STUDIES ON THE MICROTUBULES IN HELIOZOA

V. Factors Controlling the Organization of Microtubules in the Axonemal Pattern in *Echinosphaerium* (*Actinosphaerium*) *nucleofilum*

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ABSTRACT

On the assumption that the double-coiled pattern of microtubules in the axoneme of *Echinosphaerium* might be due to links of two sizes between adjacent microtubules, we disassembled microtubules with low temperature and then carefully analyzed the patterns of microtubules that formed upon the addition of heat (22°C) or heat and D_2O. Although most of the initial clusters of microtubules that formed could not be interpreted as part of an axoneme, the spacings between these microtubules were the same as that in the axoneme, 70 and 300 A. By model building we were able to show that all clusters that form, including stages in the formation of the axoneme and its 12-fold symmetry, could be explained by links of two sizes (70 and 300 A) and the substructure of the microtubule. We could demonstrate these links with improved staining methods. We suggest that nonaxonemal assemblies of microtubules may be eliminated by the natural selection of the most energetically stable configuration of microtubules, all others undergoing disassembly under equilibrium conditions. Model building further supports this suggestion since the model axoneme possesses more links per tubule than any other cluster found.

INTRODUCTION

Evidence that microtubules may be of importance in the development and maintenance of biologic anisotropy has been presented in earlier papers in this series (Tilney and Porter, 1965; Tilney and Porter, 1967; Tilney et al., 1966; Tilney, 1968 *a*) and elsewhere (Porter, 1966; Tilney, 1968 *b*; Byers and Porter, 1964; Tilney and Gibbins, 1966). It becomes of considerable interest, therefore, to determine the factors that control the distribution of microtubules, for ultimately these factors must account for the development of the asymmetric form specific for a particular type of cell. In particular, we were interested in investigating the axonemes of *Echinosphaerium* (*Actinosphaerium*) *nucleofilum*, for these structures appear to maintain the asymmetric form of this protozoan. Each axoneme, as demonstrated by Tilney and Porter (1965), is composed of a bundle of microtubules organized, as viewed in transverse section, into two interlocking coils

(Figs. 8–11). The tubules are separated from their neighbors in each coil by 70 A and from those in adjacent coils by 300 A. Further, the axoneme itself has 12-fold symmetry, a fact which is particularly obvious in large axonemes. Thus the microtubules, while remaining in the double-coiled pattern, are arranged into 12 wedge-shaped sectors.

We suspected that this highly ordered pattern of microtubules might result, in large measure, from specific types of interactions between adjacent microtubules. Because the separations between adjacent microtubules in the axoneme are almost consistently 70 or 300 A, we were led to inquire if the axoneme in fact may be assembled by interactions between the microtubules brought about by two sizes of macromolecular bridges linking them together. In order to test this supposition we set out to investigate what clues could be obtained by studying the reassembly of microtubules and axonemes after their disassembly with mild agents, such as low temperature (Tilney and Porter, 1967). It seemed feasible to try to favor the equilibrium conditions for tubule assembly. Under such conditions the microtubule monomer and the postulated linking macromolecules would be induced to assemble rapidly. By careful analysis of the aggregates of tubules thus formed, we hoped that information obtained from "normal" and "abnormal" organizations of microtubules could be interpreted to account for the precise pattern of microtubules in the axoneme. Thus by inducing the organism to rapidly assemble its axonemes we hoped that the earliest aggregates that formed might be uncorrected by whatever mechanisms the cell has at its disposal to eliminate or reorder abnormal patterns, and thus we would be able to obtain information on the factors that normally govern the axonemal pattern. For this purpose we exposed *E. nucleofilum* to low temperature, 0°C, for a period sufficient to insure disassembly of all the microtubules (Tilney and Porter, 1967). We then placed the organisms at room temperature (22°C) or applied deuterated water (D_2O) at 22°C. Both D_2O and heat are known to favor the polymerized state (Tilney, 1968*b*). *E. nucleofilum* was fixed and processed for electron microscopy shortly after the organism had been placed either at room temperature or in the presence of D_2O. Thin sections were then examined for clusters of microtubules.

MATERIALS AND METHODS

The Organisms

Cultures of *E. nucleofilum* were obtained from Carolina Biological Supply Co. (Elon College, Burlington, North Carolina). They were cultured in a wheat medium (Looper, 1928) made up in pond water to which ciliates were added as a food source.

Treatment with Low Temperature

5-ml aliquots of culture medium containing *Echinosphaerium* were pipetted into small Petri dishes (5 cm in diameter). The dishes were placed in an ice-water bath in a 3°C cold room. Small pieces of frozen medium were added to the dishes; this insured that the organisms were maintained at 0°C. The duration of the cold treatment was from 2½ to 3 hr.

Recovery Following Low Temperature Treatment

The pieces of frozen medium were removed, and the Petri dishes were placed on the bench at room temperature. The graph (Fig. 1) shows the rate at which the medium containing the organisms warmed up. (The small size of the organisms obviated any significant differential between their temperature and that of the medium.) These measurements were made with a thermistor, calibrated by using a large volume of water at temperature increments of 5°C. The fixative was added to the Petri dishes at the desired times following removal from the cold.

Experiments with D_2O

Petri dishes containing specimens that had been maintained at 0°C from 2½ to 3 hr were removed

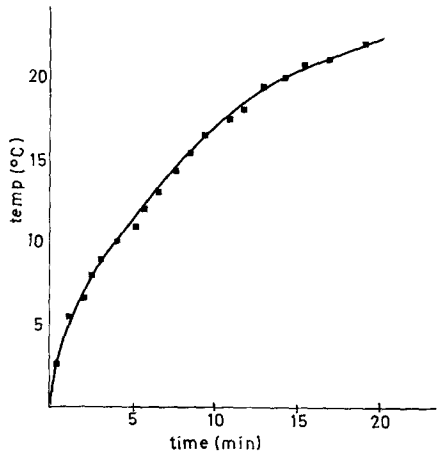

FIGURE 1 Graph depicting the rate of increase in temperature of the culture medium containing organisms plotted against time.

from the ice bath, and D_2O at 22°C was immediately added. Enough D_2O was added to the fluid in the Petri dishes to make the final concentration 70–75%. Just prior to fixation the organisms were concentrated by the removal of most of the liquid. The fixative was then added to the Petri dishes. Subsequently the organisms were pipetted into centrifuge tubes in which further fixation, washing, and dehydration were carried out.

Procedures for Electron Microscopy

Specimens were fixed by the addition of buffered glutaraldehyde to the Petri dishes containing the organisms. At final concentration, dishes contained 2% glutaraldehyde with 0.0015 M $CaCl_2$ in 0.05 M phosphate buffer at pH 7.0. The organisms were fixed in this solution at room temperature for 1–1½ hr. They were then washed in 0.1 M phosphate buffer with 0.0015 M $CaCl_2$ and postfixed in 1% OsO_4 in 0.1 M phosphate buffer for 45 min. They were dehydrated rapidly and embedded in Epon 812. Specimens were cut with a diamond knife on an LKB ultramicrotome, stained successively with uranyl acetate and lead citrate, and examined with a Hitachi HS 7S or Philips EM200 electron microscope. Toward the end of this study, in an attempt to visualize the postulated macromolecular links, we followed the glutaraldehyde or osmium tetroxide fixation with a 3-hr wash in 2% uranyl acetate at 0°C. The organisms were dehydrated rapidly and embedded in Araldite 502. Thin sections were stained for 30 min with 2% $KMnO_4$, rinsed briefly in 2% citric acid, then stained with lead citrate.

