HOW MICROTIUBULE PATTERNS ARE GENERATED

The Relative Importance of Nucleation and Bridging of Microtubules in the Formation of the Axoneme of *Raphidiophrys*

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ABSTRACT

The axonemes of *Raphidiophrys* converge near the center of the cell in an electron-opaque material, the centroplast. In order to establish whether this material acts not only to nucleate the microtubules which form the axonemes but also to give the axoneme its characteristic pattern, the microtubules were disassembled with low temperature and stages in their reformation were studied. It was shown that even though the microtubules appear to be nucleated from the centroplast, pattern formation first appeared at a distance from the centroplast. Thus, the axonemal pattern could not be attributed to any prepattern in the centroplast. Rather, the pattern appears to arise by specific interactions between tubules brought about by bridges. It was concluded that each tubule could bind to a maximum of four other tubules and that once one bridge attached to a tubule it specified the binding positions of the others, thus giving the characteristic axonemal pattern of *Raphidiophrys*.

INTRODUCTION

Patterned arrays of microtubules occur in a wide variety of cells or portions of cells. Examples include the mitotic spindle, cilia and flagella, the axostyle of certain parasitic flagellates (Grimstone and Cleveland, 1965), the cytopharyngeal basket of ciliates (Tucker, 1968), the tentacles of *Suctoria* (Rudzinska, 1965), and the axonemes present in pseudopodia of Radiolaria (Holland et al., 1967) and Heliozoa (Kitching, 1964; Tilney and Porter, 1965). From studies on the formation of the ciliary or flagellary axoneme (Renaud and Swift, 1964) it has been shown that the basal body of the cilium apparently acts as a template, not only nucleating the assembly of the axoneme but also giving the axoneme its pattern based upon the preexisting architecture of the basal body. One wonders how other patterns of microtubules arise or even how the basal body or centriole is formed since, in all cases but those involving the formation of cilia and flagella, no morphologically distinguishable pattern exists before the formation of arrays of microtubules. Is there a nondistinguishable template for these different patterns of microtubules or is pattern generated as suggested by Tilney and Byers (1969) by interactions between microtubules?

In many cells one end of every microtubule is inserted into an "electron-opaque material" whose precise characterization has not been made. It has been suggested that this material may act to initiate or nucleate the assembly of microtubules (see reviews of Porter, 1966; Tilney, 1968; Pickett-Heaps, 1969). More recently, experimental evidence has been presented to verify these postulates in at least one cell type (Tilney and Goddard, 1970). If pattern is brought about by a template
in the other systems mentioned above, the pattern would presumably be present in this electron-opaque material.

In this communication we report on some experiments designed to demonstrate whether or not patterns of microtubules arise as the result of a cytoplasmic template composed of a patterned array of nucleating sites. The ideal system to test experimentally these alternatives is a cell in which the patterned arrays of microtubules all form from a morphologically distinguishable region of the cell. The centrohelidian, Raphidiophrys (Hovasse, 1965), was selected because in this organism all of the axonemes converge on a granule located in the center of the cell. If the template hypothesis is correct, the template must be integrated into this granule.

Our approach was to depolymerize the microtubules with low temperature and to fix Raphidiophrys at various stages during the reassembly of the axonemes. We would then determine: (a) if the central granule behaves as a nucleating site for the microtubules, and (b) if the microtubules which first reassemble from this site do so in the axonemal configuration. Unknown to us at the time we undertook this project was that the pattern of microtubules in Raphidiophrys does not resemble that in other Heliozoa, i.e., Echinophastarium (Tilney and Porter, 1965) or Actinophrys (Kitching, 1964). This actually turned out to be to our advantage as we are now forced to interpret the control of axonemal pattern in terms of perturbations in the structure of the microtubule itself based upon bridging.

