THE AMEBA-TO-FLAGELLATE TRANSFORMATION IN TETRAMITUS ROSTRATUS

II. Microtubular Morphogenesis

DARRYLL E. OUTKA and BYRON C. KLUSS
From the Department of Biochemistry and Biophysics, the Iowa State University of Science and Technology, Ames, Iowa 50010, and the Department of Biology, California State College at Long Beach, Long Beach, California

ABSTRACT

Tetramitus exhibits independent ameboid and flagellate stages of remarkable morphological dichotomy. Transformation of the ameba involves the formation of four kinetosomes and their flagella. The arrangement of these kinetosomes and associated whorls of microtubules extending under the pellicle establishes the asymmetric flagellate form. While no recognizable kinetosomal precursors have been seen in amebae, and there is no suggestion of self-replication in dividing flagellates, developmental stages of kinetosomes have been identified. These are occasionally seen in association with the nucleus or with dense bodies which lie either inside of or close to the proximal end of the prokinetosome. Outgrowth of flagella involves formation of an axoneme and a membrane. From the distal tip of the kinetosome microtubules grow into a short bud, which soon forms an expanded balloon containing a reticulum of finely beaded filaments. The free ends of the microtubules appear unraveled; they are seen first as single elements, then as doublets, and finally are arranged into a cylinder. Growth in length is accompanied by a reduction in the diameter of the balloon. The concept that the formation of the kinetic apparatus might involve a nuclear contribution, followed by a spontaneous assembly of microtubules, is suggested.

Tetramitus rostratus shows remarkable ability to proceed along alternative life paths by correlative changes in its body plan; this results in a major morphological dichotomy. One form is an active, limacine ameba, with a monopodal progression. This ameba can transform into a remarkably different flagellated form, with modifications essentially in two parameters; for motility there is a kinetic apparatus well anchored to the cell’s cytoskeleton; for feeding there is a special, microtubule-supported geometry involving an asymmetric, anterior feeding groove continuous with an internal cytostomal canal. Thus, in approximately 2 hr an organism devoid of microtubules changes into one whose most characteristic ultrastructural feature is that ubiquitous component of eucellular systems, the microtubule. Tetramitus is, therefore, a very favorable organism for the study of the formation of basal bodies (kinetosomes), flagella, and cytoskeletal microtubules.

The original description, in 1852, of an organism recovered from swamp water and described as Tetramitus rostratus is credited to Perty. Bunting (1922, 1926) performed the first major study of the organism, and established that the life cycle included cystic, ameboid, and flagellate stages. Perhaps because Bunting found her strain in the rat cecum, Tetramitus has generally been considered coprophilic, and the majority of work has been done on strains recovered from contaminated...
environments, e.g. cecal contents of the rat and cockroach (Rafalko, 1951), feces of the human (Aragao, 1916) and the bat (Outka, 1965), and dung water (Hollandae, 1942). However, Sandon (1927) reported the widespread occurrence of T. rostrata in soil and water samples from various parts of the world.

The entire life cycle involves three stages; a cyst, an ameba, and a flagellate. Only the more plastic ameboid stage has been observed to encyst or excyst. Pure cultures of amebae and of flagellates have been maintained separately for over 4 yr. During this time, we have seen no evidence for syngamy, as reported by Bunting (1922, 1926). In small, hanging drop cultures, experimental conditions can be arranged so that up to 80% of amebae will transform within a 4 hr period. In contrast, reversion to the ameboid stage by flagellates has been seen only as a highly infrequent, asynchronous occurrence in certain dilute, bacterized media (Outka, 1964), and little is known about its cause.

The flagellate stage reproduces by asymmetric fission and, since the body is asymmetric (rostrum on one side, cytostome curving to the opposite side posteriorly), there is the possibility of right- or left- "handed" forms. Hollandae (1942), as a part of the most definitive work on Tetramitus up to that time, concluded that a given flagellate can produce either right or left orientations at division. This suggests an extremely intriguing sophistication in morphogenesis, which challenges an ultrastructural explanation.

Naegleria gruberi is the closest relative of Tetramitus that has been studied in detail. Naegleria flagellates, unlike those of Tetramitus, can neither feed nor divide, and reversion occurs spontaneously within a few hours. The morphological changes at transformation are less complex than in Tetramitus. These changes have been carefully studied at the ultrastructural level by Schuster (1963) and Dingle and Fulton (1966). In these papers, no centrioles, kinesomes, or recognizable kinesosomal precursors were found in the pretransforming ameba, and the earliest kinesosomes found in transforming stages seemed complete.

Kinesosomes have not been observed in non-transforming Tetramitus amebae. This report deals with light and electron microscopic observations of transformation in Tetramitus, especially as regards the origin of kinesosomes, flagella, and microtubules. A brief description is also given of the morphology of the mature ameba and mature flagellate, which represent, respectively, the beginning and the culmination of the transformation process.

**MATERIALS AND METHODS**

The strain (TrB) of Tetramitus used was that of Balamuth, which was originally collected from a "sterile" human urine specimen (Brent, 1954). Monoxenic cultures of flagellates were maintained with Esherichia coli as the bacterial associate on a dilute yeast-peptone-glucose medium, or else pure cultures of flagellates were grown in a more concentrated yeast-peptone-liver medium containing autod degraded bacteria (formula to be published elsewhere) modified from a medium described by Brent (1954). Amebae were grown with E. coli on solid media as previously described (Outka, 1965), or in a yeast-peptone-liver medium containing a serum-fraction supplement devised for Naegleria by Balamuth (1964). Transformation was achieved simply by placing into test tubes washed amebae in Hpm medium (Outka, 1965), with the addition of living E. coli. These test tubes were rotated at 2 1/4 rpm on a modified tissue-culture roller-drum maintained at 30° ± 1°C. Tubes were removed at intervals beginning at about 2 hr before the appearance of the first flagellates, covering thereby a time span of 14–20 hr after initial harvesting and washing. Flagellates were concentrated by allowing cultures to settle for 15–20 min in Petri dishes. The supernatant was decanted and centrifuged, and the pellet was washed three times. The organisms were fixed in Palade’s fixative to which 0.01% CaCl₂ had been added. Cells were dehydrated in alcohols, embedded in Maraglas or Epon, and sectioned with a diamond knife on a Porter-Blum MT-1 or a Reichert Om-U2 microtome. Sections were stained in 1% uranyl acetate solution in 50% alcohol for 30 min, followed by a 15 min exposure to the lead tartrate procedure of Millonig (1961) or a 5 min exposure to the procedure described by Venable and Coggeshall (1965). They were then examined with either a Hitachi HS-6 microscope at 50 kv, or an RCA EMU-3f microscope at 100 kv.

