MICROTUBULE BIOGENESIS AND CELL SHAPE IN *OCHROMONAS*

I. The Distribution of Cytoplasmic and Mitotic Microtubules

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ABSTRACT

In the first of two companion papers which attempt to correlate microtubules and their nucleating sites with developmental and cell division patterns in the unicellular flagellate, *Ochromonas*, the distribution of cytoplasmic and mitotic microtubules and various kinetosome-related fibers are detailed. Of the five kinetosome-related fibers, which have been found in *Ochromonas*, two, the kineto-beak fibers and the rhizoplast fibers are utilized as attachment sites for distinct groups of microtubules. The set of microtubules attached to the kineto-beak fibers apparently shape the anterior beak region of the cell whereas the rhizoplast microtubules appear to extend into and shape the tail in vegetative cells. In mitotic cells a rhizoplast is found at each spindle pole apparently serving as foci for the spindle microtubules. These findings are discussed in relation to the less well defined attachment sites for vegetative and mitotic microtubules in other kinds of cells. It is noted that the effects of depolymerizing microtubules in vivo might be easily quantitated in whole populations since no external wall or pellicle contributes to the maintenance or the biogenesis of the characteristic cell form of *Ochromonas*.

Although microtubules from different sources may vary only slightly in structure (Porter, 1966) and composition (Olmsted et al., 1970, 1971; Fulton et al., 1971), their apparent effects in mediating developmental patterns are strikingly diverse and may range from vertebrate lens induction (Byers and Porter, 1964) to plant cell wall orientation (Ledbetter and Porter, 1963). To explain these multiple activities of microtubules, a variety of recent studies have attempted to define (*a*) the timing of the appearance of microtubules (Tilney and Gibbins, 1969; Gibbins et al., 1969; Pickett-Heaps and Northcote, 1966; Cronshaw and Esau, 1968; Outka and Klauss, 1967) (*b*) the number and distribution of microtubules and microtubule "linkers" (Byers and Porter, 1964; Tilney and Porter, 1967; Tilney and Byers, 1969; Roth et al., 1970; MacDonald and Kitching, 1967), and (*c*) the site of microtubule biogenesis and growth (Gibbins et al., 1969; Tilney and Goddard, 1970; Tucker, 1970), some or all of which may provide specificity to developmental patterns.

Especially useful in these and other studies which have provided experimental evidence for a dependence on microtubules during some phase of development have been reagents (colchicine, vinblastine) and physical agents (cold, pressure) which depolymerize microtubules in vivo. However, because microtubule participation in development is often transient, the kinetics of microtuble assembly and disassembly has not been well documented for whole organisms, and the tantalizing question of how and where polymerization is initiated remains largely unanswered

for most cells. "Nucleating sites" which presumably function in microtubule initiation are often associated with kinetosomes or centrioles but are seldom well defined structurally (c.f. Pickett-Heaps, 1969).

Populations of the flagellated unicell, *Ochromonas* offer special advantages in further clarifying the developmental role of microtubules, and in clarifying some of the requirements of microtubule initiation. Individually these cells have a distinctive teardrop (pyriform) shape yet lack either a cell wall or pellicle. Collectively or individually these cells may be reversibly converted into motile spheres by treatment with colchicine, vinblastine, and other microtubule depolymerizing agents (Bouck and Brown, 1970). These preliminary findings with *Ochromonas* have been amplified and extended in the following two papers, and efforts have been made to detail the complete microtubule complement of individual cells as well as to quantitate the effects of microtubule depolymerization using cell populations. In this first report evidence is presented for at least three independent sets of microtubules. Each is associated with a portion of the *Ochromonas* asymmetry, and each set is attached to a separate kinetosome-related site. One of these sites, the rhizoplast, is demonstrated to serve not only as an attachment site for vegetative microtubules, but also provides the site of spindle microtubule attachment during mitosis. In the second report, colchicine and pressure experiments are detailed which demonstrate an obligatory role for microtubules for the construction as well as the maintenance for the species-specific cell form of *Ochromonas*, and further suggests that the sites of microtubule attachment are indeed the nucleating sites for microtubules.