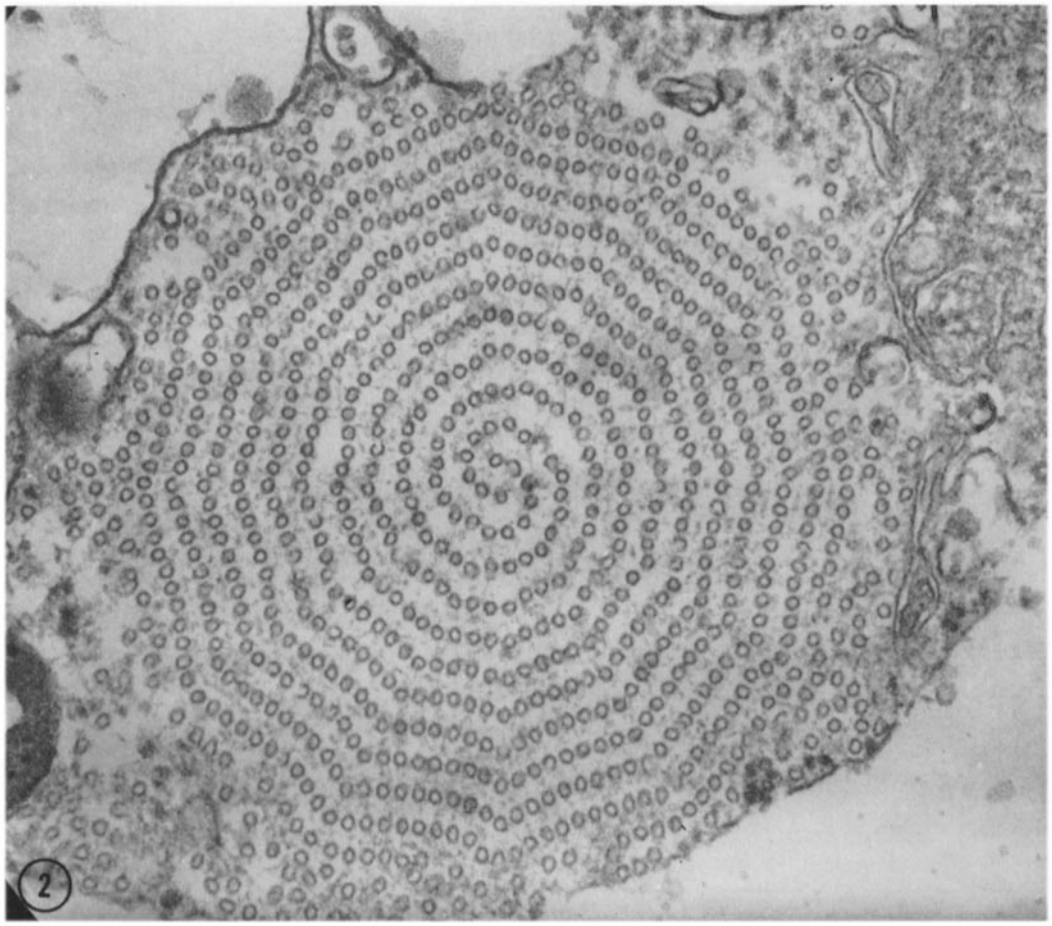

FIGURE 2 Transverse section through an axoneme of *Echinosphaerium*. Evident in this micrograph are the two interlocking coils composed of microtubules and the 12-sided symmetry of the axoneme. At the periphery of the axoneme are a number of microtubules; they are not integrated into the axoneme. × 80,000.

RESULTS

In order to orient the reader we have included a micrograph (Fig. 2) showing a mature axoneme in cross-section. This figure illustrates the two interlocking coils of microtubules; each microtubule is separated from its neighbors in the same coil by 70 A and from the nearest of those in the other coil by 300 A. The 12-fold pseudo-symmetry of the axoneme is apparent in this micrograph.

Figs. 3 and 4 are transverse sections through axopodia of untreated organisms. In the center of each axopodium is a cluster of microtubules, most of which are separated by 70 or 300 A. These clusters obviously do not resemble the typical axonemal structure seen in Fig. 2. Axopodia such as these comprise about 10% of the axopodia of normally cultured organisms.

Recovery Following Treatment with Low Temperature

In the following description of electron microscopic results we describe the distribution of microtubules in cells fixed 4, 8, and 12 min into

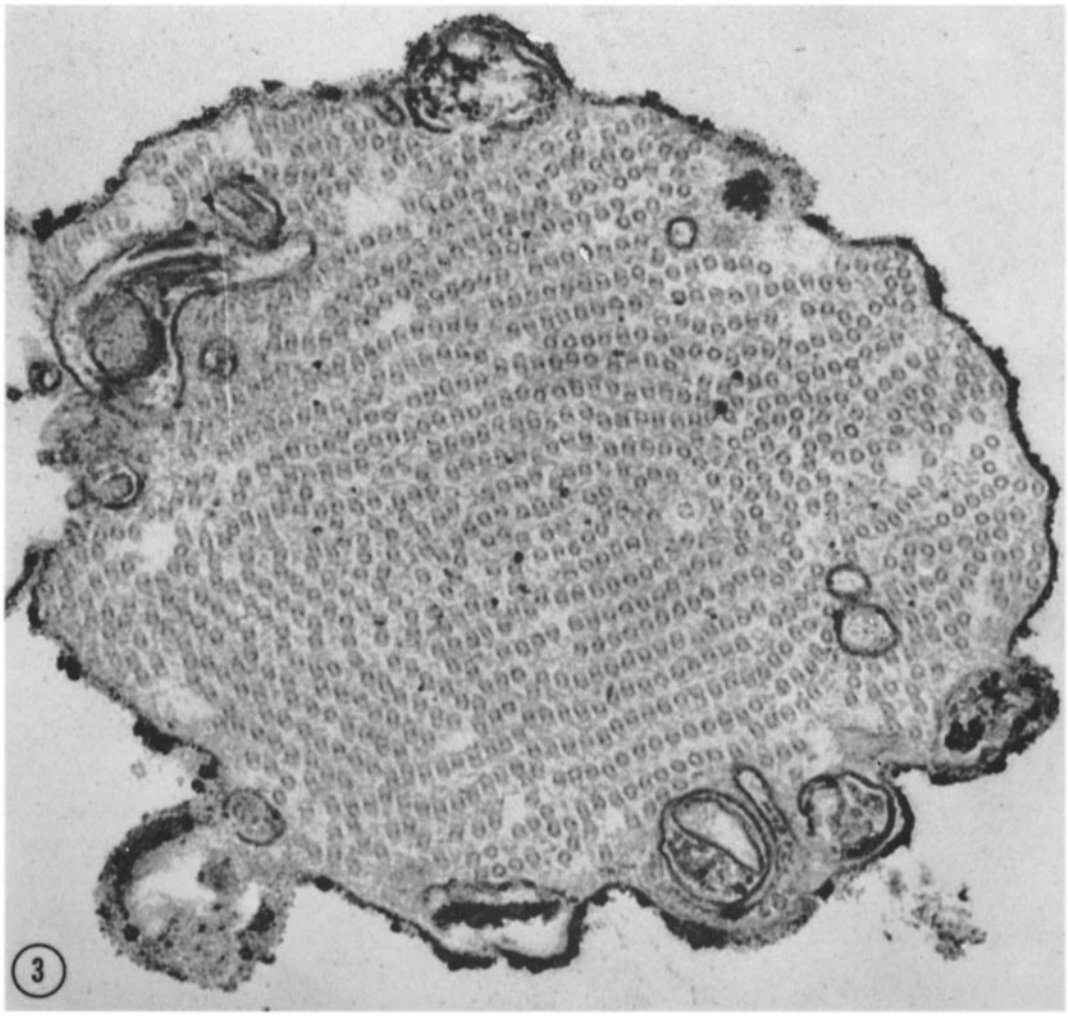

FIGURE 3 Transverse section through an axopodium of an untreated organism. Note the large numbers of microtubules oriented parallel to each other. Although these elements appear in rows, the typical form of the axoneme is absent. × 72,000.

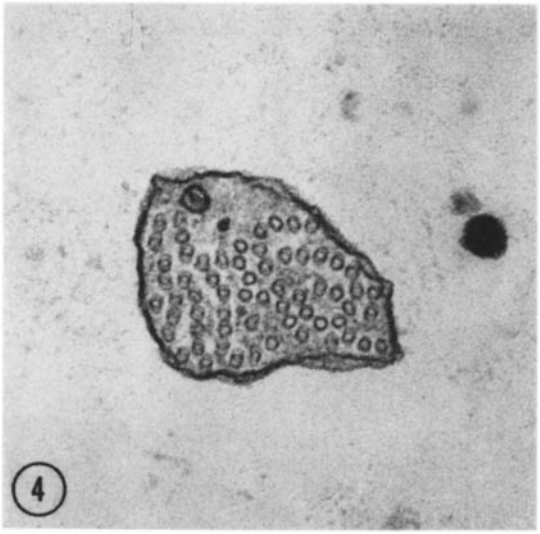

FIGURE 4 Section cut through an axopodium near its tip. The organism was not experimentally treated. × 75,000.

the recovery period. As shown on the graph (Fig. 1) the temperature of the medium containing the organisms was 10°C at 4 min, 15°C at 8 min, and 17°C at 12 min. We should remind the reader that the axopodia retract completely if *Echinosphaerium* is chilled to 4°C or below (Tilney and Porter, 1967). However, if the temperature is reduced to 5°C, the axopodia shorten but do not retract completely. Therefore, organisms 4 min into the recovery period have had actually 3 min in which to initiate the assembly of microtubules, the temperature during the first minute (Fig. 1) being below 5°C. Similarly, organisms fixed 8 and 12 min after their removal from the cold have had only 7 and 11 min, respectively, in which to recover.

