In order to dispel all fears that these high levels of colchicine might act as an osmotic shock, we
measured the osmolarity of a 1% solution of colchicine and found it to be approximately 25 m-osmoles. An equivalent osmolarity produced by the addition of sucrose to pond water has no effect upon *Actinosphaerium*.

To investigate recovery following colchicine treatment, we washed the specimens several times with pond water and let them recover in Petri dishes containing fresh pond water. The organisms were treated with colchicine for 35 min, a time after which no axopodia remained.

**Light-microscopic procedures**

Cells were observed during the colchicine treatment with bright-field, phase-contrast, Nomarski interference or polarization microscopy. The Zeiss universal microscope, equipped with Zeiss optics, was used. For polarization microscopy we found that the ×16 objective and a compensator setting of 3° proved optimal.

**Electron-microscopic procedures**

Specimens were fixed for 1½ h at room temperature in 3% glutaraldehyde in 0.05 M Sorenson's phosphate buffer at pH 7.0 to which 0.0015 M CaCl₂ had been added. The cells were washed briefly in two changes of 0.1 M buffer to which the CaCl₂ had been added and post-fixed for 1½ h in 1% OsO₄ in 0.1 M buffer at 4 °C. They were dehydrated rapidly and embedded in Epon 812. Sections were cut on a Servall MT2 Porter-Blum ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate and examined with an RCA EMU 3 F or a Siemens Elmiskop I electron microscope.

**RESULTS**

*Observations on the axopodia of untreated living specimens*

A central core or axoneme is present within each axopodium, extending from the tip of the axopodium to the medullary region of the cell body (Fig. 1A). This structure is birefringent.

Two types of particles, mitochondria and dense granules, the latter believed to be involved in prey capture (for a complete description, see Tilney & Porter, 1965), stream up and down the axopodium peripheral to the axoneme but within the limiting plasma membrane. These particles have characteristic movements; they appear to move in linear tracks, each track being separated from an adjacent one by 0.3 μ or less. The separation is sufficient to allow particles to pass one another going in opposite directions with no apparent interference in their velocities. But what is most unusual about the motion of these particles is that they are constantly changing speed and direction; they will move centripetally for a few seconds, then stop, fidget for a variable length of time, and then either continue in the same direction or reverse direction and move centrifugally. All this is carried out in the same track, although they are able to switch tracks during their ‘fidgeting’ movements. (These observations were made using phase-contrast or Nomarski interference microscopy. The ×97 objective was employed.)
Observations on colchicine-treated living specimens

Effect upon the axonemes and axopodia. A diminution in the amount of birefringence in the axonemes can be seen within 5 min after the application of the colchicine. By 10 min this is particularly striking, not so much in the portion of the axoneme present within the cell body but in that portion of the axoneme that remains in the axopodium. The length of the axoneme is reduced as well, so that by 10 min most of these structures are less than one half their untreated length. The length of the axopodium follows closely the length of the axoneme such that a 50% reduction in axonemal length shows a concomitant reduction in axopodial length.

By 20 min few axopodia remain and all that is left of the axonemes are their short bases, which are confined to the cell body (Fig. 1B). Extra axonemal bases or bases with an amount of birefringence greater than what was present before colchicine treatment were never observed. Rather the axonemal bases are less birefringent than they were before treatment. The few remaining axopodia are never more than one half their untreated length. A slender birefringent axoneme is present within each, extending centripetally into the medulla.

By 1 h no axopodia are seen. The axonemal bases remain, however (Fig. 1C). The orientation of these bases relative to each other and to the free surface of the organism appears to be altered in some cells; in others these bases remain oriented.

The effect upon streaming in the axopodia. The motion of particles within the axopodia is not interrupted by treatment with colchicine; the particles continue to move in tracks, often stopping, fidgeting, and reversing direction. As the axopodia begin to withdraw, the time that each particle spends moving centripetally increases even though individual particles can move centrifugally for several microns while particles in adjacent tracks are moving toward the cell body. In this way the streaming granules accumulate in the cortex of the cell body. Even after 15 min in colchicine, a time when few of the axopodia remain, streaming particles can still be found in the remaining cell extensions. These particles are sparsely distributed, however, such that spaces of 20 μ or more between particles are not uncommon.