Materials and Methods

Ochromonas danica was grown in defined medium as described by Aaronson and Baker (1959) and modified by Dubnau (1961). A 16-h light and 8-h dark schedule was used for most of the studies reported below, although *Ochromonas* grows well in total darkness. A large surface-to-volume ratio is required in unaerated cultures so that only 400 ml of medium was added to each 3-liter Fernbach flask (Corning Glass Works, Corning, N. Y.) for maximum uniform growth. Growth (in numbers of individuals) was assayed by direct count with a Levi hemocytometer (Clay-Adams Inc., Parsippany, N. J.). This method was found to be more reliable than measuring increases in total protein or total chlorophyll. The maximum density of cells after 3 days of growth was found to average about 5×10^6 cells/ml. Additional culturing under our conditions resulted only in an increasing number of moribund or pleomorphic cells, perhaps because of secretion of urea into the medium (Lui and Roels, 1970). For this reason all experiments and observations were performed on cells harvested 3 or 4 days after inoculation.

For electron microscopy cells were collected by centrifugation and resuspended in 3% glutaraldehyde (vol/vol) buffered with 0.1 M sodium phosphate at pH 7.0. After 2 h of fixation in glutaraldehyde the cells were rinsed four times in buffer alone and then postfixed for 2 h in cold buffered 1% (wt/vol) osmium tetroxide. Dehydration in a graded acetone series was followed by infiltration with Spurr's (1969) hardest resin mixture, and then polymerization at 60°C for 24 h. Sections of hardened blocks cut with a DuPont diamond knife were collected on Formvar-coated, copper grids, stained in uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Philips 300 electron microscope.

For light microscopy a Zeiss 1.30 NA planapochromat bright field objective and interference contrast (Nomarski) optics were utilized. Prolonged examination of slides particularly of dividing cells was facilitated by ringing the cover slip with silicone fluid (360 Medical fluid; Dow Corning Corp., Midland, Mich.). Individual flash photographs were obtained with a Zeiss microflash unit, the images recorded on Kodak Plus-X 35mm film, and the negatives developed in Diafine (Acufine, Inc., Chicago, Ill.).

RESULTS

External Appearance

Ochromonas is characterized by two anterior flagella which are different in length, orientation, and external appendages (Bouck, 1971), and by an asymmetric, approximately pear-shaped (pyriform) cell body. The complete absence of symmetry is the result of several more or less distinctive distortions of the cell surface which are generally well fixed in relation to two arbitrarily defined longitudinal axes. These longitudinal axes are in turn determined in relation to the eyespot which, because of its highly visible color, refractile properties, and eccentric location at the anterior of the cell, serves as a convenient reference point. Thus the side of eyespot displacement is herein the frontal or ventral view. Examining the cell lengthwise from the ventral position then defines the right and left portions of the cells as well as the dorsal area (Fig. 1).

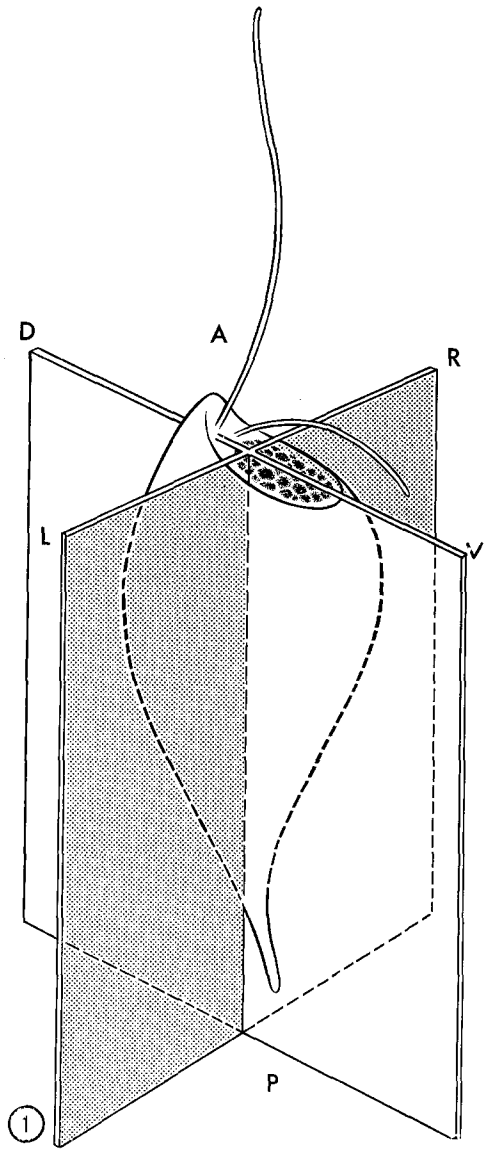

FIGURE 1 Schematic diagram of *Ochromonas danica* showing arbitrarily defined reference axes.