MATERIALS AND METHODS

On October 10, 1970, pond water containing Spirogyra and Elodea was collected from Lake Lacawac, Lacawac Sanctuary, Wayne County, Pa. Rice grains were added and 3 wk later large numbers of Raphidiophrys could be found together with many other species of protozoa. Subcultures were made. Subculturing must be carried out every 10 days, for after that time other protozoa take over the cultures. At least one organism, an unidentified amoeba, appears to ingest Raphidiophrys.

Light microscopy was carried out using Nomarski interference microscopy or polarization microscopy with a rectified condenser. Living specimens were observed under polarized light at room temperature and during cooling with use of the rapid cooling stage of Inoué et al. (1970).

Fixation for electron microscopy was carried out in 3% glutaraldehyde in 0.05 M phosphate buffer at pH 6.8 with 0.0015 M CaCl₂ added. Fixation was carried out for 1 hr at room temperature. Organisms were postfixed for 45 min without an intermediate wash in 1% OsO₄ in 0.1 M phosphate buffer and 0.0015 M CaCl₂ at 0°C, dehydrated rapidly in acetone, and embedded in Araldite. Thin sections were cut with a diamond knife on a Servall Porter-Blum ultramicrotome II, stained with uranyl acetate and lead citrate, and observed with a Philips 200 electron microscope.

For fixation at low temperature or at intervals during rewarming following low temperature administration, organisms in 1 ml of their culture fluid were pipetted into a graduated centrifuge tube and placed in an ice-water bath. For fixation at 0°C the glutaraldehyde fixative was precooled to 0°C for 1 hr. For fixation during rewarming the centrifuge tubes were removed from the ice-water bath and immediately placed in a beaker containing water at room temperature. This brought the temperature of cooled protozoa to room temperature more rapidly. At intervals a centrifuge tube was removed and fixative was added. The glutaraldehyde fixative was used at room temperature, and osmication and the early stages of dehydration were carried out at 0°C.

RESULTS

Living Cells

Raphidiophrys measures about 25 μ in diameter with axopodia up to 100 μ in length. As in the other Heliozoa, particles saltate along the axopodia. In living organisms it is possible to see the centroplast with Nomarski interference microscopy (Fig. 1) or polarization microscopy. From this body birefringent axonemes extend out into the axopodia. The nucleus is difficult to resolve in living cells. Surrounding the cell body and extending a short distance out from the base of the axopodia are tiny spicules. The cytoplasm contains vacuoles, the larger of which contain ingested prey. The cortex of the cell is often vacuolated. Numerous refractile granules are also present, most of which are located in the cortex.

Fine Structure

The centroplast is located near, but seldom at, the exact center of the cell. It is characteristically encircled by the Golgi apparatus, which makes the finding of the centroplast, even in organisms in which the axonemes have been caused to disassemble, relatively easy (Fig. 2). The nucleus lies outside the Golgi sphere and is thus eccentrically positioned; in fact, in small cells it lies near the cell
surface. It is irregular in contour, explaining why it is difficult to find in living cells. The axonemes, which are located near the eccentric nucleus, frequently pass between lobes in the nucleus. In fact, it appears as if the highly irregular contour of the nucleus results from its being molded by the axonemes.

The cytoplasm of the cell is extremely dense; it contains a surprisingly large number of free ribosomes and rough surfaced endoplasmic reticulum (ER) (Fig. 2). The latter, as well as mitochondria, seem to be excluded from the centrosphere, although free ribosomes are common there. Fat droplets and electron-opaque granules are frequent inclusions of the cortex. The extracellular spicules commonly present surrounding the cell body in living cells are also found in the vacuoles in the cortex where they appear to be in the process of formation. Also present in the cortex but more commonly encountered in the axopodia are the haptocysts (Bardele, 1969).

As shown in Fig. 2 the axonemes extend from the axopodia through the Golgi sphere to eventually terminate in a mass of dense, coarsely granular material, the centroplast. This material surrounds the basal ends of the microtubules; it has no precise fine structure (Fig. 3). Peripheral to the dense mass of material is a cotton-like substance in which vesicles and ribosomes are comparatively rare (Fig. 2). At the edge of this material but within the Golgi sphere appear large numbers of ribosomes and vesicles. The centroplast then appears to be composed of an inner sphere of dense material and an outer mass of a cotton-like substance. This description coincides nicely with the light microscope description of Dobell (1917) on a related centrohelidian, Oxnerella, and our own observations on living cells.