**RESULTS**

**Terminology**

Several apparently synonymous terms exist for the component parts of the flagellar apparatus and its associated structures. For some of these structures, the terminology of Chatton’s group, originally applied to ciliates but equally useful for flagellates, seems simplest and most convenient.
and will therefore be used here (for a review, see Pitelka and Child, 1964). Thus, a kinetid is made up of a kinetosome or basal body with its attached flagellum and of any additional intracellular fibrils at or near the kinetosome. A kinetome is a special grouping of kinetids within a cell. A striated fiber similar to the kinetodesmal fibril in ciliates is present in Tetramitus, but the synonymity is less certain; therefore, the classical term for this structure in flagellates, rhizoplast, will be used. Finally, Schuster (1963) used the term "spur" for a whorl of microtubules appearing laterally to the kinetosome of Naegleria; we have used the same word for a similar structure in Tetramitus.

The Morphology of Transformation

The general morphology of the flagellate is diagrammatically illustrated in Fig. 1. Tetramitus (Greek: tetras, four; mitos, thread) has four kinetids which comprise the structural and morphogenetic center of the flagellate. The kinetosomes are designated $K_1$ through $K_4$, and lie so that their contained tubular fibrils are parallel. $K_1$, $K_2$, and $K_3$ are always in a row, whereas $K_4$ is out of line and to one side of the other three. The four kinetosomes are connected anteriorly to the axoneme and proximally to a striated rhizoplast ($R_2$). The latter in turn contacts the microtubular elements that comprise the cytostome ($CM$). Nucleus ($N$), mitochondria ($M$), food vacuoles ($FV$), which accumulate posteriorly and the contractile vacuole ($CV$) are shown. According to Hollande (1942), one of the antero-ventral (anterior, flagellated end; ventral, surface containing cytostomal groove) lips is enlarged into a flaplike rostrum, which thereby establishes a right- or left-handed asymmetry to the body. Presumably this rostral extension helps direct food into the cytostomal groove. Studies of thin sections of clonal cultures give the impression that this expanded region is probably related to the primary asymmetry of the kinetome.

Figs. 2-5 are phase-contrast photomicrographs of living organisms. Fig. 2 shows the general appearance of a limacine ameba, with a large nucleolus surrounded by the less dense nucleus, a posterior contractile vacuole, and other inclusions. Fig. 3 shows newly forming flagella arising from kinetosomes which typically appear in the region of the contractile vacuole. Fig. 4 illustrates a later stage in transformation in which lips are formed, but the elongate, asymmetric geometry has not yet been established. Fig. 5 is a micrograph of a mature flagellate, as seen from the dorsal side. Four flagella emerge from the kinetosomal area, although the details of their relationship with the individual kinetosomes cannot be resolved. A short portion of the rhizoplast can be seen extending proximally from the kinetosomal region.

The flagellate stage measures from 10 to 30 $\mu$ in length and from 7 to 11 $\mu$ in width. Cysts measure 10–16 $\mu$ in diameter. Amebae approximate the mass of the flagellate stage.

Ultrastructure of the Mature Flagellate

Fig. 6 is a longitudinal section through $K_1$, $K_2$, and $K_3$ in approximately the same orientation as diagrammatically illustrated in Fig. 1. Microtubules supporting the pellicle are especially numerous and closely spaced at the top (dorsal) and bottom (ventral) anterior lips. A part of the
FIGURES 2-5  Phase-contrast micrographs of living organisms illustrating different stages in the life cycle.

FIGURE 2  Trophic ameba from axenic culture. Note very dark, spherical nucleolus surrounded by light nucleus. Contractile vacuole is represented by single, large, clear sphere at top of picture. Direction of movement is downward in the picture. $\times 1600$.

FIGURE 3  Early stage of transformation from bacterized culture. Three flagella are present; one of these is very short. Their origin near the contractile vacuole is typical. Small, dark spheres (measuring about 1 $\mu$) in the cytoplasm are mitochondria. $\times 1400$.

FIGURE 4  Later stage in transformation from bacterized culture. At upper left, anterior lips are forming, near four vacuoles which will eventually fuse to form a single contractile vacuole just before discharge. Two intracytoplasmic axonemes are visible (arrow), one running under the contractile vacuole and the other along the outer surface. $\times 1600$.

FIGURE 5  Mature flagellate stage from particulate-containing medium. Dorsal surface (side opposite cytostomal groove) nearest viewer. Note kinetosomal area, lips, contractile vacuole. Three flagella are arranged in a row, with the fourth lying slightly to the right. $\times 1600$.

cytoplasm-filled cytostome as outlined by microtubules is seen below the nucleus. Other microtubules appear beneath the pellicle as the plane of section passes obliquely through the posterior surface. Fibrous material appears to join $K_1$ with the dorsal lip. A portion of the striated rhizoplast connects with the cytostomal microtubules ventrally to the nucleus.

Fig. 7 is an anterior, transverse section through the kinetome, and illustrates the orientation of the kinetosomes and microtubules in relation to the contractile vacuole and supporting lips. The arrow indicates an intracellular axoneme which suggests that this may be an early division stage and thus modified somewhat in shape.