In addition to the flagella the three prominent asymmetries of the cell are: (*a*) the *beak* which comprises the elevated portion of the cell found in the dorsal, anterior region (Figs. 1 and 2). Near the ventral base of the beak the long flagellum extends directly forward from its point of attachment. The smaller flagellum emerges at an acute angle to the long flagellum, arches over the eyespot on the anterior surface, and terminates near the ventral side of the cell. The position of the beak relative to the flagella and eyespot is similar to that of the "proboscis" found in the spermatozoids of brown algae (Manton and Clarke, 1951) and other algae. However, the beak of *Ochromonas* is not as prominent as the proboscis nor does the underlying pattern of microtubules of the two structures appear to be homologous (see below). (*b*) The *platform* is found also at the anterior surface but ventral to the beak and to the emerging flagella. The platform overlies the eyespot and has elevated margins which constitute the platform *ridges*. The ridges thus form the right and left margins of the ventral anterior portions of the cell. (*c*) From the anterior beak and ridges the cell swells posteriorly and then tapers into a highly characteristic *tail* (Figs. 1, 2, and 15 n). The tail which varies in length in different individuals appears to be involved in swimming movements, probably serving in part as a stabilizer since the asymmetric cell rotates eccentrically during forward locomotion. The tail appears to be more labile than other portions of the cell, and may be withdrawn during cell division or under unfavorable conditions.

At no region of the cell is there evidence for either a cell wall or pellicle. Cell shape is therefore influenced to some extent by particles moving within the cell, and by the size and distribution of major organelles. Cannibalism which involves engulfment of a complete *Ochromonas* by another is not uncommon in older cultures and indicates that a cell can undergo considerable distortion when necessary.

Internal Anatomy

A variety of earlier studies (especially, Gibbs, 1962 *b*; Schuster et al., 1968; and Hibberd, 1970) which have detailed the structure and distribution of the major membraneous organelles in *Ochromonas* have been verified during the course of this work. Most importantly, the nucleus, chloroplast, eyespot, Golgi complex, and vacuole are relatively fixed in position and can therefore provide reliable markers for identifying the plane of random thin sections cut through cells. The nucleus is located near the anterior end of the cell and is surrounded in part by lobes of the chloroplast. The close association between nucleus and chloroplast is due to an extension of the nuclear membranes which envelop the chloroplast entirely (Gibbs, 1962 a), giving rise to an intramembrane continuum, the

perinuclear continuum. The perinuclear continuum appears to be important in these cells in the production of flagellar mastigonemes (Bouck, 1971). Posterior to the nucleus is found a prominent vacuole which probably contains soluble reserves (Pringsheim, 1952). The single Golgi complex, eyespot, kinetosomes, and various "rootlets" extending from the kinetosomes are all found at the anterior of the cell. The platelike collection of granules comprising the eyespot are found within a lobe of the chloroplast which extends up to and flattens slightly against the platform surface (Fig. 2).

Kinetosome-Related Structures and Microtubules

With the exception of the axonemal microtubles of the flagella none of the cytoplasmic microtubules are directly connected to or continuous with the kinetosome microtubules. However, all microtubules do appear to be associated with several structures which are themselves associated with kinetosomes. Thus since these kinetosome-related structures may function as organizing centers for microtubules, their nature and distribution is of considerable interest. Of the several "fibrous" structures which converge on one or the other of the two kinetosomes, two have longitudinal periodicities (striations) and the remainder exhibit no visible evidence of ordered substructuring (c.f. Fig. 2′ for distribution of microtubules and fibrous structures).

STRIATED FIBERS: A *kineto-kinetosome*[1] fiber appears to connect one side of the proximal end of the short flagellum with the lateral margin of the other kinetosome (Fig. 7). This fiber tapers towards the long flagellum kinetosome and consists of electron-opaque materials alternating with electron-transparent regions of varying thickness. A similar kind of fiber has also been described between the kinetosomes of the biflagellated unicell *Chlamydomonas* where it was suggested it might play a role in flagellar coordination (Ringo, 1967).