Alterations in organelle distribution. We have already pointed out that after 1 h in colchicine the axonemal bases in some of the cells do not retain their radial orientation. Likewise the nuclei can shift position, some even being found near the surface of the cell. (The latter observation is most clearly made with Nomarski interference where the nuclei can be easily distinguished from small vacuoles of comparable size.) Distinctions between the cortex and medulla become increasingly difficult to make. The vacuoles have increased in size and thus cells imprisoned by a coverslip become increasingly flattened. There seems to be a reduction in vacuole number as well. With even longer periods in colchicine the vacuoles continue to increase in size, eventually rupturing.

The production and behaviour of colchicine-induced 'knobs'. Short rounded projections or knobs were occasionally encountered in untreated cells at the point of intersection of the base of an axopodium with the cell surface. These structures, which measured about 1 μ in length, became more prominent both in number and
Colchicine and microtubules of Actinosphaerium

in external dimensions during treatment with colchicine, often attaining a length of 2–3 μ and a width of 1–2 μ (Fig. 2). Their shape undergoes constant alterations, from simple finger-like projections to highly complex multi-branched structures; the latter are connected to the organism proper by slender stalks. The movements of these knobs are reminiscent of the movements of the pseudopodia of amoeba. Birefringence could not be detected within these knobs, the only birefringence associated with them being a property of the limiting membrane. Particles stream into, whirl around, and return from these knobs. They are not confined to tracks as in the axopodia.

Electron-microscopic observations

Ten minutes of colchicine treatment. By this time most of the axonemes and axopodia are one half their untreated length, although some are even shorter. Transverse sections through the axopodia reveal, in most cases, an axoneme and peripherally situated granules and mitochondria. If sections are cut near the base of the axopodia so that the number of microtubules in treated and untreated cells can be reproducibly compared, then it becomes apparent that the number of microtubules in treated axopodia has been reduced. In a few cases we found only a portion of an axoneme remaining, almost as if half had been surgically removed (Fig. 3). Since serial sections were not cut, it is unclear if these sections of ‘half axonemes’ represent specific spots along the axoneme, where the tubules had depolymerized locally, or if the ‘half axonemes’ extend the full length of an axopodium. Small numbers of microtubules not associated into the double coil and situated near the limiting membrane were present, as in untreated cells.

In sections cut near the cell surface, we frequently encountered processes (Fig. 4) which contained a central mass of a finely filamentous material and peripherally situated dense granules. No microtubules were found in these processes. We do not know if these profiles represent sections through a knob or an axopodium in the final stage of retraction. The latter appears more likely for two reasons: first its large diameter, too large for a ‘typical’ knob, and secondly its similarity to the ‘beads’ described during pressure-induced retraction of the axopodia (Tilney et al. 1966). It is probable that the finely filamentous material results from the breakdown of the axoneme.

The cell surface in section is irregular; some of its angulations must correspond to the knobs visible by light microscopy. There is nothing unusual about these irregularities, most of which contain the dense granules, and all of which are completely lacking in microtubules. The dense granules and mitochondria which, in untreated specimens, stream up and down the axopodia concentrate in the cell cortex, upon retraction of the axopodia, in two well-defined layers. The former are invariably found just beneath the limiting membrane with the latter occupying a slightly deeper position. (Fig. 5, although taken from a cell treated with colchicine for 20 min rather than 10 min, illustrates this point clearly.)

Alterations in the spacing and morphology of the microtubules in a few of the axonemes in the cortex of the cell were observed after this brief exposure to colchicine. These early changes were particularly noticeable in longitudinal section, where
some of the tubules presented an irregularly curved, almost undulatory profile. This feature becomes more pronounced with longer periods in colchicine, as will be mentioned in more detail below.

The medulla is morphologically indistinguishable from that of untreated specimens.