4 MIN: Except in the most superficial layer of cortical cytoplasm, microtubules, each about 220 A in diameter, were present throughout the cytoplasm. The arrows in Fig. 5, a section cut through the medulla, indicate these elements. (It should be noted that the extracellular space in the left of this micrograph is a portion of a medullary vacuole, *not* the free surface of the organism.) Frequently the tubules appeared single, but small clusters of two to six were present as well. No pattern could be discerned in their distribution relative to the free surface or to the center of the cell. Representative examples of the clusters are illustrated in Fig. 6. It is convenient to refer to some of these clusters by the geometric figures they present in cross-section. Many of these configurations, such as the isosceles right triangle and the row of three equally spaced tubules with an additional tubule adjacent to the central one, were never found in the axonemes of untreated cells. The axoneme is defined as the double-coiled array of microtubules depicted in Fig. 2. An equilateral triangle of three microtubules was not encountered at this stage. In general the microtubules in the clusters were separated by 70 A. Never did the walls of the tubules approach each other more closely than this distance. It should be remembered that 70 and 300 A are the separations of the microtubules in a mature axoneme. A 300-A spacing, as differentiated from spacings other than 70 A, was rarely found in treated organisms, although an exception to this can be seen in the rhomboid and rectangle (Fig. 6).

8 MIN: As in the preceding stage, microtubules although common were conspicuously absent from the most superficial cortical layer. Generally the microtubules occurred in clusters (Fig. 7), some of which appeared to represent the initial stages in axoneme formation. When they occurred singly or in small clusters, they were unoriented, but in general, when larger clusters of microtubules were encountered, they appeared to be radially oriented with respect to the free surface of the cell and to the cell center. Thus when several axonemes were encountered in the same section, they appeared nearly parallel to one another, although exceptions were found (Fig. 7).

Many of the small clusters resembled those present after 4 min such as the triplets, parallelograms, and rhomboids. Larger clusters occurred as well (Figs. 8 and 9); they included circles which may or may not have tubules in their centers, axonemes in which three pairs of tubules (rather than two) overlap in the center, axonemes in which one or more extra microtubules appear to be added to one of the interlocking coils, clusters in which some of the tubules are cut normal to their long axes and others oblique, and complex patterns of tubules (Fig. 8). Furthermore we found several instances in which two small axonemes, both coiling in the same direction, appeared to be coupled together (see Fig. 8 for an example of one of these). These nonaxonemal assemblies of tubules occurred frequently. Clusters believed to be stages in axoneme formation are depicted in Fig. 9. Of greatest interest, however, is that the

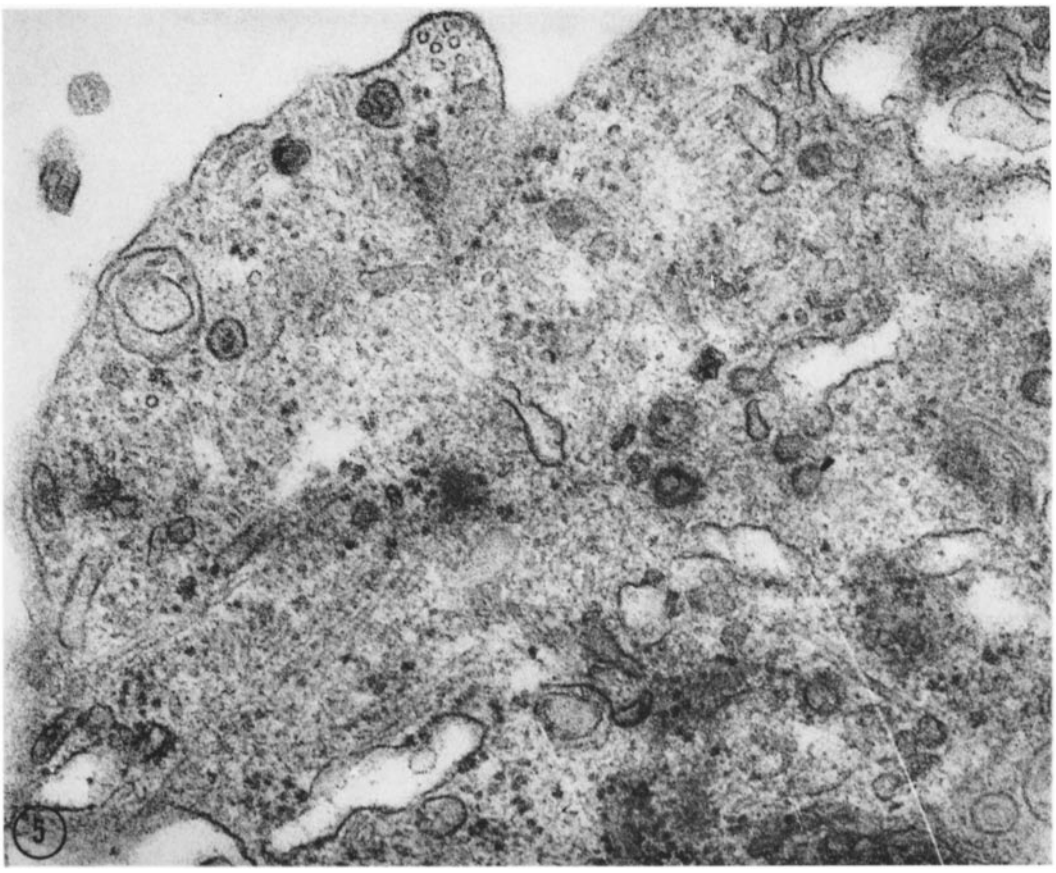

FIGURE 5 Section cut through the cell body of *Echinosphaerium* fixed 4 min after its return to room temperature, following treatment with low temperature. Of particular interest in this section are the large numbers of microtubules (see arrows) randomly oriented in the cytoplasm. A small cluster is seen near the upper margin. The extracellular space on the left hand side of the micrograph is a portion of a vacuole. × 55,000.

tubules in all these clusters, whether or not they resembled axonemes, were generally separated by 70 or 300 A, the same separations found in untreated axonemes. Tubules closer together than 70 A were not found, nor were any tubule clusters in the form of equilateral triangles encountered.

12 MIN: Short axopodia were first visible at this time. Within each was an axoneme composed of organized arrays of microtubules. In several instances the axonemes could be traced from the axopodia to the medullary region. Individual tubules within this structure could be followed for several microns before disappearing from the section. Random arrays of microtubules were also encountered; in some instances they were present at right angles to a formed axoneme. Later stages in axoneme regrowth and axopodial development have already been presented (Tilney and Porter, 1967).

The Effect of D_2O at 22°C on Specimens Treated with Low Temperature

No axopodia reformed in the presence of D_2O regardless of the duration of treatment. If, however, the D_2O treatment was longer than 30 min, the effect on *Echinosphaerium* was irreversible.