Transverse sections of the axonemes, irrespective of whether they are cut near the centroplast (Fig. 4), through the cell body (Fig. 5), or through the axopodia (Fig. 6) show a patterned array of microtubules. At first sight the microtubules appear to be arranged into cylinders, the wall of each cylinder being composed of six microtubules. In actual fact this pattern of microtubules resembles hexagonal packing and would be hexagonal if a central tubule were to be added to the center of each cylinder. There are no geometric reasons why a central tubule could not be added (see Fig. 18). As argued by Tilney and Byers (1969), the geometric arrangement of microtubules in Echinocystis could be accounted for by two lengths of bridges connecting adjacent microtubules. Even though these bridges could not be seen initially (Tilney, 1968), by model building it was shown that the pattern of microtubules present in Echinocystis could be interpreted by having adjacent tubules bridged by one or the other of these hypothetical links. Subsequently,
bridges have been seen in the positions expected (Tilney and Byers, 1969; Roth et al., 1970). We believe that the axonemal pattern in *Raphidiophrys* can also be accounted for by bridges. We will at this point depart somewhat from the usual format in a scientific paper for the purpose of brevity. We will present micrographs of the pattern of microtubules in the axoneme before, during, and in the early stages following experimental treatment. Next to these we will show a tracing of the micrograph using circles of fixed diameter. The circles will be connected by the hypothetical bridges.

![Figure 2](image-url)

**Figure 2** Low magnification electron micrograph of the centrosphere. The axonemes all converge on the centrosphere. The Golgi apparatus (*Go*) surrounds the centrosphere. A portion of the nucleus (*N*) can be seen. × 41,000.
Bridges can be seen in some of our micrographs and so undoubtedly they exist, but with present techniques they are difficult to preserve routinely.

As shown in Figs. 4-6 adjacent microtubules in the axoneme are separated by about 70 Å. This distance represents the length of the postulated bridge. In Figs. 5 and 6 we have included tracings of the micrographs. The tracings were made by projecting the negative through an enlarger and drawing a circle wherever a microtubule was located. Thus the tracings are accurate renditions of the original micrograph. We have connected all of the tubules which are separated by the length of the postulated bridge. Close examination of the tracings reveals that all of the microtubules are bonded to a maximum of four others. Furthermore, the bridges are not spaced randomly around the circumference of the tubule; instead, they invariably form two pairs on opposite sides of the tubule such that each bridge forms a 60° angle with its closest adjacent bridge on one side and a 120° angle with its nearest neighbor on the other.

In actual fact the pattern of microtubules in the axoneme is even more complex than this. As can be seen from the micrographs and the tracings, the microtubules lie in rows. The tubules in one...
row (see line drawn on Fig. 7) are separated from one another by the dimensions of a bridge, in an adjacent row by two times the bridge distance plus the diameter of a microtubule. Such a pattern is predicted by the nearly hexagonal packing. Yet a careful examination of a row shows that the individual microtubules do not lie on a straight line drawn down the center of a row, but instead zigzag above or below the line. If a microtubule is made up of an odd number of subunits such as 13, as is suggested for certain plant microtubules (Ledbetter and Porter, 1964), and in other cases (Ringo, 1967), then this zigzag pattern becomes understandable.