**Twenty minutes of colchicine treatment.** By this time most of the axopodia have fully retracted. On one occasion we managed to obtain a section through one of the few remaining axopodia. Within the centre of it was an axoneme composed, as usual, of microtubules arranged into the double-coiled configuration; peripheral to this axoneme were several dense granules. The only other processes that remained were processes extending for very short distances from the free surface of the cell body. These contained dense granules and a central mass of finely filamentous material; they probably represent sections through the knobs.

The cortex becomes more distinctive at this stage (Fig. 5). Unlike the untreated cells where the surface cytoplasm (that is, the cytoplasm that borders the free surface on one side and the outermost vacuoles on the other) is very thin, generally less than 1 μ in thickness, the surface cytoplasm of colchicine-treated specimens is thicker. This thickened region appears to result from the accumulation of the axopodial cytoplasm which, as was mentioned in the preceding stage, becomes stratified. What produces and maintains this stratification is by no means clear.

The axonemes which remain, with the exception of those infrequent ones which are found extending out into the few short axopodia, extend from the cortex into the medulla. As in the untreated specimens their medullary ends are either found in a pocket in one of the nuclei or lying free, adjacent to one of the nuclei. The cortical ends seldom penetrate the thick surface layer of cytoplasm.

Most prominent and of greatest interest are the changes in the axonemal microtubules. These elements, at least in the cortical portion of the axoneme, no longer present straight, linear profiles when viewed in longitudinal section, but rather appear curved, almost undulatory (Fig. 6). Furthermore, the spacing between tubules is no longer uniform so that adjacent microtubules, when examined in longitudinal section, will at one point be separated by distances of 50 Å or less, while at points 0.1 μ further down their length will be separated by distances of 250–300 Å (Fig. 6). Thus a cross-section of an axoneme will show some of the microtubules cut normal to their long axes, others at a variety of oblique angles. (This point is well illustrated in Fig. 7, even though this is a section from a cell treated with colchicine for 1 h.) These alterations in the linearity of the microtubules are generally not present in the medullary portion of the axoneme.

Small regions about 1 μ in diameter and composed of a finely fibrillar material were found in many of the cells. (Fig. 8, even though from a cell after 1 hr of colchicine treatment, shows an example of such a region.) These regions were not membrane-delimited although small bits of membrane were sometimes seen around portions of them.

**One hour of colchicine treatment.** All the axopodia have been retracted and many of the vacuoles, both in the cortex and in the medulla, have increased in size although decreased in number. In a few instances, in fixed specimens, we noted that the
vacuoles were virtually absent, and the organisms flattened. Since vacuole-less organisms were not found prior to fixation it is possible that the total loss of vacuoles resulted from the fixation procedure. The specificity of the dense granules for the layer of cytoplasm nearest the free surface is reduced so that some are present even in the deeper layers of cytoplasm.

The microtubules in the axoneme appear irregularly curved along their entire length. When the axonemes are examined in cross-section, they can still be resolved into the twelve sectors (Fig. 7), a characteristic of untreated cells (Tilney & Porter, 1965). The spacing of adjacent rows in each sector is nearly constant for that sector, but comparisons between adjacent sectors in the same axoneme show considerable variation (see arrows, Fig. 7). Thus each sector appears to behave as a unit.

In some axonemes many of the microtubules in the double-coiled configuration appear to have broken down or possibly to be in the process of breaking down, for one finds in cross-sections only a portion of the wall of the tubule (Fig. 9) in the position normally occupied by a microtubule. These tubule fragments have an identical wall thickness to that of untreated microtubules and generally retain the curved profile of a tubule. In some cases no sign of the former tubule remains, there is only an amorphous material distinguishable from the ground substance of the cytoplasm. Of particular interest is the fact that axonemes, which clearly appear to be breaking down, as in Fig. 9, contain tubules which measure about 320 Å in diameter rather than 220 Å in diameter. A similar type of tubule was found in cells treated with low temperature (Tilney & Porter, 1967).