The *rhizoplast* (or *kineto-nuclear* fiber) is found in *Ochromonas* as in other cells (reviewed in Pitelka, 1969) extending from the kinetosome region towards the nuclear surface where it may run some distance along but does not make contact with the surface (Figs. 8, 9, and 10). The rhizoplast consists of fine fibers with crossbandings consisting of amorphous materials at more or less regular intervals (c.f. also Hibberd, 1970). The rhizoplast of *Ochromonas* has two very different surfaces: the upper is associated with the Golgi complex (parabasal body), whereas on the lower surface of the rhizoplast microtubules are found (Fig. 9). These microtubules (the rhizoplast microtubules) often appear to be attached at the crossbanded regions of the rhizoplast, and from there they extend towards the cell posterior and probably terminate in the tail (c.f. Brown and Bouck, 1973, adjoining paper which provides experimental evidence to demonstrate the loss and regain of rhizoplast microtubules is coincident with tail retraction and formation). The rhizoplast microtubules comprise a distinct set of microtubules in *Ochromonas* which are not visibly associated with other fibrous structures nor are they intermixed in the anterior regions of the cell with microtubules from the beak.

UNSTRIATED FIBERS: The *kineto-mitochondrial* fibers of which there may be several in a given cell, appear as strands attached to the kinetosomes at one end but end blindly in the cytoplasm. These fibers are often several microns in length, and are readily identified by their association with groups of mitochondria (Figs. 3 and 6). Mitochondria appear closely appressed to the fiber surface throughout most of their length, suggesting an energy transfer function. Conceivably the kineto-mitochondrial fiber is a primitive example of the elaborate mitochondrial (Nebenkern) associations found in sperm tails of more highly evolved organisms. Microtubules are not found associated with the kineto-mitochondrial fiber.

Kineto-stigmatic fibers originate near the kinetosome of the short flagellum (c.f. also Rouiller and Fauré-Fremiet, 1958), and sweep in an arc around the eyespot towards the ventral (frontal) surface (Fig. 11). These fibers are varied in structure For example one fiber appears to consist of two closely appressed plates, open along their margins or with fine dense materials along the margins (Fig. 7). This fiber in longitudinal section often has the trilaminar appearance characteristic of a membrane but is thicker and apparently may have free ends (Fig. 12). Another of the kineto-stigmatic fibers appears as a paired microtubular-like structure which passes between

[1] Terminology is meant to convey only some idea of the structures associated with both ends of the fiber rather than any functional interaction.