In Fig. 8 we have traced the pattern of microtubules of Fig. 5 and have put over the tracing tubules made up of 12 and 13 subunits. Of particular interest is the fact that the bonding pattern of the bridges which connect adjacent tubules is less strained in the cluster in which 13 subunits are used rather than 12. This can be recognized by carefully observing a single microtubule. If it is composed of 12 subunits, two of the bridges connected to an individual tubule could bond to
Figure 5 Transverse section through an axoneme in the cortex of *Raphidiophrys*. To the right is a tracing of the micrograph; connections are drawn between adjacent microtubules. Note that each tubule, except at the periphery of the axoneme, is bonded to exactly four others and that in all cases the bridges are present as two pairs on opposite sides of the tubule. X 140,000.

Figure 6 Transverse sections cut through two axopodia. Beneath the micrographs are tracings of the microtubules making up their respective axonemes. X 120,000.

either of two subunits on the adjacent tubule. If the tubule is composed of 13 subunits, on the other hand, there is no choice: the nearest subunit on the adjacent tubule is obvious. Thus, the zigzag pattern appears as an expression of an odd number, presumably 13 subunits.

Unlike the situation in *Echinopharrarium* (Tilney and Porter, 1965; Tilney and Byers, 1969; Kitching and Craggs, 1965; Roth et al, 1970), in *Raphidiophrys* we do not find any free microtubules, that is microtubules which are not ordered into the axonemal pattern. Furthermore, with rare exceptions the microtubules at the periphery of an axoneme are in a position such that each could be bridged to at least two others.

The number of microtubules per axoneme is variable. This is due partly to the fact that the axoneme tapers as it extends toward the tip of the axopodium (see Figs. 5 and 6). Even so, adjacent axonemes in the same section differ in the number of microtubules per axoneme, sometimes by ten or more. Also, certain cells appear to have larger axonemes than do others, even though the relative distance from the centroplast is similar.
Treatment with Low Temperature

About 20 min after the initiation of 0°C the birefringence of the axonemes in the cell body disappears. Weak residual birefringence can still be seen in the axopodial bases, but this is best attributed to the spicules. Gradually the axopodia withdraw.

Organisms Fixed after 1.5 Hr at 0°C

A careful search through the cytoplasm of a number of organisms failed to reveal any microtubules, except for a few in the centroplast region. As pointed out earlier, this region can be readily identified by the encirclement of the Golgi apparatus and the amorphous material characteristic of the centroplast. In some organisms no microtubules were found; in others small numbers of microtubules (up to a dozen) could still be discerned (Fig. 9). In one cell a few of these extended into the Golgi region. In cases where residual microtubules remained, they were not associated into the axonemal pattern.

Rewarming after Treatment with Low Temperature

After 15 min of rewarming, birefringent axonemas could be located in the cell body. For these observations we used the cooling stage of Inoué et al. (1970). Rewarming on this stage occurs within a few seconds. Rewarming of 1 ml of fluid containing cells, however, is slower so that organisms fixed after 15 min of rewarming in the fluid have actually spent less time at 22°C.

Fixation after 10 Min of Rewarming after 1½ Hr at 0°C

The degree of recovery varies somewhat from organism to organism. In some organisms there are few microtubules; in others many have repoly-

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**Figure 8** The axoneme presented in Fig. 5 was traced and, where the microtubules appeared, a tubule composed of 12 or 13 subunits was placed. The purpose of this figure is to show that the zigzag pattern of tubules depicted in the preceding figure can be explained by assuming that each microtubule is composed of 13 subunits rather than 12. Thus, the bonding pattern of the bridges which connects adjacent tubules is less strained in the cluster in which 13 subunits are used rather than 12. This can be recognized by carefully observing a single microtubule. If it is composed of 12 subunits, two of the bridges connected to an individual tubule could bond to either of two subunits on the adjacent tubule. If the tubule is composed of 13 subunits, on the other hand, there is no choice; the nearest subunit on the adjacent tubule is obvious. Thus, with 12 subunits two of the bridges from a single tubule do not extend at right angles to a tangent drawn to the circumference of a tubule at the point of bonding, yet with 13 subunits the bridges almost always lie perpendicular to tangents drawn on the circumference of the bonded tubules.
merized (Fig. 10). In both instances few were found peripheral to the Golgi sphere. Furthermore, one end of each microtubule makes contact with or would make contact with the centrosphere if a serial section were cut. The axonemal pattern could not be found even though clusters of microtubules could be found. Generally five or fewer microtubules were present in each cluster. Pairs, equilateral triangles, straight rows, four's arranged in the form of a Y, and five's in the form of an X were present.