Droplets, 1–2 μ in diameter, which contain a finely fibrillar material are found dispersed throughout the cytoplasm (Fig. 8). These droplets are incompletely membrane-limited and are most frequently located midway between the cell surface and the central vacuole.

Recovery following colchicine treatment

Within 1/2 h of removal from colchicine the axopodia begin to regrow. Within each reforming axopodium a birefringent axoneme could be identified, extending from the tip of the reforming axopodium into the medullary region.

Movement of the whole organism over the substrate begins when the axopodia have regrown to about one half their normal length; they appear to be incapable of movement when the axopodia are withdrawn. The possible mechanism of this movement has already been discussed (Tilney & Porter, 1965).

Cytoplasmic streaming in the axopodia begins very early in axopodia formation, in fact, particles move out into the axopodia as these structures grow. As in the untreated specimens, only the dense granules, mitochondria, and small vacuoles move out into the axopodia.

Specimens were fixed when the axopodia were a fraction of their untreated length. In all cases sections cut through the axopodia revealed arrays of microtubules; peripheral to these tubules were mitochondria, dense granules, or vacuoles which contained a portion of a dense granule. In most cases microtubules in the axopodia were not organized into the double-coiled configuration but rather were found in
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curved rows (Fig. 11). The spacing of adjacent microtubules within each row was uniform. Furthermore, between rows, we frequently found an amorphous layer similar in characteristics to the layer which exists between rows in the axonemes of untreated cells. The presence of rows of microtubules and the amorphous layer between the rows suggests that this might be an initial stage in axoneme development. As will be described shortly, such an axonemal pattern is never found in the cell body where microtubules, when associated, are invariably found in the double-coiled configuration. Occasionally we found axonemes in axopodia in which the microtubules were organized into the double-coiled configuration of untreated cells. An example of such an axopodium is illustrated in Fig. 10. Small numbers of microtubules were found peripheral to the axoneme proper.

The microtubules in longitudinal sections cut through axonemes, whether it be deep within the cell body or through the short regrowing axopodia, appear perfectly straight, never bent or wavy as during colchicine treatment (Fig. 12). In transverse section through the axonemes of the cell body the microtubules are organized in the double-coiled configuration, although individual microtubules in this double coil are either completely missing or only portions of the wall of the tubule are found (Fig. 13). In this respect these axonemes appear similar to the picture one gets during prolonged colchicine treatment (Fig. 9), although the diameter of the tubules is different during recovery, each measuring about 220 Å. The spacing of the microtubules in the axonemes and their organization into twelve sectors was identical to the controls. In the medullary region, small granules (these include both ribosomes and slightly larger granules of unknown function) and small vesicles were occasionally found within the axoneme proper, between adjacent rows of microtubules (Figs. 13, 14).

We were struck by the large numbers of microtubules in the medullary region and in a deeper portion of the cortex. Some of these were unassociated with one another, but the majority were grouped into axonemal configurations. As shown in Fig. 14, up to twelve axonemes could be found near a single nucleus. Such a number was never seen in untreated cells, where a maximum of two or possibly three were found in the neighbourhood of a nucleus. The number of microtubules not associated into axonemes was also greater than in untreated cells; in most instances these 'free' elements parallel the axonemes.

In most instances all the axonemes within a single field coiled in the same direction, albeit clockwise or counterclockwise (Fig. 14). In a few cases we found axonemes coiling in opposite directions in the same section; in these cases the clockwise and counterclockwise coiling axonemes were scattered together.