10 MIN OF D_2O: Microtubules were scattered throughout the cytoplasm. They were frequently in clusters that were not oriented relative either to the free surface or to other clusters. In longitudinal section the microtubules, even those

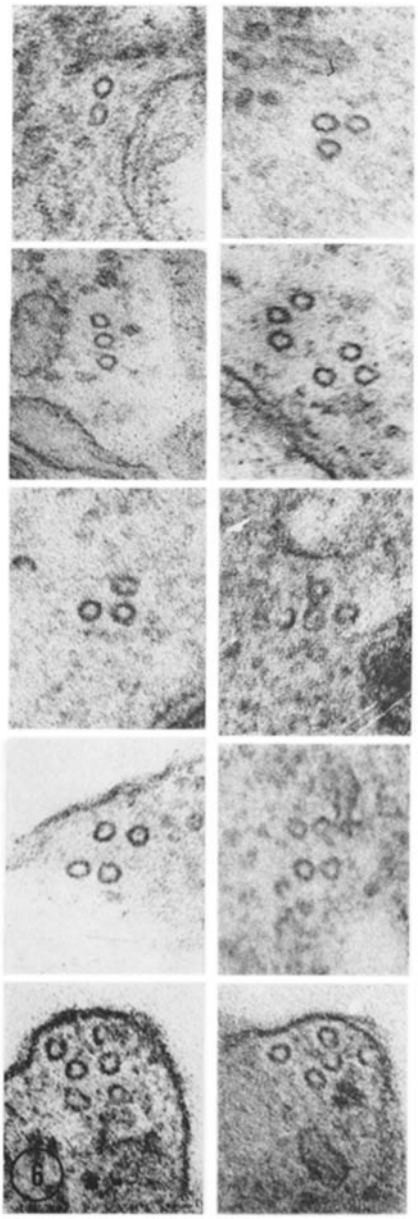

FIGURE 6 These micrographs illustrate clusters of microtubules that are present in cells fixed early in the recovery period (4 min) following treatment with low temperature. Of particular interest are the clusters of three which form a right triangle, the cluster of four in which one microtubule projects from the center of a linear cluster of three, and the bottom two micrographs. None of these configurations could be interpreted as part of an axoneme. × 100,000.

in an aggregate, did not always present parallel profiles. Similarly, in transverse section the spacing between tubules in the cluster was frequently variable, although we encountered pairs, triplets, or short rows of tubules in which the tubules were separated by 70 A. Often other tubules were present in the same cluster or row, but with a spacing greater than 70 A. Tubules were never closer than 70 A. A 300-A spacing did not occur frequently. No axonemes or clusters that might be interpreted as early stages in axoneme formation were found.

15 MIN OF D_2O: Although many microtubules were still randomly dispersed, there was a greater tendency for the tubules to parallel one another. Thus, fields several microns in diameter could be found containing many microtubules oriented approximately parallel (Fig. 10). As before, the tubules were spaced at least 70 A apart. We found clusters which formed the following variety of figures: triangles, irregular circles, straight and curved rows, and rows that appeared to branch and connect to other rows (Figs. 11 *a*, *b*, *d*, *e*, *h*). Many of these clusters formed very complex patterns. In these clusters the spacing between adjacent tubules was 70 A. Several equilateral triangles were found.

35 MIN OF D_2O: By this time larger clusters of tubules were found interspersed between numerous unassociated tubules and smaller clusters similar to those described in the preceding stages. Some of these clusters resembled small axonemes which contained at most 50 microtubules. Whereas the organization of the microtubules in the center of the double coil was identical with that present in axonemes of untreated cells, at the periphery (Fig. 11 *m*) there were marked variations in pattern. Rows of tubules were seen extending at right angles or at oblique angles to a tangent drawn to the last row in the double coil. Furthermore there were numerous examples of tubules which, instead of being separated from the preceding row in the double coil by 300 A, were separated by only 70 A (arrows point to some of these in Fig. 11 *m*), but in almost all instances the tubules in the axoneme were separated from each other by either 70 or 300 A; seldom were intermediate distances found. When more than one axoneme was encountered in the same section, they appeared to be parallel.

We found a number of configurations of tubules that we interpreted as intermediate stages in axoneme development. All of these deviated

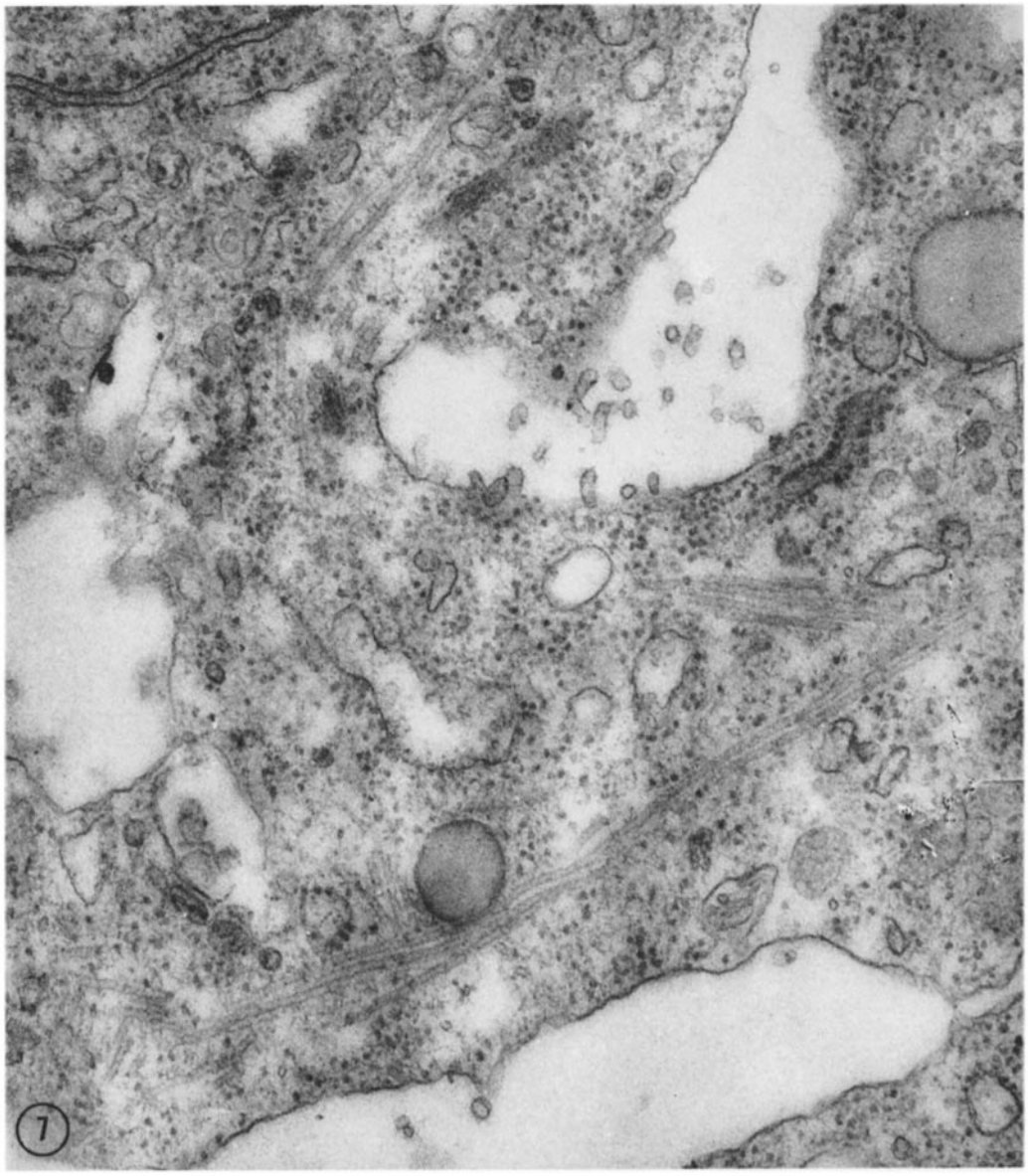

FIGURE 7 Section through the cell body of an *Echinosphaerium*, which, following treatment with low temperature, had been placed at room temperature for 8 min. Clusters of microtubules are apparent in this micrograph. These clusters are randomly oriented with respect to each other. A portion of a nucleus appears in the upper left hand corner of this plate. × 55,000.

from normal small axonemes (many resembled some of the configurations presented in Fig. 6); nevertheless, the tubules were generally separated by 70 or 300 A, although separations of 300 A (examples in Fig. 11 *e*) occurred much less frequently than 70 A separations.