15 Min of Recovery after Treatment with 0°C

By this stage many more microtubules have reassembled. As before, all the microtubules are oriented such that one end could make contact with the centroplast (Fig. 11). Even so, the arrangement of microtubules in the centroplast region appears unorganized (Fig. 11). The clusters have increased markedly in size, sometimes including more than 40 microtubules (see Figs. 12 and 13). The clusters are very small near the centroplast, however, indicating that adjacent microtubules tend to converge away from the centroplast. In longitudinal sections we have seen instances in which adjacent clusters in the cortex do indeed merge, presumably being bridged together.

Careful analysis of the clusters (see Figs. 12 and 13 for our tracings) demonstrates that many of the tubules in the clusters are separated by the postulated bridging distance. Particularly common are
clusters of 2's, 3's in the form of equilateral triangles (two examples can be found in Fig. 12), 4's in the form of Y's (one in Fig. 11, four in Fig. 12, one in Fig. 13), and 5's in the form of X's (Fig. 13). In Fig. 13 we have illustrated one example of a cluster which very closely resembles an axoneme. Most of these tubule patterns are arranged such that the bridges which could form to connect adjacent microtubules would do so such that each tubule is bridged to no more than four others; the bridges then would appear as two pairs on opposite sides of a tubule. The included tracings (Figs. 11-13) help to illustrate this point. Admittedly, there are also examples of individual microtubules in the clusters which have the bridge spacing but which do not conform to this precise pattern of bridging. These instances are rare and are generally located at the periphery of the tubule cluster.

20 Min of Recovery after Treatment with 0°C

The statements made thus far adequately describe this stage even though more microtubules...
are present. Furthermore, we should mention again that all of the microtubules appear to radiate from the centroplast.

25 Min of Recovery after Treatment with 0°C

By this stage axopodia are common although short. The axonemes have increased in diameter and the over-all pattern of the centroplast is similar to that of the untreated cell, although the pattern is not yet as precise (compare Figs. 2 and 14). However, sections cut through the periphery of the centroplast (Fig. 15) reveal that even after this extensive recovery time the axonemal pattern found in untreated organisms is not present in the centroplast. There are tremendous numbers of microtubules here but their arrangement is seldom the axonemal one (Fig. 16). Transverse sections through axonemes peripheral to the centroplast, on the other hand, reveal in most instances a pattern of microtubules similar to the pattern of an untreated axoneme (Fig. 17). These patterns of microtubules also illustrate that each tubule is bridged to a maximum of four others and in the pattern described above. It thus appears that the bases of the axonemes in the centroplast region are one of the last places for the axonemal pattern to reappear.


DISCUSSION

The Centroplast as a Nucleating Site

At the earliest stage in the recovery period, a stage when there are no axopodia, the microtubules seldom extend beyond the center of the cell demarcated by the Golgi sphere. They are oriented such that one end of each microtubule, in an adjacent section could, or in the observed section does make contact with the centroplast. As the

Figure 12 Section cut near the centroplast of Raphidiophrys. This organism was treated with 0°C for 1 1/2 hr, warmed for 15 min, and then fixed. Many microtubules can be found. They appear as singlets, pairs, triplets in the form of equilateral triangles, Y's composed of four microtubules, and X's composed of five microtubules. Certain of the clusters are traced. Bridges are added to the tracings if adjacent tubules are separated by the bridge distance. × 140,000.
FIGURE 13. Transverse sections through clusters of microtubules found in the cortex of *Raphidiophrys*. As in Fig. 11 and Fig. 12, the organisms were subjected to 0°C for 1½ hr, rewarmed for 15 min, and then fixed. Beneath the micrographs we have traced the microtubules and connected neighbors by bridges. Note that many of these patterns closely resemble the axonemal pattern or the basic unit of the axoneme which appears as five tubules in the form of an X. X × 140,000.