Some additional ultrastructural details of the kinetome may be seen in Fig. 8. The kinetids are of two different kinds: those with ($K_2$ and $K_3$) and those without ($K_1$ and $K_4$) a spur. Spurs are chains of tubular fibrils consisting of a dozen or more units. Each scoop-shaped whorl of fibrils appears to be reinforced by a supporting matrix on the inward side; this gives a characteristic, trilaminar appearance (arrows). The kinetosomes have the typical nine peripherally arranged sets of triplets, with subfibris A and C (inset) joined by a connecting fibril. Interconnecting fibrous elements join the kinetosomes together (most clearly illustrated between $K_2$ and $K_3$); areas of increased electron opacity surround them. The inset to Fig. 8 shows a more proximal section of a kinetosome and thus illustrates the details of the "cartwheel."

Fig. 9 shows microtubules arising from the region of $K_2$ and extending under the surface presumably to join the group of subpellicular fibrils supporting one of the cytostomal lips. The emerging picture is one of the kinetome firmly
anchored in an elaborate cytoskeleton, consisting of subpellicular and cytostomal microtubules reinforced by the rhizoplast (Rz in Fig. 9). The inset shows a flagellum in cross-section; *Tetramitus* flagella have arms on the peripheral doublets and generally adhere to the classical picture.

**Morphogenetic Ultrastructure**

The ameba, usually limacine when in locomotion, lacks specific body shape (Fig. 2). This condition is correlated with the absence of subpellicular microtubules. The earliest recognizable stages of transformation present an ameboid organism with kinetosomes and flagella (Fig. 10, organism on right; on the basis of likelihood, the organism on the left in Fig. 10 is an ameba). The kinetosomes first appear near the contractile vacuole at the posterior end of the motile ameba (Fig. 3); they normally are positioned near the membrane, and microtubules grow outward from the kinetosomes. It is noteworthy that there are no subpellicular or cytostomal microtubules in the posterior two-thirds of the body at this stage. The inset to Fig. 10 shows only one subpellicular microtubule (arrow). This condition of a few subpellicular microtubules located anteriorly correlates with the absence of the mature flagellate body form. Some other features of interest include the dense material surrounding the kinetosome, some cytoplasmic microtubules on the left, and the slightly tilted axosome from which the central axonemal pair of microtubules originate.

There is also present, in the ameba (like the organism on the left) and the early transforming organism seen in Fig. 10, an abundance of small globular elements of undetermined composition which give the cytoplasm an extremely dense ap-
appearance, so dense as to almost obscure the mitochondria. During this study it became apparent that there was in general an inverse correlation between the degree of cytoplasmic density and the maturity of the flagellate. In a given section, early transforming organisms exhibited a dense, ameboid cytoplasm whereas an immediately adjacent organism with reduced cytoplasmic density possessed many microtubules and the typical flagellate shape. Chemical identification of the responsible granular material has not been made, but the assumption was that, under the particular transformation conditions, synthesis of one or more ameba-specific products was discontinued in those cells undergoing transformation.

Further study showed that the techniques used for inducing transformation of Tetramitus in large numbers were not very good as regards synchrony. While it is possible to obtain up to 80% flagellates in 4 hr in small, hanging drop preparations, yields in roller-tube cultures were only 10-30% in a similar period. These could be concentrated by allowing the amebae to attach to glass and by decanting the supernatant, a procedure which tended to reduce the number of very early transforming organisms. But no matter how the preparation was made, there was always present a mixture of cell types. Besides amebae (Fig. 2) and typical transformers (Figs. 3 and 4), there were also mature flagellates undergoing division and transforming stages also in division. As more micrographs were obtained, an effort was made to approximately correlate the amount of cytoplasmic density, the number of cytoplasmic microtubules, and the degree of flagellar development. Flagellates in logarithmic growth phase from a mature flagellate culture (no ameba present) were studied as a control. The pattern of flagellar and axonemal development appeared identical in all cases, and the idea is now held that this pattern is “stereotyped,” in that transforming amebae and dividing flagellates use similar processes. Since mature flagellates have low levels of cytoplasmic density,
Figure 8. Higher power view of section through four kinetosomes, showing arrangement of the kine-
tome. Striated rhizoplast runs on the left of three kinetosomes. Curved chains of microtubules (spurs)
(arrows) are seen emanating from \(K_2\) and \(K_3\). Interkinetosomal connections are visible especially be-
tween \(K_3\) and \(K_4\).

Inset, section through a more proximal region of the kinetosome at the level of the cartwheel. In this
view the observer is looking from the base of the kinetosome distally (clockwise skewing of triplet fi-
brils), which is the reverse of the previous orientation in Fig. 8. \(\times 82,000\).

consistently better micrographs can be obtained. Figs. 22–30 in this paper (to be described shortly)
are micrographs from mature flagellate cultures; they contain details which would probably have
been obscured in most of the transforming ma-

Fig. 11 is a transverse section through four de-
veloping flagellar axonemes. The axoneme near-
est the body is nearly complete; it has seven periph-
eral doublets in position; the eighth is out of
position; the ninth is missing. Immediately to the
right of this incomplete flagellum is a “double”
flagellum, containing one complete axoneme plus
several doublets and one or two single micro-
tubules. The organization of the latter into a com-
plete axoneme has received composite documenta-
tion in dozens of micrographs. Such cross-sections
containing one or two partially completed
axonemes are often seen in varying degrees of
development toward the \(9 + 2\) pattern. This de-
velopment is a proximo-distal process, as well as a
time-related one. Thus, the farthest removed,
membrane-bounded flagellum in Fig. 11 contains
both doublets and “singlets,” and represents a
more distal section through a developing flagel-
llum: the earliest development stage of the four
if one takes into account both the proximo-distal
and time parameters. The inset of Fig. 12 il-
ustrates a still earlier stage in a better plane of
section, showing fewer double and single micro-
tubules. In all of these flagella, the limiting mem-
brane is some distance from the microtubules,
and the cross-sectional dimensions are considerably
greater (up to \(700-800 \mu\)) than the normal

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FIGURE 9  Longitudinal section through three kinetosomes. Note microtubules arising near $K_2$ and continuing under the pellicle toward the ventral surface. These microtubules are presumed to be extensions of the chain of microtubules (spur) from $K_2$. $K_2$ exhibits a dense central area in proximal portion presumed to be the region of the cartwheel. Rhizoplast, $R_z \times 30,000$.