In the first paper in this series (Tilney & Porter, 1965) we demonstrated that the axonemes were organized relative to each other, such that the plane of bilaterality produced by the region of overlap between the two intertwining rows of the double coil of one axoneme was approximately parallel to the plane of bilaterality of its neighbouring axonemes. In the organisms recovering from colchicine treatment this inter-axonemal organization was absent, so that the planes of bilaterality of adjacent axonemes were randomly positioned relative to each other (Fig. 14).
It is, at the present time, not clear where the axonemes first form. Do they, for example, form near the nucleus or in the cell cortex? And, during recovery, are the axoneme bases, which remain during the colchicine treatment, broken down entirely or are the microtubules, which compose them, unbent and added on to? We cannot answer these questions definitively, but we have determined that during recovery the microtubules found within axonemes were never observed to be bent, nor did the spacing between rows appear to vary. Furthermore, the increase in axoneme number was most obvious in the medullary region, and in particular near the nuclei. Tiny axonemes consisting of a half-turn or less (Fig. 15) were common around nuclei, never separated more than 1 μ from the nuclear envelope. It is not unreasonable, therefore, to expect the axonemes to reform in the medullary region.

As during colchicine treatment, we encountered many large, incompletely membrane-limited droplets (Figs. 8, 16). Each of these droplets contained a finely fibrillar material and a few granules, each about 300 Å in diameter, nothing else. In recovering cells, some of these filaments appeared as if radiating from the granules (Fig. 16). When one carefully examines a forming axoneme in the medullary region (Fig. 17), one finds a greater concentration of this finely fibrillar material in this region than throughout the rest of the cytoplasm. In particular, this material was found near the dense material which signifies the basal end of the axoneme. The possible significance of these observations will be discussed.

**DISCUSSION**

*The role of the microtubules in the formation and maintenance of the axopodia*

From these observations and the fact that the plasma membrane by itself in no instance is known to support rigidly long protoplasmic extensions, we conclude that the microtubules are related not only to the maintenance of these needle-like processes but also to their growth. Therefore, as observed, if the microtubules are caused to disassemble, these extensions ought to retract and not reform until these tubular units reassemble. These conclusions are in agreement with other studies on Actinosphaerium in which we showed that other agents, also antimitotic in nature, namely hydrostatic pressure (Tilney et al. 1966) and low temperature (Tilney & Porter, 1967), give similar results.

In order to test the hypothesis that the microtubules are important in the production of cell shape in multicellular systems as has now been demonstrated in the heliozoan *A. nucleofilum*, Tilney & Gibbins (1966) undertook experiments on the formation and differentiation of primary mesenchyme cells of *Arbacia punctulata*. From their experiments it appears that, as in Actinosphaerium, the microtubules in this multicellular embryo are related to the production of cell shape. It seems reasonable to predict that in many other cell types as well, the microtubules may be influential in the determination of cell shape.
Microtubules, cytoplasmic movements, and the plasmagel

We suggested in the second paper in this series (Tilney & Porter, 1967) that the microtubules give shape and orientation to the plasmagel with which they are co-existent. By doing so they may define regions of solation, regions in which cytoplasmic movements can take place. In the axopodia of Actinosphaerium, individual particles appear to be restricted to 'tracks', such that their movements are linear yet bidirectional. In fixed preparations of axopodia we have invariably found small numbers of microtubules peripheral to the axoneme proper (Tilney & Porter, 1965). From the distribution of these extra-axonemal tubules it is not unreasonable to suggest that they might be instrumental in the regulation of granule movement in tracks. The results of this study strengthen this interpretation, for extra-axonemal tubules are found in the retracting axopodia and during retraction the particles continue to stream in 'tracks'. Another example of movement of granules in 'tracks' has been described in the melanophores of teleosts (Bikle, Tilney & Porter, 1966; Green, 1966; Rebhun, 1963), where, as in Actinosphaerium, the microtubules are also present in orientations in which they could regulate the granule movement (Bikle et al. 1966; Green, 1966).

A second type of streaming is the movement of particles into and out of the knobs. Whereas this is also a complex movement, it is more closely related to a general flow of cytoplasm than to the motion of individual particles in 'tracks'. Similar knob-like processes and movement of particles within them have been described by Mizurski (1949) in colchicine-treated fibroblasts in tissue culture. Since in living Actinosphaerium the knobs show no birefringence which could be attributed to an axoneme, and since thin sections of regions presumed to be cut through the knobs show the complete absence of microtubules, we must conclude that these tubular units are not involved in either the formation of the knobs or the motion of particles within them.