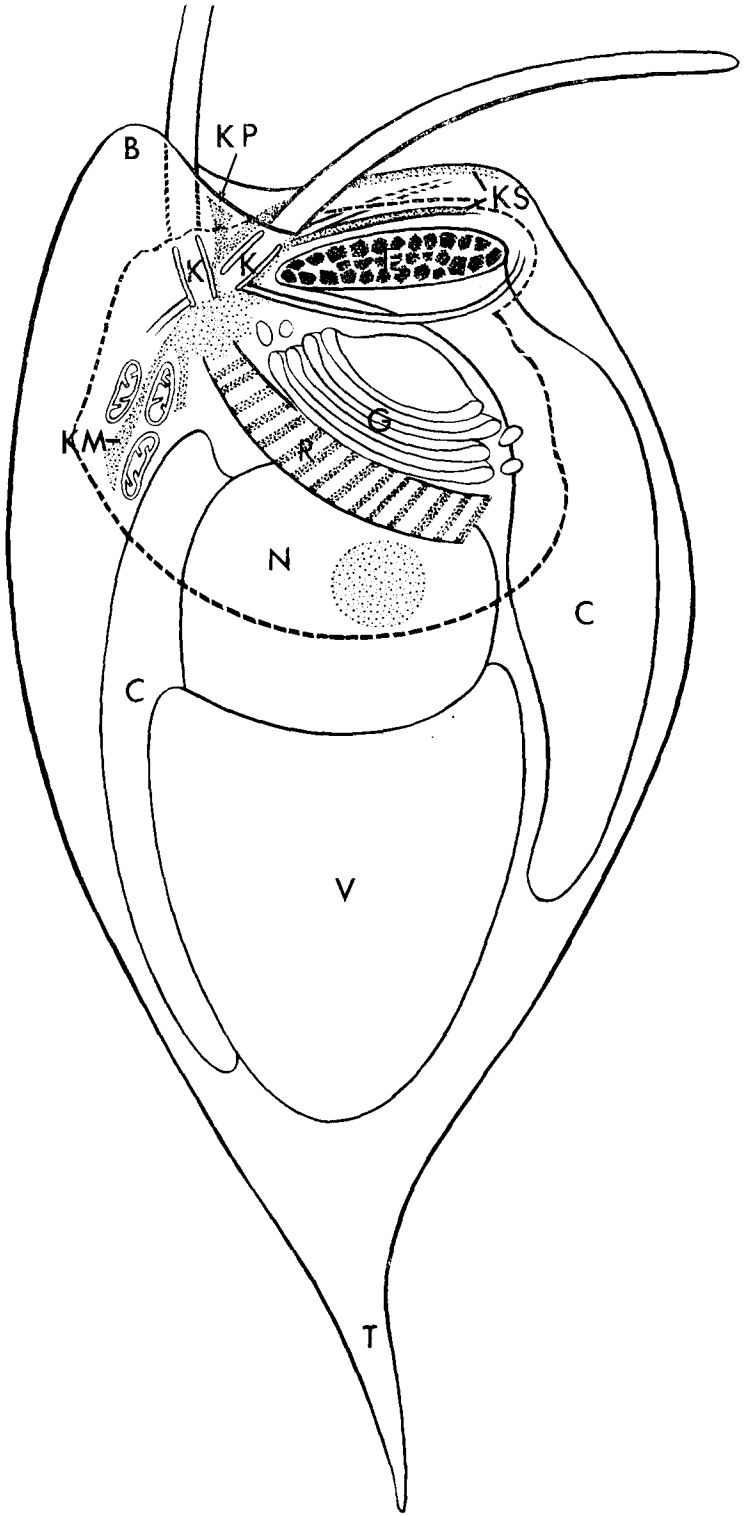

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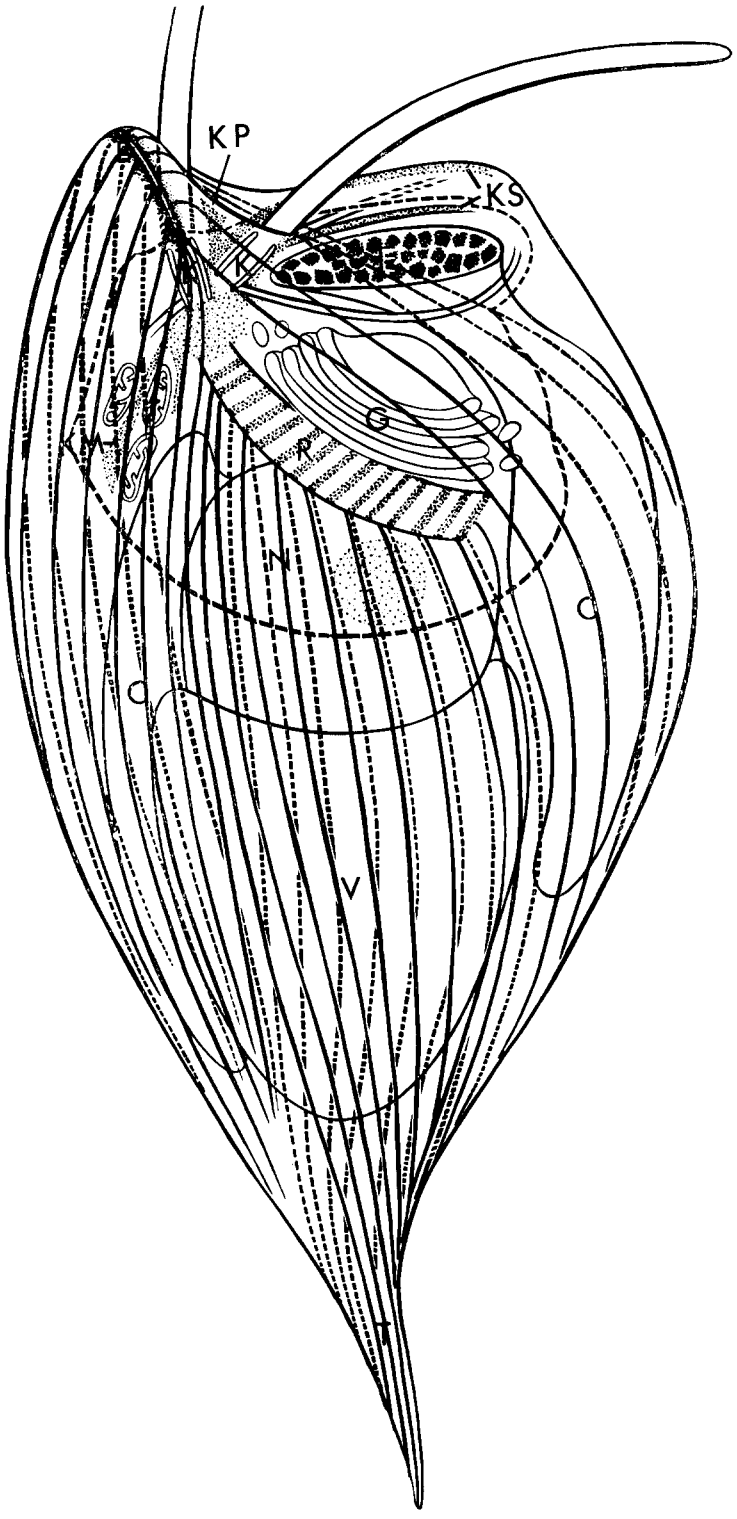
FIGURE 2 Diagrammatic representation of major components of *Ochromonas* as elaborated in text. *B*, beak, *C*, chloroplast, *E*, eyespot, *G*, Golgi complex, *K*, kinetosome, *KM*, kineto-mitochondrial fiber, *KS*, kineto-stigmatic fibers, *KP*, kineto-surface fiber, *N*, nucleus, *R*, rhizoplast (kineto-nucleus fiber), *T*, tail, *V*, vacuole.