The Presence of Links in Untreated Cells

When we had nearly completed the experimental work for this study we were able to develop better techniques for the staining of thin sections of *E. nucleofilum*. This enabled us to demon-

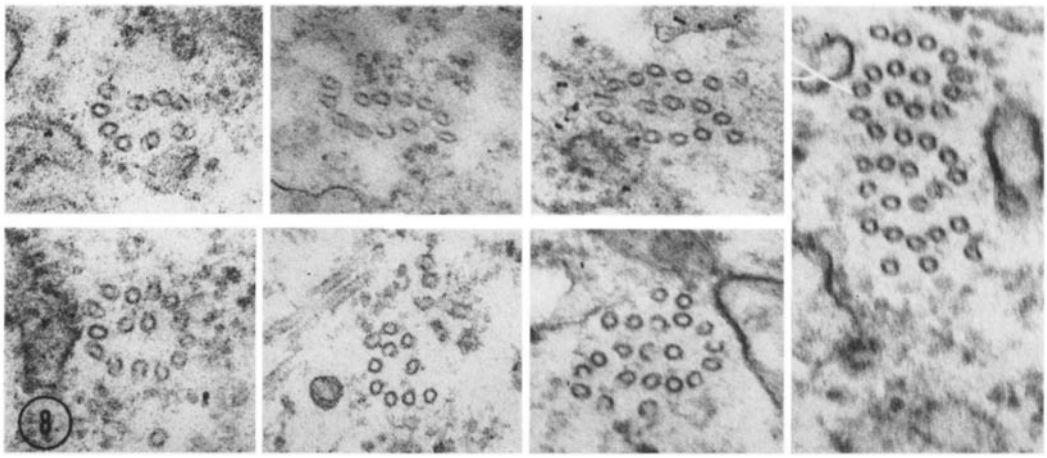

FIGURE 8 These seven micrographs illustrate representative clusters of microtubules which are found in cells 8 min in the recovery period, following treatment with low temperature. All these clusters differ from small axonemes in one or more respects. × 70,000.

strate links of two sizes connecting adjacent tubules. Fig. 12 is a representative example. The long link (300 A) can be seen clearly.

DISCUSSION

The Use of Model-Building to Relate the Patterns of Microtubules Found During Axoneme Reformation to the Substructure of the Tubule and to Links Connecting the Tubules

Before it is possible to draw any conclusions on the factors that might influence, if not control, the pattern of microtubules in the axoneme, we must analyze in detail the clusters of microtubules present in organisms that have been induced to rapidly reassemble axonemes. In particular we were led to inquire whether the morphology of the axoneme might result from interactions between adjacent tubules, interactions that take the form of two sizes of macromolecular linking units (70 and 300 A). Thus by carefully studying the spacing of tubules within the reforming aggregates we might obtain clues about the organization of the axoneme. This analysis can be most easily carried out with scale models that are built to represent sections of the clusters. In these models microtubules are represented by small cylinders about 1 cm in diameter in whose wall are 12 equally spaced grooves (Fig. 13). These grooved cylinders can be joined together by two sizes of plastic links meant to represent the 70 and 300 A spacings. These links also correspond to the links mentioned by MacDonald and Kitching (1967) for the mature axoneme, the short link (70 A) representing their "tangential" link which connects adjacent tubules within each of the two interlocking coils, and the longer link (300 A) being equivalent to their "radial" and "secondary" links which join the tubules in adjacent coils. The radial and secondary links differ by only 20 A, a value that does not seem significant in view of the difficulties encountered in the fixation of the heliozoa (Tilney and Porter, 1965) and in making precise measurements. It will later become obvious that it is not necessary to have two different sizes of long links. The 12 grooves in the cylinders represent the subunits that are thought to make up the wall of each microtubule. Although there is some disagreement about the exact number of filamentous subunits that make up the wall of microtubules, assuming that we are dealing with a similar structure in plant and animal cells (Tilney, 1968 *b*; Porter, 1966), most investigators agree that this number lies between 11 and 13 (Kiefer et al., 1966; André and Thiery, 1963; Barnicot, 1966; Pease, 1963; Gall, 1966; Porter, 1966; Grimstone and Klug, 1966; Behnke and Zelander, 1967). In negatively stained preparations it is impossible to be sure of the number of filaments, for when a tubule is dried on a grid the filaments tend to superimpose. In fortuitous sections cut

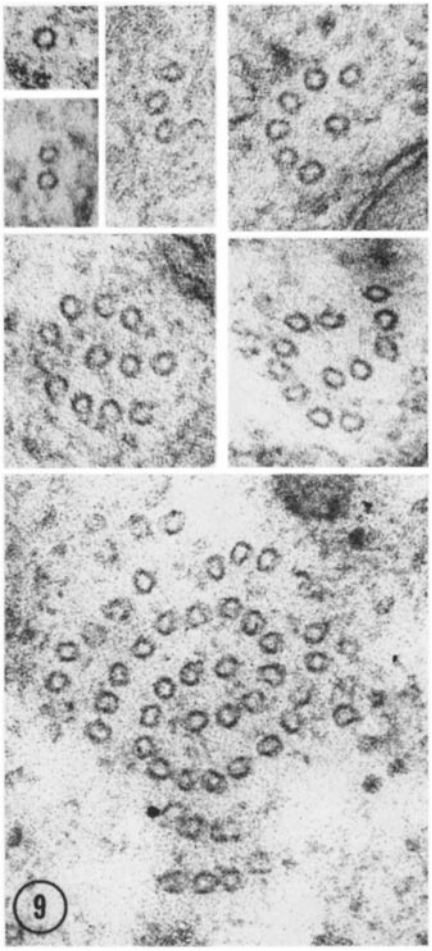

FIGURE 9 In this figure we have included what appears to be stages in the formation of an axoneme. These micrographs were selected from cells fixed 4 and 8 min after removal to room temperature following low temperature treatment for 3 hr × 100,000.

through two higher plant cells, Ledbetter and Porter (1964) were able to demonstrate that the walls of these microtubules are composed of 13 subunits. There is, however, no a priori reason to expect all microtubules to be composed of exactly the same number of subunits. The best available evidence for *Echinosphaerium* indicates that there are 12 rather than 13. Two observations back up this statement. First, the 12- rather than 13-fold symmetry of the axoneme can be interpreted as indicating that there are 12 subunits in the wall of each microtubule of *E. nucleofilum* rather than 13. This point will become more apparent later when we demonstrate that the 12-fold symmetry of the axoneme might result from interactions between the long links (300 A) and tubules of adjacent rows. We suggest that these links attach to different subunits. If each microtubule had 13 subunits rather than 12, and if each link attached to a separate subunit, then the axoneme might more logically show 13-fold symmetry. Second, although they presented no evidence MacDonald and Kitching (1967) stated that "Markham rotations of a limited number of transverse sections of microtubules suggest that the wall of a microtubule comprises 12 subunits." Actually for these models the number of subunits, albeit 12 or 13, is unimportant, as is further discussed later.

In Fig. 14, using epi-illumination, we projected the micrographs of many of the small clusters of tubules illustrated in the Results onto a piece of cardboard. Black circles, meant to represent tubules, were pasted in the exact locations where the tubules were found in the micrographs. Beneath these we constructed models with the units illustrated in Fig. 13. In every case it was possible to construct a model which corresponded closely to the tubule clusters in the micrograph, even though most of these clusters could not be interpreted as being a fragment of an axoneme. We, therefore, concluded that these patterns may well have arisen as a result of the combining properties of the units (tubules and links) themselves.

Additional information can be gained from these models. Since each microtubule appears to be composed of 12 or 13 longitudinal rows of identical filamentous subunits, it would be possible to form maximally only 12 or 13 links around the circumference of a single tubule, assuming, of course, that there is only one link per subunit. Thus links on the same tubule would not form angles smaller than 30° (360/12). (If there are 13 subunits, then the angle would be approximately 28°; a difference of 2° could not be distinguished with the accuracy of our measurements.) Taking into consideration the diameter of a tubule, it would not be possible for two short links on the same tubule to form an angle as small as 30° and also connect to other tubules without having an overlap of the two linked tubules. If the walls of the two linked tubules were to touch, the angle between the links would be 45°. Likewise, if the walls of two tubules that were connected to a third tubule by two long links were to touch, the links on the third tubule would form an angle of approximately 25°. Such situations have never