Inset, cross-section of mature *Tetramitus* flagellum showing nine peripheral doublets (with arms) and the central pair. Matrix components are visible between central pair and peripheral doublets, $\times 52,000$.

As has been mentioned, microtubules also function as a cytoskeleton, and are particularly numerous at the anterior lips, where they impose a special geometry which serves to direct food into the cytostome. Fig. 13 illustrates how effective these microtubules can be in forming an asymmetric lip. They seem to be spaced corresponding to the amount of support they must give. Additional electron-opaque material occurs in specific areas (arrows). The microtubules not associated with the shoulders often have "wisps" of fibrous material extending toward the membrane and inwards on the opposing side, giving an appearance similar to the matrix material in the flagellum. That this matrix area excludes ribosomes and other cytoplasmic constituents results in a region of low electron opacity. The subpellicular microtubules lie beneath the pellicle a fixed distance of 20–30 $\mu$, about the same distance that separates...
FIGURE 10 Section through an ameoba (left) and a very early transforming stage (right). Note the uneven appearance of the peripheral outline of both organisms and the density of the cytoplasm. $\times$ 15,000.

Inset, higher magnification of anterior region of transforming ameoba. Slightly oblique, longitudinal section through flagellum, including terminal plate representing distal end of kinetosome, and just above the slightly tilted axosome from which the central flagellar microtubules originate. Only one sub-pellicular microtubule can be seen (arrow). $\times$ 40,000.
FIGURE 11  Section of transforming organism through three ballooning flagella containing three developing axonemes and presumptive elements of a fourth. Left flagellum is nearly complete, containing eight peripheral doublets, with one of these out of position. Central flagellum contains one nearly complete axoneme, plus single and doublet filaments not yet arranged into a cylinder. Right flagellum contains few single and double microtubules. Note two small electron-opaque membrane-associated areas at bottom of right flagellum, and one each on opposing surfaces of central and left flagella. X 109,000.

The peripheral doublets of the axoneme from the flagellar membrane.

Fig. 14 illustrates a chain of microtubules in association with a kind of “amorphous material” (MP) seen later in Fig. 21. The 20-30 m zone of low density is evident as a clearer area before the MP begins on either side. It will be noticed in Figs. 11–14 that, according to the plane of section, a substructure is visible in the wall of the microtubule. The arrow indicates a possible stage in formation of a microtubule, in which five presumptive subunits appear to be assembled into a partial circle. The diameter of these subunits is 35–45 A, with about a 50 A center-to-center spacing. The microtubules thus conform to the general picture of a typical eucellular microtubule (Porter, 1966). The microtubules become more widely spaced as they assume a subpellicular position. Amorphous material of unknown chemical composition is seen on both sides of the chain.

Intracytoplasmic Axonemes

The transforming ameba-flagellate at mid phase has more subpellicular microtubules than are seen in the early stage (Fig. 10). A small proportion of the transforming population may also show inferior flagellar axonemes lacking the flagellar membrane. Presumably these are misdirected axonemes which grow inward instead of outward in the usual fashion, although the possibility that these represent flagellar resorption cannot be excluded. Under the phase-contrast microscope, these intracellular axonemes can be seen to beat slowly and intermittently. Two of them are just visible in Fig. 4 (arrow). Fig. 15 shows fine-structure cross-sections of all four axonemes in various stages of assembly. When these axonemes are compared with external flagella seen in Fig. 11, it seems clear that the same developmental processes are illustrated. In Fig. 15 (one of three serial sections), axoneme 1 has eight peripheral doublets, axonome 2 has five (cut obliquely and more clearly illustrated in adjacent serial sections), axonome 3 has eight with the ninth out of position just at the end of the leader, and axonome 4 has six doublets but lacks a central pair. The central pair is probably present in the cytoplasm just below the axoneme, as seen in the inset to Fig. 15 (arrow).

The least complete intracellular axoneme recognized is shown in Fig. 16. It is composed of only three doublets plus a central pair. An oblique
FIGURE 12 Section through cytostomal region of transforming organism. Microtubules show a reasonably consistent spacing of about 50 μ (edge-to-edge). Fibrillar interconnections between the microtubules can be seen in many places. Cross-striations, tilted 10–20° from the vertical, appear in several areas of different microtubules. × 60,000.

Inset, transverse section through developing flagella showing doublet and single microtubules. Note subpellicular electron-opaque areas on right and left sides. × 100,000.

section containing five doublet filaments and a central pair is shown in Fig. 17. Figs. 18, 19–20 show respectively six, seven, and eight doublets arranged around a central pair. Intracytoplasmic axonemes normally appear with the central pair and with peripheral filaments arranged as doublets; this may relate in part to the difficulty of detecting random double or single axonemal microtubules in the cytoplasm.

The transforming ameba shows a distinct polarity, which begins with the first appearance of flagella and which becomes progressively more pronounced, so that an estimate of the plane of section can be made. Serial sections show a progressive loss of peripheral doublets distally from the kinetosomes. Fig. 21 shows two incomplete axonemes, each with seven doublets. Particularly noteworthy is the doublet of the left axoneme which is skewed toward the radius (arrow). Presumably this is the newest addition which has not been incorporated into the normal orientation. This axoneme is remarkably similar to the one in the proximal axoneme in Fig. 11. Three sets of chains of cytoplasmic microtubules are present and associated with a region of cytoplasmic density, designated MP. There is a similarity between these chains of microtubules and their associated electron-opaque material (MP) and the arrangement seen in Fig. 14.