recovery period is increased, the microtubules, although longer, are still oriented with respect to the centroplast. From these observations we conclude that the microtubules must initially assemble at the centroplast and, as the time of rearming increases, grow in length. The centroplast then appears to nucleate tubule assembly. Similar conclusions have been presented (Tilney and Goddard, 1970) for the ectodermal cells of sea urchin blastulae on the basis of a similar experimental approach. It should be noted that in both of these cases the nucleating sites are composed of an electron-opaque material which is without visible substructure. In the literature there have been numerous reports demonstrating that the tips of many microtubules are inserted into "dense material." Speculation has been presented suggesting that this material "initiates" or orients microtubule assembly (see Porter, 1966; Tilney, 1968; and Pickett-Heaps, 1969), but thus far there have been few instances of any experimental verification of these speculations. Our two studies, then, provide more evidence that these speculations are well founded and that one might expect that the dense material indicates the site of microtubule nucleation in many systems.

The Centroplast as a Template Controlling Axonemal Pattern

We have shown that the microtubules do not initially reappear in the axonemal pattern. Rather, they reappear as doublets, as equilateral triangles, as Y's, and as X's. With time, more of these clusters begin to take on the morphology of the axoneme present in untreated *Raphidiophrys*. This pattern, interestingly enough, appears first at a distance from the centroplast. Thus, although the centroplast does appear to nucleate microtubule assembly it does not appear to be associated with the development of the axonemal pattern. Instead, pattern production appears to develop gradually by a hit-or-miss mechanism which seems to function best at a distance from the centroplast. Our conclusion conflicts with that of Tucker (1970) on the formation of tubule patterns in *Nassula*. Tucker concludes that the dense material gives pattern to the tubules or acts as a template. Although his work is ex-
Section cut through the centroplast of a *Raphidiophrys* which had been cold-treated and then allowed to recover for 25 min before fixation. Note the axonemes converging on the centroplast. The pattern at the centroplast is not well ordered. $\times 120,000$.

cellent, we believe that he has misinterpreted his results. One could just as easily interpret his findings by the same principles as we do ours, as will be outlined below.

**Control of Axonemal Pattern**

Linear structures (microtubules) separated by a constant spacing for extended distances must be either joined together by connections or held together by some outside force. If they are held together by some external means, they would become hexagonally packed—the most compact packing possible. There is a biological example of this phenomenon. At the tips of cilia the nine outer doublets form nine singlets. These apparently lose their bridges, for they become closely packed; in fact, the packing becomes hexagonal (see Reese, 1965, for olfactory cilia of the frog; Tilney, unpublished observations). Since the arrangement of microtubules in the axoneme of *Raphidiophrys* does not show hexagonal packing, it must be concluded that the pattern is not maintained by a force.
peripheral to the microtubule cluster. It is possible that the pattern might arise by some structure lying parallel to the microtubules within the axoneme proper which binds to adjacent microtubules. The most logical would be a linear structure present in the center of each cylinder of six. This possibility can be eliminated by carefully studying the periphery of the axoneme where the pattern is maintained, although only a portion of the cylinder exists, and by the fact that there is no evidence in the micrographs to suggest such a structure.

What we would like to propose for the control of axonemal pattern in *Raphidiophrys* is that once one bridge or possibly a pair has attached to a microtubule it specifies the only other possible attachment sites on the circumference of that microtubule. Then by a random process, a hit-or-miss mechanism, these other sites would become gradually utilized. As has been argued repeatedly for self-assembly mechanisms, the assemblage will take on the state of minimum free energy. Thus, we would expect that in time all the four possible bridge sites would be filled.