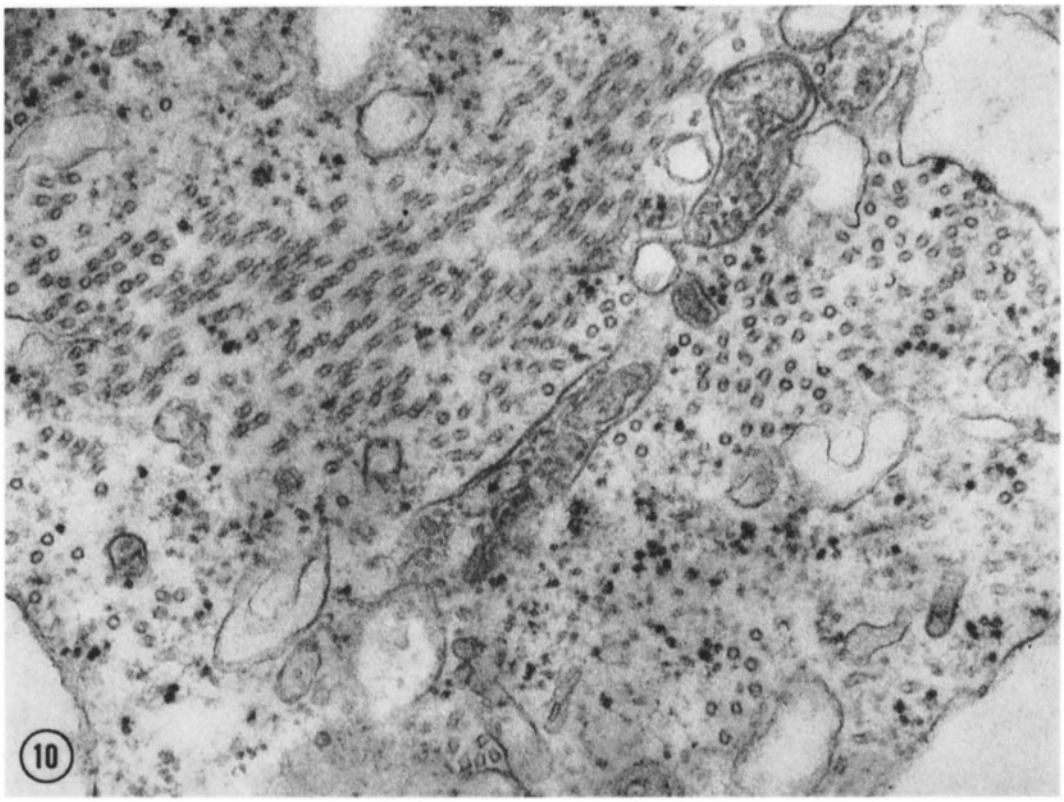

FIGURE 10 Section cut through the cell body of *Echinosphaerium* which, following low temperature treatment, was immersed into 70% D_2O at room temperature. The organisms were fixed 15 min later. Of greatest interest are the large numbers of microtubules present in this micrograph; they lie approximately parallel to each other. × 55,000.

been seen; tubules are always separated by at least 70 A. In Fig. 14 we have illustrated five instances in which different configurations of triplets were encountered. Below these are our models. In each of these triplet combinations different sets of grooves must be used or, in terms of a microtubule, the links are bonding to different subunits. In the other clusters illustrated in Fig. 14, it is also apparent that links on the same tubule would bond to different subunits. In some of these, for example the parallelograms, both short and long links are present.

In Fig. 15 we have copied more complex clusters of microtubules. The corresponding models again demonstrate that these configurations can be constructed with the model units, and thus these tubule patterns can be interpreted as resulting simply from intrinsic properties of the building blocks. In these clusters the characteristic spacing of the long link is more frequent. Some of these clusters resemble what would be interpreted as early stages in axoneme formation, yet they are abnormal in one or more respects. For example, we have illustrated two instances where three rather than two pairs of microtubules overlap in the center of the axoneme, and axonemes in which microtubules diverge from the outermost row of the double coil at a sharp angle to the axoneme proper. We have further included an example of what appears to be two interconnected small axonemes coiling in the same direction. In these cases and in clusters of microtubules that do not resemble axonemes, i.e. circles with or without tubules in the center, links on the same tubule do not form on the same subunit. When two or more long links are present on the same tubule, the minimum angle between the links is 30°. A long and a short link form an angle of at least 60°.

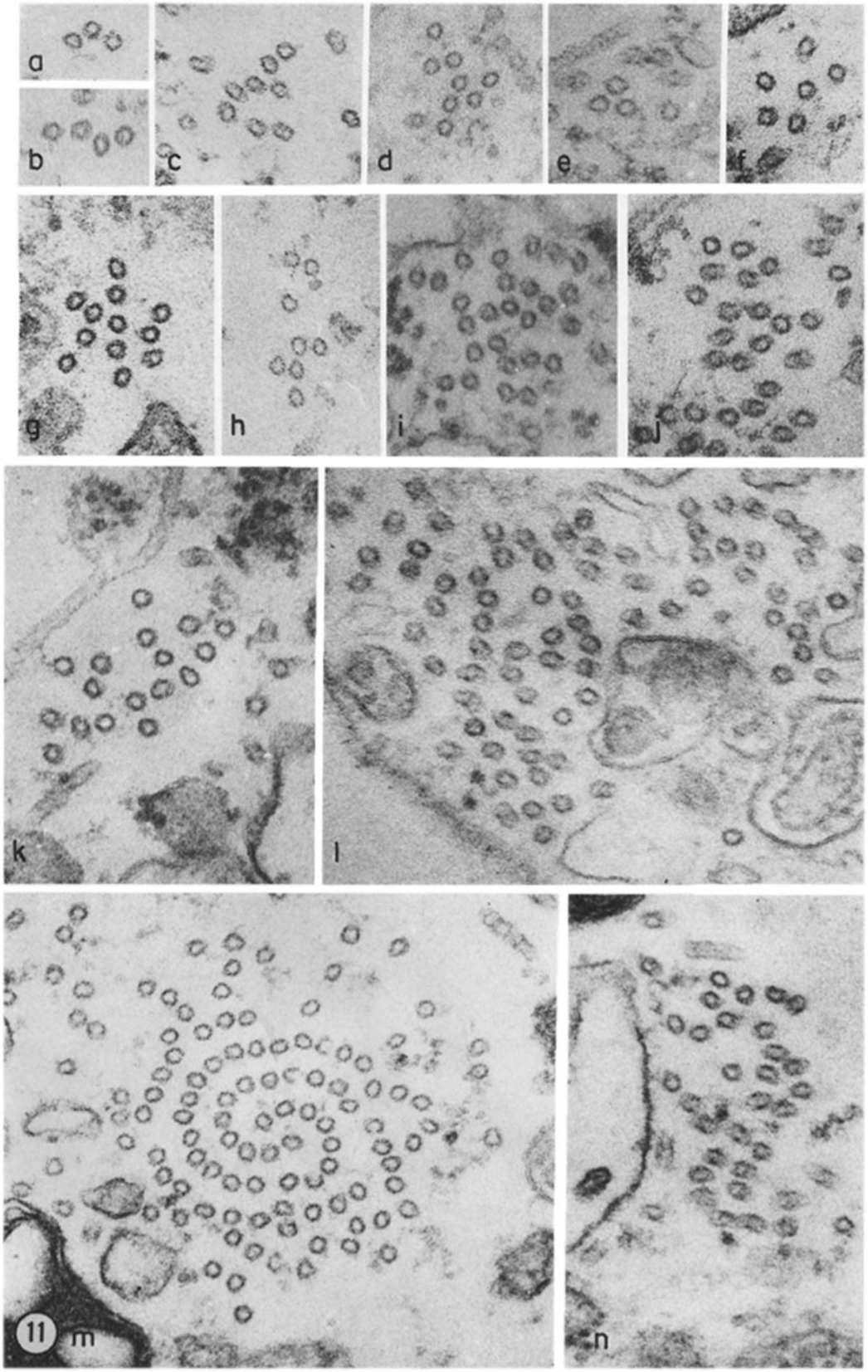

FIGURE 11 In this figure are representative clusters of microtubules present in cells fixed 15 min after immersion in 70% D_2O at room temperature following low temperature treatment. The arrows illustrate points of departure from the perfect axoneme (see text). × 100,000.

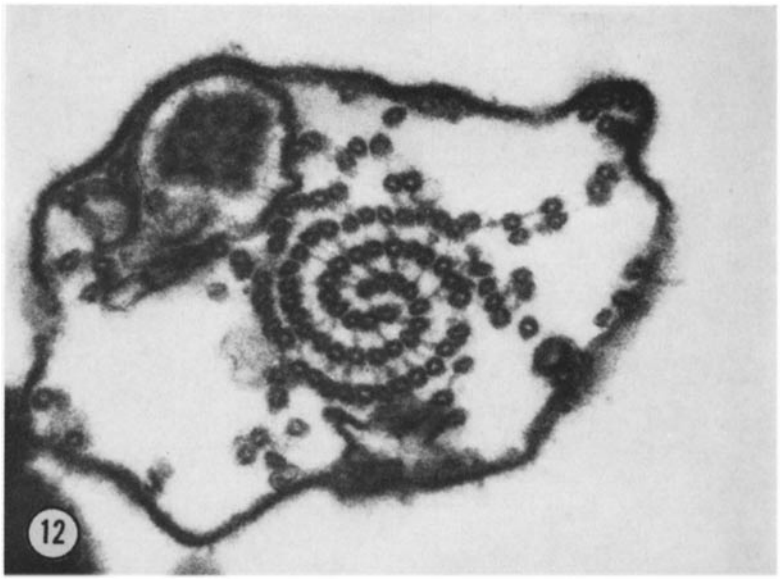

FIGURE 12 Transverse section through an axopodium of an untreated *Echinosphaerium*. This Araldite section was stained with potassium permanganate and lead citrate. The long links connecting the tubules of adjacent rows appear clearly. Note also the long links connecting some of the tubules peripheral to the axoneme proper. × 120,000.