The Assembly of the Kinetome

relationship with the nucleus: A set of four serial sections cutting through all four kinetosomes at an early developmental stage had provided some information about the kinetome assembly. Probably the most outstanding feature shown is the direct connection between the nucleus and the base of one kinetosome (K3) by means of an extremely long nuclear process (Fig. 22). Except for this process, the nucleus is typical for Tetramitus and reveals a double nuclear membrane, ribosomes attached to the outer membrane, and four pores in surface view in the lower right corner. The plane of section has presumably missed the nucleolus. At higher magnification (Fig. 23) the double membrane extension can be traced to a dense mass occupying the center of K3; this mass in turn extends well into the kinetosomal cylinder.

assembly of the kinetosome: In Fig. 24, which is a serial section to Fig. 22 and 23, sections through K1 (above) and K2 are shown. K2 is believed to represent a developmental stage, with a long (0.65 μ) microtubule on the
The assembly of the flagellum: In Fig. 23, $K_4$ represents a mature kinetosome, in association with a flagellar bud. The distal part of $K_4$ shows three members of the right triplet and two of the left, with no others visible. The kinetosome is closed at its distal end by a terminal plate, which has the appearance of a pyramid at this stage, with the apex of the pyramid perhaps representing the axial granule of Gibbons and Grimstone (1960). From this apex one of the central pair of axonemal microtubules arises. At the upper ends of the right triplet, at the level of the terminal plate where one of the triplet subfibrils of the kinetosome should end, an aggregation of loosely organized material occupies the position where new doublets will form or are forming. On the left side of the flagellar bud, in line with the left kinetosomal microtubule, a short microtubule is present; it seems poorly formed. The relationship between the longitudinally skewed microtubule extending proximally from the tip and the developing axoneme is enigmatic.

A later stage in development is represented by the axoneme arising from $K_1$ (of the same organism as shown in Figs. 22 and 23), illustrated in Figs. 24 and 25. In Fig. 24, the upper end of $K_1$ is continuous with the base of a flagellar axoneme extending into a balloon-shaped, membrane-bounded flagellar unit. Such balloons have been identified in cross-section (Fig. 11) and are believed to be the next stage in development after the flagellar bud seen in Fig. 23. An aggregation of electron-opaque material occurs on the left inner margin of the membrane. The interior of the balloon consists of a loosely arranged series of tiny filaments, many of which in Fig. 25 appear to have a substructure with a 35-50 Å periodicity. The obliquely sectioned end of the most distal, right microtubule in Fig. 24 coin-
FIGURE 15 Section through a transforming organism showing four intracellular axonemes (1, 2, 3, and 4). All axonemes are incomplete. One axoneme (3) shows the ninth doublet (leader) present but quite far removed from its normal position. Another axoneme (4) contains six peripheral doublets, with the central pair missing. X 40,000.

Inset, enlargement of axonemal region (4) showing the central pair (arrow) some distance away in the cytoplasm. X 66,000.

cides with a small terminal spiral (S), which is thicker than any of the other filaments in the balloon.

Fig. 25 is a serial section to Fig. 24 showing the termination of the axoneme originating from K1. The left microtubule ends in a long, thin filament (F) which extends nearly to the flagellar tip. The microtubule immediately adjacent appears single except for a small region in the middle, where it is present as a complete doublet (D). The short member of the doublet has expanded ends.

Fig. 26 represents a section through one side of an axonemal cylinder from a different organism. The central doublet continues almost to the tip. The upper doublet extends nearly halfway into the flagellar bud, then seems to reappear near the tip as a somewhat poorly formed, single microtubule. The lower microtubule exhibits a similar proximo-distal discontinuity. In fact, the microtubules of developing axonemes (Figs. 25-27) appear unusually nonlinear, "wavering" from side to side and in and out of the plane of section.

Potential sources of microtubular proteins: Since microtubules are one of the most electron-opaque components of the cell, any large aggregation of subunits prior to assembly

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Figures 16–20 Transverse sections through intracellular axonemes illustrating different numbers of peripheral doublets. Fig. 16, three peripheral doublets; × 80,000. Fig. 17, oblique section with five doublets; × 80,000. Fig. 18, six doublets; × 21,000. Fig. 19, seven doublets; × 21,000. Fig. 20, eight doublets, × 22,000.

Figure 21 Section through two incomplete axonemes in transforming stage. Axoneme on right is cut somewhat obliquely, but contains seven doublets. Axoneme at left center also contains seven doublets. Doublet at arrow is out of position with respect to its orientation and also its distance from its nearest neighbor. Microtubules are present as short chains. They frequently seem to be associated with an amorphous material (MP) which may represent unassembled microtubular protein. X 49,000.

should be visible. Positive identification of unassembled microtubular protein would require correlative observational and chemical methods. To our knowledge, at this time there are no methods with sufficient sensitivity which would allow this correlation in Tetramitus. Without this, one can only infer a functional relationship based on location and appearance. Nevertheless, it is important to identify potential aggregations of unassembled microtubular protein, if they exist, as a basis for future work. In this paper, the designation MP, without any implication that chemical information is available, will be used to indicate electron-opaque areas which might represent accumulation of such material.

Two general categories of potential sources of microtubule protein (MP) can be distinguished. The first is a more or less diffuse cytoplasmic aggregation, which is closely associated with chains of microtubules (Figs. 14, 21), or may be somewhat more compact and spherical, as illustrated in Fig. 27. The second type has greater density, is more “granular,” and seems most often associated with the base of “young” kinetosomes, as illustrated in Figs. 24, 29, 30. The latter are similar in appearance to the dense mass occupying the center of K3 in Fig. 23, which has a persisting
nuclear association. This second category is especially interesting, and is well illustrated in four micrographs.

In the first of these (Fig. 24), an MP “body” (MP) lies at the base of $K_2$. From this body a broad ribbon apparently extends upward to the left side of $K_2$. It may be only coincidental that the spiral (arrow), tentatively assumed to represent a stage of assembly of $K_3$, also arises on this side.

Fig. 28 is a very thin longitudinal section of a kinetosome and flagellar bud. At the base of the kinetosome is a spherical MP body which appears continuous with microtubule-like elements in the center of the kinetosome.

Fig. 29 illustrates a presumptive prokinetosome on the right. At the base of this kinetosome a pair of microtubules apparently are joined to an MP body (arrows). The kinetosome can be identified as $K_4$ with a portion of $K_5$ and the rhizoplast visible on the left. Note the distal “unraveled” appearance of the microtubules of $K_4$.