To summarize then: we appear to have two types of cytoplasmic movements in Actinosphaerium, one of which appears to be completely independent of the microtubules.

Possible sites of interaction of microtubules with colchicine which relate to abnormalities in the spacing and the linearity of the microtubules not fully disassembled by the colchicine

Within 10–15 min after the application of colchicine we observed that some of the microtubules in the axonemes of the cell body were no longer absolutely straight as in untreated specimens, but presented an irregular, almost wavy profile. This characteristic grew increasingly marked with longer periods in colchicine, so that after 1 h most of the axonemal tubules even deep in the medullary region presented irregular contours. We have seen no morphological connexion either between the microtubules within each coil or between the microtubules in adjacent coils. It is likely that connexions exist, for in untreated cells the spacing between the microtubules in the axoneme is precise. Furthermore MacDonald & Kitching (1967) have recently claimed to have seen connexions both between tubules in each coil and between the tubules in adjacent coils.
The observations on the non-linearity of the microtubules during colchicine treatment are best explained by assuming that this plant alkaloid bonds to the ligands connecting the microtubules to each other when they are associated in the form of an axoneme, thereby affecting the spacing of the tubules relative to each other. This of course does not rule out the possibility that colchicine might bond to both the tubule proper as well as to the ligands. (Furthermore, colchicine may bond to other regions in the cell, as, for example, the microtubule-initiating system which is considered in the last section of this discussion.)

Products of microtubule disassembly and their behaviour during microtubule reformation

Examination of processes presumed to be retracting axopodia shows that within the position formerly occupied by the axoneme is a finely fibrillar material. This fibrillar material appears irrespective of the agent causing the retraction, whether it be low temperature (Tilney & Porter, 1967), hydrostatic pressure (Tilney et al. 1966), or colchicine. Similar fibrillar material is present within the cell body, where it concentrates in globules approximately 1 μ in diameter. In cells subjected to low temperature this material, referred to as ‘matrix substance’ (Tilney & Porter, 1967), seldom concentrated into globules in the cell body but rather was dispersed throughout the cytoplasm. We suspect that the tendency of this finely fibrillar material to aggregate in the form of globules is related to the speed of axoneme breakdown; if it is rapid, the tendency is high as in the hydrostatic pressure and colchicine experiments; if it is much slower as with low temperature, then this material tends to disperse throughout the cytoplasm.

One wonders whether this finely fibrillar material is a product of microtubule disassembly or if it is something else, perhaps related to the amorphous layer, which in favourable preparations is always found between adjacent rows of tubules in the double coil. That it is related to the axoneme seems reasonably clear, for this material appears directly after the axoneme or a portion of the axoneme has broken down and in an intracellular location presumed to be just vacated by the axoneme. Furthermore, it is absent in fully recovered specimens or in untreated cells. The only indication that this material may be derived from the tubules themselves rather than from the material between rows in the axoneme comes from the work of Pease (1963) and Barnicott (1966), in which it was shown that the ends of the microtubules, when dried on a grid and negatively stained, fray at their tips giving rise to fine fibrils each about 40–50 Å in diameter. If these fibrils were to become jumbled following disassembly of the microtubules, they would ultimately take the appearance of the material seen in these globules. During recovery, similar fibrillar material was present in considerable amounts in close association with the basal ends of the axonemes near a dense material that seems to signify the basal tips of the microtubules. This distribution lends support to the notion that the fibrillar material is used in the assembly of the axonemes, perhaps even in the assembly of the microtubules themselves.