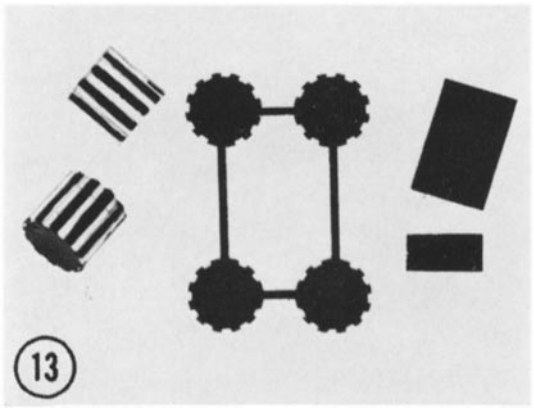

FIGURE 13 This figure illustrates the units we used to construct the models depicted in the following four figures. The tubules are represented by the cylinders in which are grooved 12 slots meant to represent the subunits of the microtubules and thus the bonding sites for the links. The links are of two sizes representing the short (70 A) and the long (300 A) link. These links fit into the grooves in the cylinders.

In Fig. 16 we have made models of most of the tubule clusters illustrated in Fig. 9. These represent what appear to be stages in normal axoneme development. As can be readily seen,

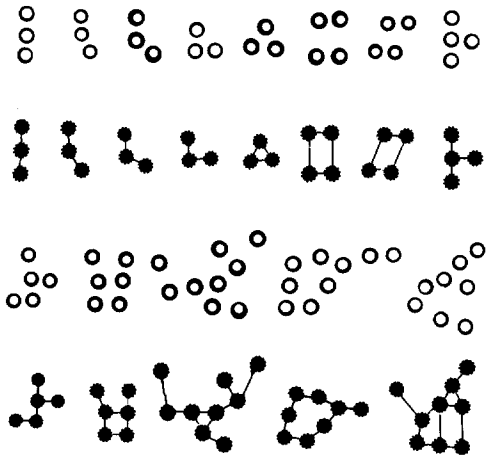

FIGURE 14 In this figure we have included tracings (rows 1 and 3 of electron micrographs, illustrated in Figs. 6, 8, 9, and 11. These small clusters of microtubules are found in cells either early in the recovery period following treatment with low temperature or when cold treated cells were immersed for 15 min in D_2O at room temperature. Beneath these tracings (rows 2 and 4) we have constructed a model, using the units figured in Fig. 13. It can easily be seen that these clusters, most of which could not be part of an axoneme, can be interpreted as resulting from interactions between the subunits of a microtubule and the postulated links.

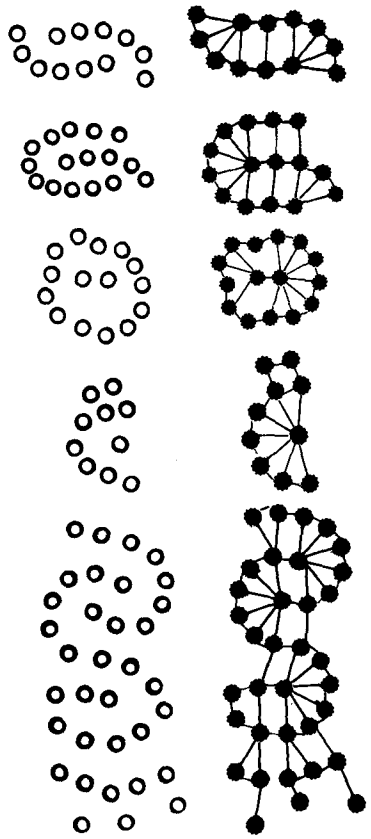

FIGURE 15 In this figure we have included tracings of some of the tubule clusters depicted in Figs. 8 and 11. We have constructed as in the previous figure a model which interprets these tracings. It should be pointed out that all these clusters deviate from the perfect axoneme in one or more respects. Nevertheless a corresponding model using our model units can be made and thus these clusters can be interpreted as resulting from the substructure of the tubule and of two sizes of links.

these patterns might also be interpreted as being a property of the tubule substructure and two sizes of links.

From the above considerations with respect to models, let us summarize the rules that appear to be imposed by the nature of the building blocks (tubules and links) on the patterns of microtubules encountered in organisms either treated with D_2O at 22°C or recovering normally following treatment with low temperature. First, microtubules, if associated, are separated from their neighbors by 70 or by 300 A, the lengths of the two sizes of links. Second, the minimum angle formed by two long links on the same tubule is 30° and by two short links is at least 60° (as during D_2O treatment), assuming that these links are connected to other linked tubules. Third, tubules within a cluster are ordered relative to each other so that links could not bond to the same subunit. This induced pattern ultimately might be related to the substructure of the tubule.

The Control of Axonemal Pattern

Now we can ask the question, are these rules mentioned above sufficient to account for the construction of an axoneme. In Fig. 16 we have constructed models of the tubule clusters that appear to represent early stages in the formation of an axoneme. We have inserted links between tubules in the same positions as they are found in our micrographs, as well as in the published drawing of MacDonald and Kitching (1967). It is readily seen that these tubule clusters can be formed given only links of two sizes and tubules with 12-fold symmetry.

The 12-sided quasi-symmetry of a large axoneme can also be interpreted as a result of the links and the substructure of the tubules. We will demonstrate this point in Fig. 17. The upper half of this figure is a tracing of half of the axoneme depicted in Fig. 2; the lower half was constructed with the

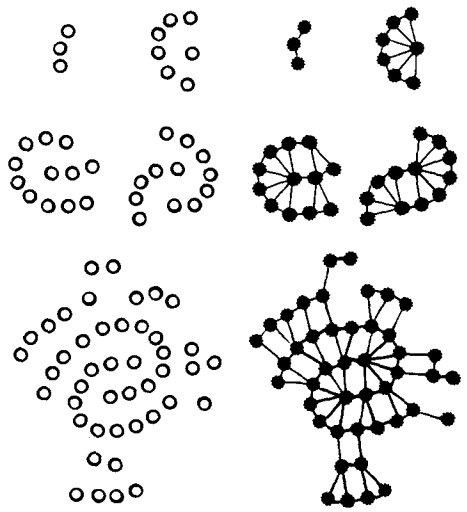

FIGURE 16 On the left hand side of this figure are tracings from most of the micrographs depicted in Fig. 9. To the right we have constructed the appropriate model. This figure depicts what we feel may be stages in the formation of an axoneme.

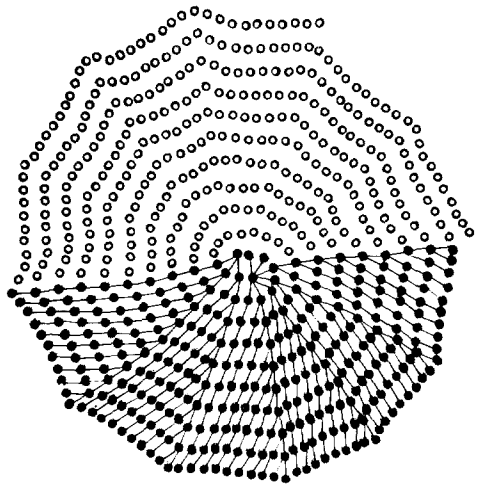

FIGURE 17 The upper half of this figure is a tracing of half the axoneme presented as Fig. 2. On the lower half we have built, using the units illustrated in Fig. 13, a model of half of the axoneme. This figure is included to illustrate that the 12-sided symmetry of the axoneme can be interpreted as resulting from the parallelism of the long links within each sector. The tubules along the radii contain three long links, two of which are separated by 30°. Thus the long links of adjacent sectors are positioned at 30° angles with respect to each other. This pattern of long links is the same as that now seen in our micrographs (see Fig. 12).

model units. We have made this model with the same number of long links indicated by MacDonald and Kitching (1967). The tubules along the radii, and only along the radii, are joined to other tubules by three long links, while those in a sector are joined by two long links. It should be noted that the long links on the same tubule always form 30°, 150°, or 180° angles. Because the long links within a sector are all parallel to each other and thus form a 30° angle with the long links of adjacent sectors and of the radii of adjacent sectors, each row of tubules within a sector, instead of lying on an arc of a circle, actually forms the chord. This spider web pattern, then, is a product of the parallelism of the long links in each sector, which in turn is determined by the proportionality between the lengths of the links and the diameter of the tubules. It is apparent, therefore, from this figure that it is unnecessary to have two sizes of long links as suggested by MacDonald and Kitching (1967).