Fig. 30 shows a similar MP body extending into the lumen of another prokinetosome (probably $K_5$ in this case). From the distal sides of this body a pair of microtubules appears to originate (arrows). In this orientation, these microtubules readily become a part of the kinetosomal cylinder.

MITOCHONDRIA AND THE RHIZOPLAST:

A characteristic of the MP aggregations is that they are not membrane-bounded. Membrane-bounded bodies do occur in the vicinity of the kinetome, and it is difficult to decide whether such bodies are mitochondria, modified mitochondria, or something else altogether. In Fig. 22, $M_1$ is reasonably typical of a protozoan mitochondrion with tubular cristae. $M_2$ has few tubular cristae and is very dense; $M_3$ has no identifiable tubular cristae at all. Nevertheless, on the basis of the possession of an external membrane, one assumes that all of these represent mitochondria in different physiological states. Such mitochondria have frequently been observed in close association with the rhizoplast.

The rhizoplast of *Tetramitus* has a nonuniform periodicity, as to both spacing and direction. In Fig. 23 the periodicity is 130 Å, with the wide bands measuring about 90 Å and the narrow bands about 40 Å. The average periodicity of 10 samples was 120 Å, with the wide bands averaging 80 Å and narrow bands 40 Å. The wide bands exhibited considerably greater variation in size than did the narrow bands.

DISCUSSION

*Tetramitus rostratus* has good potential for studies of microtubules. The extreme morphological dichotomy coupled with the capability of maintaining either the ameba or the flagellate as a separate stage provides a differentiating system equivalent to that of a multicellular organism. The direction of the differentiation is a functional one, resulting in a body form specialized for phagotrophy while swimming. The principal morphogenetic processes involve not only the formation of four flagella, but also the assembly of a cytoskeleton which simultaneously creates the phagotrophic shape and anchors the lashing flagella. The key to the differentiation is the kinetome and its cytoplasmic elaborations. The four kinetosomes and their flagella, added to the two spurs and the rhizoplast, provide the backbone of the transformation. Since the flagella, rhizoplast, and spurs all arise in close association with the kinetosomes, the first requirements for transformation are four kinetosomes.

Much has been written about the origin of kinetosomes. They have been linked to several kinds of precursors, including centrioles (Hennebugy, 1898; Lenhossek, 1898; Sotelo and Trujillo-Cerón, 1958), procentrioles (Gall, 1961), pericentriolar dense particles (Bernhard and de Harven, 1960), a 100 m/s circular profile (Ehret and de Haller, 1963), and lost chromosomal kinetochores (Pollister and Pollister, 1943). Lwoff (1950) claimed that in ciliates kinetosomes arise from preexisting kinetosomes by division, whereas Bradbury and Pitelka’s (1963) ultrastructural studies suggest more of an “organizational” function for the old kinetosome. In most of the suggestions of precursors, the concept of a requirement for a nucleic acid template to initiate, through however many steps of RNA and protein synthesis, the specific triplet morphology is implicit. Evidence for DNA in the kinetosome itself has recently been given by Argetsinger (1965), Hoffman (1965), and Randall and Disbrey (1965), among others.

But *Tetramitus* poses a unique problem in this regard, because kinetosomes are of two kinds: those with a spur, and those without. Kinetosomes usually seem to arise in pairs (Pitelka, 1963), and early in this study it was assumed that $K_1$ and $K_4$ were one pair, while the other pair, $K_2$ and $K_3$, were arranged with their microtubular triplets parallel in an antero-posterior axis and at right
FIGS. 22 and 23, first of three serial sections through the developing kinetome of a mature flagellate.

**Figure 22** Longitudinal section through a short $K_3$, presumed to be an early developmental stage, and a completed $K_4$, with its very early flagellar bud. Especially noteworthy is the very elongate nuclear process connecting to the base of the young $K_3$. Nucleus (N) contains typical double membrane with external ribosomes, and four nuclear pores obliquely sectioned at proximal end. Some of the spur of $K_3$ appears at the upper left, along with some rhizoplast material. Microtubules of one of the lips appear on the right. A mitochondrion (M) appears just below the rhizoplast and $K_3$. Three other mitochondria are shown, ranging from a type with typical tubular cristae (M1) to progressively less typical internal morphology (M2, M3). $\times 34,500$. 

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Figure 23 Enlargement of the region of kinetid of Fig. 22. K$_3$ contains microtubules of different length (MT$_1$, MT$_2$). MT$_1$ lacks an inner wall proximately; MT$_2$ appears frayed distally (see text). The double nuclear membrane is continuous with a dense mass at base of K$_3$ which extends upward into interior. Flagellar bud appears at the termination of K$_4$ (marked by terminal plate and axosome at the level of the cell surface). From the axosome one central pair of microtubules extends about halfway into the bud. From the right triplet, diffuse material, corresponding to the position where new axonemal microtubules will form or are forming, extends into flagellar bud. The left side is interrupted in its extension, with short microtubular piece on the left. X 115,000.
FIGURE 24 Serial section to Figs. 22 and 23 showing portions of the other two kinetosomes (K₁, K₂). The tip of K₁ is continuous with a balloon-shaped expansion representing a later stage than the bud of K₄. Its interior is filled with a reticulated mass of fine filaments, into which the obliquely sectioned developing axoneme extends. The tip of the right distal doublet, as it leaves the plane of section, is continuous with a filamentous spiral (S). The lower kinetosome (K₂) is believed to be an intermediate developmental stage. Just below the kinetosome is a body (MP) from which a ribbon of material appears to extend into the base of K₂ on the left side. This ribbon may be continuous with a helical or spirally arranged extension (arrow) which is assumed to represent a stage in the assembly of the left side of K₂. X 61,500.