After rather lengthy exposure to colchicine, some of the axonemes which had remained during the treatment appeared to be breaking down. Our observations consisted of finding incomplete or C-shaped tubules in the position in which a micro-
tubule should have been. Furthermore, the remaining tubules in these axonemes had increased in diameter and now measured approximately 320 Å. In an earlier paper, Tilney & Porter (1967) found similar ‘enlarged’ tubules in cells in which the axonemes had been broken down by low temperature, and suggested a mechanism whereby the 220-Å microtubule could transform into these enlarged tubules. Since similar types of tubules have been found in cells whose axonemes break down with a chemical agent as with low temperature, we suggest that these enlarged tubules may be a frequent disassembly product of the microtubules. The finely fibrillar material or the matrix substance may be a further disassembly product, the 320-Å tubule being only an intermediate stage in the breakdown process. The ‘incomplete’ or C-shaped tubules were found not only during disassembly, but also when the axonemes were reforming. The presence of these incomplete tubules, in both cases, suggests that we are describing stages in the disassembly and reassembly of the microtubules. Behnke (1967) has recently described similarly appearing C-shaped tubules during treatment of blood platelets with low temperature. An alternative interpretation is that these incomplete tubules result from the effect of the fixative on microtubules which for one reason or another are unstable (in the case of the colchicine treatment, the alkaloid had already affected the tubules by altering their linearity, and in the case of axopod reformation, we are catching the tubules in an intermediate stage, not in their final polymerized condition).

Recovery: the reformation of pattern

One of the most interesting aspects of these studies centres around the reformation of pattern both within an axoneme and between adjacent axonemes. It was pointed out in the first paper in this series (Tilney & Porter, 1965), that not only do the microtubules within each axoneme appear in a highly consistent form, but also pattern exists between axonemes. From this one might expect that sections cut through organisms during recovery would show a similar pattern to that present in untreated specimens. This did not prove to be the case, because the axonemes now appeared to be randomly orientated with respect to each other. Furthermore, many new axonemes had formed, and numerous microtubules unassociated with one another in the axonemal pattern were also present.

One wonders if the pattern of the untreated cell is regained at some future time in the life of the cell or if this colchicine-induced pattern, which does not appear to affect the viability of the organisms, might not be capable of producing clones of new varieties of A. nucleofilum. The studies of Beisson & Sonneborn (1965) and Sonneborn (1964) and the earlier studies of Lwoff (1950) and Tartar (1961) show that the cortical cytoplasm of protozoa contains a pattern which is autonomous. Furthermore, the precise order of development of many eggs such as the mosaic-developing eggs (see Wilson (1925) for details), strongly suggests that the cytoplasm of many cells is highly determined. A loss of this pattern, as appears to be the case in this system after colchicine treatment, may thus be of general interest.

One explanation for the loss of pattern following colchicine treatment may lie in the action of this drug on regions in the cytoplasm which initiate or organize the
polymerization of microtubules. Such regions include the centriole, the basal body, the kinetocore, the midbody, the cell plate, and satellites either associated directly with the centriole or basal body or unassociated with these bodies. At any moment during the life of the cell only a certain proportion of these centres have tubules attached to them; at a subsequent stage, either developmentally (Gibbins, Tilney & Porter, 1967) or during mitosis different centres have tubules attached to them, former centres now being entirely free of tubules. A detailed discussion of these statements can be found in the review of microtubules by Porter (1966) or in the paper by Gibbins et al. (1967).

That these regions or bodies may be affected by colchicine can be seen from the work on chick fibroblasts (Stubblefield & Brinkley, 1966), where cells released from colcemid (a close relative of colchicine) undergo a marked increase in ciliogenesis. Likewise in Actinosphaerium with a production of 2–3 times as many axonemes after colchicine treatment as before, it is possible that one of the actions of the drug may be on a system of centres which influence the pattern of microtubules. Thus this plant alkaloid may affect the duplication of centres leading to increased numbers of cilia or axonemes as well as affecting the position of the centres in the cell. A change in either of these parameters would lead ultimately to alterations in pattern such as those seen in the apparent lack of inter-axonemal organization.

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REFERENCES

GIBBINS, J. R., TILNEY, L. G. & PORTER, K. R. (1967). The role of the microtubules in the formation and development of the primary mesenchyme in Arbacia punctulata I. (Submitted for publication.)
Fig. 1. Micrographs taken with the polarizing microscope of the same organism during treatment with colchicine. The round bright spots are ingested ciliates. This organism became increasingly flattened during colchicine treatment. \( \times 160 \).