As mentioned earlier, large clusters of microtubules oriented parallel to one another but not organized into the typical axonemal configuration were found in a small percentage of axopodia from untreated organisms, yet the tubules were generally spaced at the usual distances of 70 or 300 A from one another. Many of the tubules were aligned in parallel rows such that the short links would form approximately at 180° angles. Thus even in nonaxonemal patterns occurring in untreated cells, or cells recovering from treatment with colchicine (Tilney, 1968 *a*), the rules of tubule aggregation mentioned above apply.

The Elimination of Nonaxonemal Assemblies

Although the intrinsic properties of the building blocks (tubules and links) appear to be sufficient to account for the axonemal pattern and thus make it unnecessary to consider any of the current theories dealing with the regulation of tubule patterns by specific organizing centers in the cytoplasm (Porter, 1966; Inoué and Sato, 1967; Gibbins et al., 1969), there are still some unexplained observations. Most important is the fact that in the early phases of axoneme development nonaxonemal assemblies of microtubules are commonly encountered. Since these configurations occur infrequently in cells fully recovered from induced axonemal disintegration and in untreated cells, such assemblies (right triangles, rhomboids, imperfect axonemes, etc.) must occur transiently, then being replaced by the correct assembly. Thus there must be a mechanism that can eliminate these incorrect assemblies. A mechanism that most easily accounts for the appearance and disappearance of these transient clusters is a selection of the most favorable (energetically) bonding pattern, which we assume is the axoneme, while all other (less stable) patterns depolymerize in time. In support of this concept is the observation based on the models that in all configurations of tubules thus far encountered in *E. nucleofilum* the axoneme appears to contain more links per tubule than any other configuration found biologically. Reconsideration of Figs. 14, 15, 16, and 17 makes this point clear. The tubules at the inner ends of the two interlocking coils contain seven links per tubule; the next six consecutive tubules have five links per tubule. From this point outward there are either four or five links per tubule. Obviously small clusters such as the parallelograms or the triplets have very few links per

tubule. Yet even larger clusters, as the imperfect axonemes, have fewer links per tubule than a normal axoneme. For example, in an axoneme that contains three pairs of overlapping central tubules, rather than two, the middle pair can have only four links per tubule, rather than five (compare Figs. 15 and 16). Yet since patterns of three overlapping pairs are frequently found, such a pattern of tubules must be relatively stable. It is obvious from the models that the other configurations of tubules have fewer links per tubule, some significantly fewer.

This hypothesis is strengthened by the observation that even in untreated organisms an occasional axopodium is found in which the microtubules are not present in the axonemal pattern. Likewise, at the periphery of all axonemes so far encountered in axopodia, there is always a number of associated tubules that are not in the axonemal pattern (Tilney and Porter, 1965, 1967; Tilney, 1968 *a*). We would interpret these observations as an indication that there is a continuous selection of the most stable configuration of microtubules, in untreated as well as treated cells. Continuous selection of the energetically most favorable pattern conforms with the natural behavior of *E. nucleofilum*. For example, during feeding (Kitching, 1964), during movement (Watters, 1968), and under the influence of a variety of environmental stimuli the axopodia undergo retraction and reformation. Thus the microtubules must undergo frequent disassembly and reassembly into the axonemal pattern, or the bonds between the links and tubules must be broken to change the pattern.

If there is a selection for the most energetically stable configuration of tubules, one might wonder why certain configurations, such as concentric circles with one or two tubules in the center, or hexagonally packed clusters of tubules separated by the short or long link exclusively, do not occur biologically, for these patterns would have considerable numbers of links per tubule. The absence of concentric circles surrounding one or two tubules can be explained by the sizes of the links and the 12-fold symmetry of the tubule. Given links of 70 and 300 A, *between* 10 and 11 linked tubules could form around a central tubule and *between* 13 and 14 around a central pair. This may explain why the circle surrounding two central tubules (Fig. 15) is uneven and the tubules in the center are not equally spaced. It is more difficult to explain what, biologically, prevents *E. nucleofilum* from forming either the short or the long link exclusively to form hexagonally packed clusters of tubules. We would assume that hexagonal packing using only the long link does not occur because this link always forms at a much slower rate than does the short link. The long link rarely occurs in the early stages of recovery; most of the tubule patterns that form involve the short link. The absence of hexagonally packed tubules, which are separated by short links, appears to be due to a type of steric hindrance. We pointed out that equilateral triangles of three tubules, while occurring in cells treated with D_2O (the presence of D_2O may influence the bonding characteristics of the links), do not form in cells normally recovering from low temperature treatment. Instead, short links on the same tubule are separated by a minimum of 90°. MacDonald and Kitching (1967) have indicated that the short links are thicker than the long links and always remain at a distance from them. Perhaps this visible thickness indicates that the native structures themselves are too thick to bond at narrow angles on the surface of the microtubule.

Thus our concept, that the axoneme is "selected" in preference to less energetically-favorable configurations, seems well founded both for biologically-occurring clusters of microtubules and for hypothetical arrays.

We do not wish to suggest that there are no control mechanisms other than the tubules themselves and the two sizes of links. Certainly this is not the case, for the smallest assemblies of microtubules are not oriented; yet when axonemes appear, just prior to axopodial formation, the axonemes are radially arranged in the organism. Although there are as yet no data indicating how the axonemes become oriented, it seems possible that axoneme orientation may be accomplished by rather subtle factors, such as cytoplasmic gradients.

General Biological Importance of These Observations

The preceding discussion on the control of axonemal pattern by the substructure of the microtubule, the nature of two sizes of links, and the selection of the energetically most stable configuration of tubules may have general biological implications in the control of microtubule pattern in many systems. Furthermore this concept considerably simplifies, at least in the case of

E. nucleofilum, the requirements for additional mechanisms involved in the establishment of the microtubule pattern and consequently in the control of cell shape.

Regular arrays of microtubules occur in many diverse forms of cells, and in many of these cases threadlike links have been described or can be found in the published micrographs. We believe that the integrity of many of these clusters is maintained by links and may be produced, at least in part, by considerations similar to those presented above; these might include the axostyle of the flagellate *Saccinobacculus* (Grimstone and Cleveland, 1965), "km" fiber systems of *Stentor* (Bannister and Tatchell, 1968), the cytopharyngeal basket of *Nassula* (Tucker, 1968), the tentacles of the suctorian *Tokophyra* (Rudzinska, 1965), the cortical bundles of tubules present in some euglenoids (Sommer and Blum, 1964) and ciliates (Allen, 1967, for *Tetrahymena*; Grim, 1967, for *Euplotes*), and the infraciliature of rumen (Roth and Shigenaka, 1964) and caecal protozoa (Anderson and Dumont, 1966). Tubules connected by links have also been described in many spermatozoa or stages in the formation of spermatozoa as, for example, the planaria (Silveira and Porter, 1964), the dragonfly spermatid (Kessel, 1966), and the fowl spermatid (McIntosh and Porter, 1967), as well as in other cilia and flagella (Gibbons, 1961). They have also been described between the tubules in the forming cell plate of plants (Hepler and Jackson, 1967) and between microtubules in vertebrate neurons (Palay et al., 1968). It is a reasonable assumption that the tubules may slide past one another as in skeletal muscle, as McIntosh and Porter (1967) point out in the fowl spermatid and Bannister and Tatchell (1968) in the km fiber systems of *Stentor*. In these cases and in others the links may play an active role in the motile process. Thus these links may not only be involved in the production and maintenance of tubule patterns, but also may play a fundamental role in motility.

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