FIGURE 25 A serial section to Fig. 24, showing the distal region of the flagellar balloon of K₁. On the left, running just beneath and parallel to the flagellar membrane, is a dense matrix, seen also in Fig. 24. The left axonemal component occurs as a doublet proximally, then as a single microtubule which terminates in a finer filament (F). Immediately adjacent is a doublet (D), one member of which appears to be shorter than the other. X 84,000.

angles in a transverse plane. This arrangement differs from that described by Gall (1961) and others for centrioles, and the geometry of self-replication becomes more difficult to explain if one has to account for two different types of kineto-
somes arranged in such a special relationship. It has been assumed that *Tetramitus* flagellates divide following kinetosomal replication. On the other hand, both *Naegleria* and *Tetramitus* amebae apparently lack kinetosomes (Schuster, 1963; Dingle and Fulton, 1966; Outka, unpublished observations, 1966); therefore they must make them anew.

The idea that the nucleus itself might play a role in some direct fashion has not been recently emphasized. Hollande summarized the available evidence prior to 1942 relative to the origin of kinetosomes (blepharoplasts) in flagellates. Every possibility, including the origin from the nucleus or an intranuclear centriole, was apparently offered by one or more of the early light microscopists. Hollande himself seemed to lean toward a kinetosome-division theory for most flagellates, e.g. *Euglena* (1942, page 96), but he suggested that a pair of granules migrated from the region of the nucleolus just prior to transformation in *Tetramitus* (Hollande, 1942, page 184, plate XV). Baker (1926), studying *Euglena agilis*, traced the origin of the kinetic complex from granules arising near the endosome during early prophase; the development of a new apparatus for each daughter cell was coupled with the resorption of the old one. Obviously, resolution greater than what is available with the light microscope is needed to determine whether such granules exist and, if so, whether they contain a triplet substructure.

Hollande's description of flagellate division in *Tetramitus* is reasonably typical for symmetrigenic fission in flagellates. He indicates that division is polarized about two kinetosomes. The nucleus normally undergoes a mitotic sequence following the appearance of two sets of flagella. The nucleus is not closely applied to the kinetosomes, and the spindle on which the chromosomes move is intranuclear. A connecting strand, the desmose, joins the old and new kinetosomes for a time before cytokinesis is completed. Thus the nucleus of the organism shown in Figs. 22-25 is in interphase by reason of its position in the cell-division cycle, as well as by its ultrastructural appearance. The nucleolus, which persists throughout the cell cycle, is presumably out of the plane of section; there is no evidence that it is involved with the process. Also, there is no evidence for an intranuclear procentriolar or prokinetosomal body.

The conclusion that the nucleus is associated with the kinetosome is a descriptive one. We have seen this association between a nuclear process and
FIGURE 27 An oblique section through a kinetosome and two flagellar buds of a mature flagellate. The kinetosome includes components of the spur on the left, a suggestion of a helical assembly on the right, and an MP body (MP) in the adjacent cytoplasm. × 28,500.

FIGURE 28 Longitudinal section through a kinetosome and flagellar bud. A granular MP body is present at the base of the kinetosome. Several microtubule-like elements appear to extend from the MP body into the lumen of the kinetosome. × 35,500.

Some part of a kinetome often enough to be certain of its reality; we are not certain of its significance. An important question in this regard relates to the timing of the association; it is the authors' opinion that this association is transitory and occurs only during the early developmental stages of the kinetosome. This point becomes vital to an eventual understanding of what the nucleus is doing and is worthy of elaboration.

It is remarkable that the literature contains so few confirmed ultrastructural descriptions of developing basal bodies (however, see Bradbury and Pielka, 1965). One question is what would they look like? The answer is presumably like procentrioles (Gall, 1961; Dirksen and Crocker, 1966). These really resemble very short basal bodies, perhaps the cartwheel or basal portion only. It becomes exceedingly important, then, to decide whether the kinetosome with the most obvious nuclear association (K₁ in Fig. 23) is an early developmental stage of the procentriole type (a prokinetosome) or represents an oblique section through a mature kinetosome. The authors believe that K₁ is a prokinetosome; this interpretation is based on three features, as follows.

(a) The four kinetosomes of Tetramitus are always positioned quite parallel to each other. K₁ tends to be the least restricted, deviating by as much as 15°. K₂, K₃, and K₄ rarely vary by as much as 10° from each other. Similarly, the triplet members of the kinetosomal cylinder are remarkably parallel, with almost no variation in this regard.

(b) Considerations of stereology (Elias and Pauly, 1965) can provide reliable estimates of the inclination of a line (fibers, filaments, and fibrils) according to the formula \( p = t \cdot \cos \alpha \), where \( \alpha \) is the angle of inclination to the plane of section, \( t \) is the section thickness, and \( p \) is the apparent length of the line.

If this formula is applied first to K₂ in Fig. 24, on the assumption that the section thickness is 75 nm, for the microtubule (not the spiral) on the left, the inclination is 17°; for the entire microtubule on the right, 7°; and for the straight line portion of the right microtubule, 10°. One concludes that either the microtubules of the kinetosomal cylinder are not parallel, or they are of different lengths.

If the formula is applied to the left microtubule of the K₁ flagellum in Fig. 24, the calculated angle of inclination to the plane of section is 14°. Since the axoneme is essentially a cylinder, the oblique
FIGURE 29 Longitudinal section through a prokinetosome. A granular MP body is present at the base of the prokinetosome. Two microtubules appear to extend from the MP body (arrows) into the lumen of the kinetosome. X 77,000.

FIGURE 30 Longitudinal section through another prokinetosome. A granular MP body extends into the lumen of the proximal end of the prokinetosome. A pair of microtubules extends upwards from the distal end of the MP body (arrows). X 71,500.

section of the cylinder appears as an ellipse, a geometric form markedly different from that of \( K_2 \). If \( K_3 \) were an obliquely sectioned cylinder, it would have to assume an elliptical form at its upper end. Since it does not, \( K_3 \) is therefore a cylinder composed of microtubules of different length. Thus \( K_3 \) and \( K_4 \) are parallel, and the plane of section through them is almost exactly longitudinal.