1A, 5 min of colchicine; 1B, 25 min of colchicine; 1C, 1.5 h of colchicine.

Fig. 2. A portion of the cortex of an *Actinosphaerium* which had been treated with colchicine for 30 min. Projecting from the surface are small processes or knobs into which small particles can enter. This micrograph was taken with Nomarski interference optics. \( \times 1100 \).

Fig. 3. Transverse section through an axopodium from an organism treated with colchicine for 10 min. Within the limiting membrane is a mitochondrion (m) and a portion of an axoneme. \( \times 59000 \).
Fig. 4. Ten minutes of colchicine. This process was located near the cell body. From its size it appears to be an almost completely retracted axopodium. Within this process are a number of peripherally situated granules (g) and a central mass of finely fibrillar material (J). × 59,000.

Fig. 5. Twenty minutes of colchicine. This micrograph depicts the layer of cytoplasm that separates the external environment from the most superficial vacuoles. It has become thicker at this stage due primarily to the retraction of material which makes up the axopodia. × 37,000.
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Fig. 6. Twenty minutes of colchicine. Section through the cortex of a cell to illustrate the change in the linearity of the microtubules with colchicine treatment. $\times 115,000$.

Fig. 7. Section cut through the medulla of a cell treated with colchicine for 1 h. Few vacuoles remain in this cell which, when examined with the light microscope, appeared flattened. The arrows point to two sectors of an axoneme in which the spacing between rows within each sector is constant, but in which the spacing in the same row of the two sectors is clearly different. $\times 42,000$. 
Fig. 8. One hour of colchicine. Between portions of two nuclei (n) is a mass of finely fibrillar material (s). ×40000.

Fig. 9. One hour of colchicine. Transverse section of an axoneme. Many of the microtubules in this axoneme have broken down so that it is somewhat difficult to envisage the axoneme in its entirety. Portions of the walls of some of the tubules remain. ×127000.
Figs. 10, 11. Sections through axopodia which were reforming following treatment with colchicine. The cells were fixed during the initial stages of axopodial reformation.

Fig. 10. In this nearly transverse section one can distinguish the axoneme, consisting of a double coiled configuration of microtubules, and dense granules (g). Small numbers of microtubules not organized into the double coil are located among these granules or directly beneath the limiting plasma membrane. The double-coiled configuration, although typical of untreated cells occurs uncommonly in these early stages of recovery. × 100,000.

Fig. 11. The organization of the microtubules in this axopodium is commonly found in the early stages of recovery following colchicine treatment. The granules (g) and vesicles (v) most frequently lie near the limiting plasma membrane with the microtubules appearing in rows in the central portion. × 100,000.
Fig. 12. Nearly longitudinal section cut through the cortical portion of a recovering axoneme. × 60,000.

Fig. 13. Transverse section through an axoneme within the cell body of a cell recovering from colchicine. The arrows point to 'incomplete microtubules' in which just a portion of the tubule wall is discernible. × 120,000.
Fig. 14. Section cut through the medulla of an organism in the early stages of reformation of the axopodia. Situated around the nucleus (n) are twelve axonemes. These axonemes look normal with the exception that they are randomly orientated relative to each other. The arrows emphasize this by pointing along the plane of bilaterality induced in each axoneme by the overlap of the microtubules in each of the two interlocking coils. $\times 23000$. 
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Fig. 15. Micrograph taken near the surface of a nucleus (N) during recovery. One frequently finds small axonemes around the nuclei. $\times 100000$.

Fig. 16. Portion of the cortical cytoplasm of a cell recovering from colchicine treatment. Depicted in this micrograph are numerous fine fibrils (i). The arrow points to a granule on which some of the fibrils appear to terminate. $\times 56000$.

Fig. 17. Section through the medulla of a cell in the process of reforming its axopodia. The arrows indicate the tips of the microtubules. The letter (s) indicates a finely fibrillar material believed to be similar to that in Fig. 16. $\times 77000$. 