Actually, all of the available evidence supports this hypothesis. For example, with the hit-or-miss
association of bridges to tubules the development of the axonemal pattern should take a considerable amount of time. This is observed, for even though microtubules start to reassemble from the centriplast within a few minutes after rewarming, axopodia do not begin to reappear until 20 min after rewarming. Even then, the tubules are still poorly organized. One would also predict that the pattern of the tubules at the periphery of the cluster would be the least structured after 20 min. This is also observed. Eventually, of course, perfect axonemes would be formed.

The high incidence of triangles, Y's and X's seen in the earliest stages of rewarming is particularly significant, for these clusters illustrate the maximum bridging which could occur per tubule in small clusters. For example, the X cluster should be a rather stable pattern as all the tubules would be bridged to at least two microtubules. Equally significant is the fact that all of the microtubules present in an untreated axoneme could be bridged to at least two others, even at the periphery of the axoneme. These observations would be predicted by our postulated mechanism involving a selection of the clusters which would be the most stable.

Further evidence in support of our hypothesis that the axonemal pattern is derived from four bridges arranged as two pairs on opposite sides of a tubule can be seen by examination of Fig. 18. Most significant are the patterns depicted in c and d. Although these patterns are nearly identical, d is never found biologically; careful examination of the distribution of bridges around the circum-

Figure 16  Tracing of the microtubules in the central portion of Fig. 15. Bridges have been added. There are two circles of six but otherwise the pattern of microtubules does not resemble the axonemal pattern.

Figure 17 Thin sections cut through the cortex of a Raphidiophrys (a-c) or through an axopodium (d). The organisms were cold-treated for 1.5 hr and then rewarmed for 25 min. Note that the microtubules are generally organized into the axonemal pattern or something that approaches the axonemal pattern. This is particularly evident in the axopodium seen in d. × 130,000.
ference of some of the tubules of d shows that the distribution differs from that in c, which, of course, is the true axonemal pattern. The same reasons eliminate other patterns of tubules except the pattern depicted in Fig. 18f. This arrangement of tubules has not been found biologically, presumably for two reasons. For one, this tubule pattern would collapse if a lateral force were applied. More significant is that during reformation of the axoneme we find large numbers of equilateral triangles and clusters in the form of a Y or an X. This indicates that, in the earliest clusters formed, extensive use is made of a pair of bridges separated by approximately 60° on the same tubule, forming a rather stable pattern. Thus, if a pattern of tubules such as depicted in Fig. 18f were to begin to form, the tubules at the periphery of the cluster would form equilateral triangles, X's or Y's. Once this happened, growth would cease and, by the hit-or-miss mechanism, such a pattern would be reordered.

There is evidence in other systems for specific binding sites on the circumference of a tubule. Many tubule patterns have been described in which the bridges or arms "know" exactly where to attach to the tubule. The best studied example is that of cilia where, in a group of elegant experiments, Gibbons (1963, 1965) was able to remove the dynein arms from the nine outer doublet tubules of cilia. When returned to the solution of isolated ciliary axonemes, the arms reattached to the same sites on the outer doublet tubule (on the A tubule) to which they were attached before removal. Grimstone and Klug (1966) point out that these dynein arms are never found attached to the B tubule.

Close examination of the connections to the A tubule on the outer doublet tubules of cilia reveals that the pair of dynein arms on the A tubule is situated on one side of the circumference of the A tubule, and the connections to the B tubule are found on the opposite side. Thus, each A tubule has two pairs of connections situated on opposite sides of the tubule. This is the same relationship as the postulated connections to the tubules of Raphidiophrys. Exactly what determines the specificity of binding sites on the A tubule of cilia remains unanswered. It is known, however, that, in negatively stained preparations of cilia, perturbations exist in the wall of the outer doublet tubules (Grimstone and Klug, 1966; Thomas, 1970). It seems reasonable to expect that specific bonding sites are determined by these perturbations. It behooves us to examine other tubule patterns as being ordered by specific bonding sites, rather than by other hypothetical mechanisms such as recently proposed by Roth et al. (1970).

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