If the same rationale and formula are applied to \( K_3 \) in Fig. 23, in which the section thickness can be reliably estimated as about 75 m\( \mu \), the angle of inclination calculated for \( MT_1 \) is 16°, or almost identical with the angle calculated for the left microtubule of \( K_2 \). In fact, \( K_3 \) is almost identical in appearance with \( K_3 \) except for the long right microtubule in the latter. Since there is no indication of an elliptical form to the \( K_3 \) cylinder, and since there is no reason to assume such an unusual inclination as 16° from its colleagues, \( K_2 \) and \( K_4 \), we submit that \( K_3 \) is sectioned longitudinally also, that it is probably earlier in development than \( K_3 \), and that it is therefore truly a prokinetosome.

(c) The final point in respect to the “age” of \( K_3 \) relates to the frayed appearance of the ends of microtubules, especially the distal end of \( MT_2 \). This appearance is similar to the right side of the \( K_4 \) axoneme. In both cases it seems reasonable to tentatively suggest that this appearance may also represent a developmental phase.

If one assumes that the interpretation of nucleus-prokinetosome association is correct, what might be the role of the nucleus at this time? The stage of development shown in Fig. 23, while early, is a reasonably complete one in that the four kinetids are in place, \( K_3 \) and \( K_4 \) appear linked together by fibrous material, and both the rhizoplast and spur are represented. A kinetosome of this precision and complexity must require a high degree of special “ordering.” Perhaps we need a new, dynamic concept of the role of the eucellular nucleus which would include the proper positioning of multiple elements in a complex organelle. Once positioned and “nucleated,” self-assembly of microtubules and other components could occur.

The nucleus-kinetosomal association removes any theoretical requirement for DNA in the Tetramitus kinetosome, and in fact suggests that this organelle is not self-replicating. Thus the nucleus
might substitute for and function like kinetosomal DNA suspected to be in other organisms. Randall and Disbrey (1965, page 487) have discussed some possible functions for kinetosomal DNA. These could include the control of both the synthesis of microtubular proteins and their organization into the final structure. Alternatively, only an organizational function may exist, in which case the protein could be made elsewhere in the cytoplasm. Randall and Disbrey suggest that protein chains might be polymerized into a globular form by enzymes coded by DNA, after which self-assembly could occur.

**FLAGELLAR AND MICROTUBULAR FORMATION:** The development of flagella can be followed in both transverse and longitudinal sections. Some of its characteristic features are summarized as follows.

(a) First, a membrane-bound "bud" is formed, followed by an expanded balloon. The balloon becomes filled with a fibrillar material which could conceivably contribute to the axonemal assembly. Since they are absent from mature flagella, the unique membrane-associated, amorphous, dense areas (Figs. 11; 12, inset; 24; 25) might also be implicated as precursors to microtubules or membranes.

(b) The assembly of axonemal microtubules occurs coincident with flagellar outgrowth. The assembly begins at the kinetosome and progresses distally (Figs. 11; 12, inset; 23-27), with some regional, temporary reversal of that order. Single microtubules are more common distally, and are presumed to be formed before the doublet is made.

(c) The central pair of microtubules is formed early, always before the peripheral cylinder of doublets is complete. At first the doublets are randomly arranged, but then they are positioned sequentially (Figs. 11, 15-21).

(d) A flagellum can be judged mature when it has a complete cylinder (including arms on the peripheral doublets), and when the membrane approaches to within 25-35 μm. This microtubule-to-membrane distance is the same as the distance between the subpellicular microtubules and the pellicle, or the subpellicular microtubules in regions of the anterior lips, or the "zone of exclusion" of cytoplasmic microtubules, and perhaps may be referable to a basic lateral substructure of the microtubule itself.

**POTENTIAL SOURCES OF MICROTUBULAR PROTEIN:** Microtubules can be formed very rapidly; it is therefore assumed that the process must involve an assembly of preformed protein subunits. While the "packaging" of protein for export by Golgi membranes is now well documented, little is known about the way proteins are stored for internal use. Stockinger and Circelli (1965), working on respiratory ciliated epithelium, described some "granular precursors" of only tenuous association with developing centrioles, and concluded that centrioles arose de novo. Dirksen and Crocker (1966), also studying ciliated epithelium, described two types of precursors to centrioles. One of these, the proliferative element, most nearly resembles the loosely organized MP material (Fig. 14). The other precursor, their condensation form, which becomes the center around which several new centrioles originate, closely resembles the granular MP body seen in Figs. 24 and 28-30. Whether these structures are homologous is uncertain.

For Tetramitus, one possibility is that both forms of MP are simply different states of concentration; if they really do represent microtubular protein in some sort of a precursor configuration, their function would be to be at the right place to provide the molecules for assembly. A second possibility is that granular MP does something in addition to or different than the more diffuse MP, e.g., it might contain different kinds of proteins or it might in fact modify common microtubular proteins into forms which would allow assembly into triplets and other specific variations required by the kinetosome. It seems possible that growth of kinetosomal microtubules may involve some sort of initial proximal assembly (Figs. 24, 28-30), whereas axonemal microtubules appear to be growing at the tip (Figs. 23, 25). Finally, the association between the material in the center of K₃ in Figs. 22 and 23, which resembles granular MP, and the elongate nuclear process suggests that the nucleus may be the source for granular MP or at least may make a contribution to it.

**THE RHIZOPLAST:** The other major kinetosomal organelle that is being assembled concomitantly with the microtubules is the rhizoplast which must represent a main protein commitment for Tetramitus. Since striated fibrils of this type are almost ubiquitous in the Protozoa, e.g., the kinetodesmal fibrils of ciliates, striated fibrils in flagellates, etc., it would seem possible that proteins similar to those used in the assembly of kinetosomes and axonemes might be used. The
only indications of a special relationship that we have observed is a frequently close association with mitochondria. Schuster (1963) commented on the “invariable” association of the rhizoplast and mitochondria in Naegleria, and suggested that an energy requirement might exist for the elaboration or maintenance of this structure. In spite of the fact that the rhizoplast periodicity is much different from that of collagen, there remains at least a superficial resemblance of the former to the latter in both structure and function.

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