(2')

FIGURE 2' Schematic of distribution of kineto-beak microtubules and rhizoplast microtubules superimposed over Fig. 2.

the two kinetosomes and follows an undetermined path from the center region of the cell to the eyespot region. Other elements of the kineto-stigmatic complex are poorly defined structurally, and may be found on one side (usually) of the eyespot or along both sides. A few microtubules may be found scattered in association with the fibers of the complex, but no consistent pattern has been detected, and microtubules are not a prominent feature of this part of the cell.

The *kineto-beak* fibers are significant as the other principal site of microtubule attachment. The kineto-beak fibers consist of amorphous materials closely associated with microtubules so that a portion of the beak contains microtubules and strands of amorphous materials in parallel (Figs. 3, 5, and 6). The microtubules proceed anteriorly into the beak, and then gradually change directions to form a fountain-like display. In addition to these curved microtubules others may originate abruptly at right angles to the curved microtubules apparently from the amorphous material of the fiber (Figs. 4 and 5). Consequently although microtubules of the beak may form two patterns nearly perpendicular to each other, they are associated with the same kineto-beak fiber, but are attached at different points along its curved path.

A short spur (*kineto-surface* fiber) has been found in many cells. However, it is not yet clear with which kinetosome it is associated or where on the cell surface in terminates. Presumably this fiber provides an anchor for part of the flagellar apparatus. No microtubules are associated with this fiber.

The Tail

The tail region is of special interest since it seems to have the capacity for controlled bending and because it appears to be highly labile when exposed to unfavorable conditions, usually shortening or withdrawing completely. The dominant components of the tail are microtubules (Fig. 2′, 13) which converge from some point anterior to the tail. The distribution of tail microtubules is not detectably regular, but microtubules must terminate at different lengths within the tail since there are fewer microtubules in the distal tail region where the diameter is reduced. Mitochondria are excluded throughout the tail but ribosomes and cisternae of endoplasmic reticulum may be found in the more proximal tail regions. In addition to microtubules, fine filaments are also found in the distal regions of the tail (Fig. 14).

Mitosis

During the initial phases of mitosis *Ochromonas* loses its pyriform shape first by a tail retraction, and then by a general sphering of the entire cell. Early in mitosis the paired flagellar apparatus replicates and the two sets of flagella lie adjacent to each other (Fig. 15 a). Although the precise details of this process are not yet known, it appears that the flagella grow to nearly their full length before much separation of the two sets has occurred and before prophase can be recognized (Fig. 15 a). However, by prophase the two sets of flagella have moved apart somewhat along the anterior portion of the original cell (Fig. 15 b). The chromosomes, which are individually too small to be seen with certainty in the light microscope, are aligned at metaphase in a detectable plate. As anaphase commences the two sets of chromosomes move apart (Figs. 15 b, 15 c, 15 d, and 15 e) and eventually approach the opposing poles (Fig. 15 f). During anaphase the two sets of flagella have also moved apart slightly (Figs. 15 c–15 e) but

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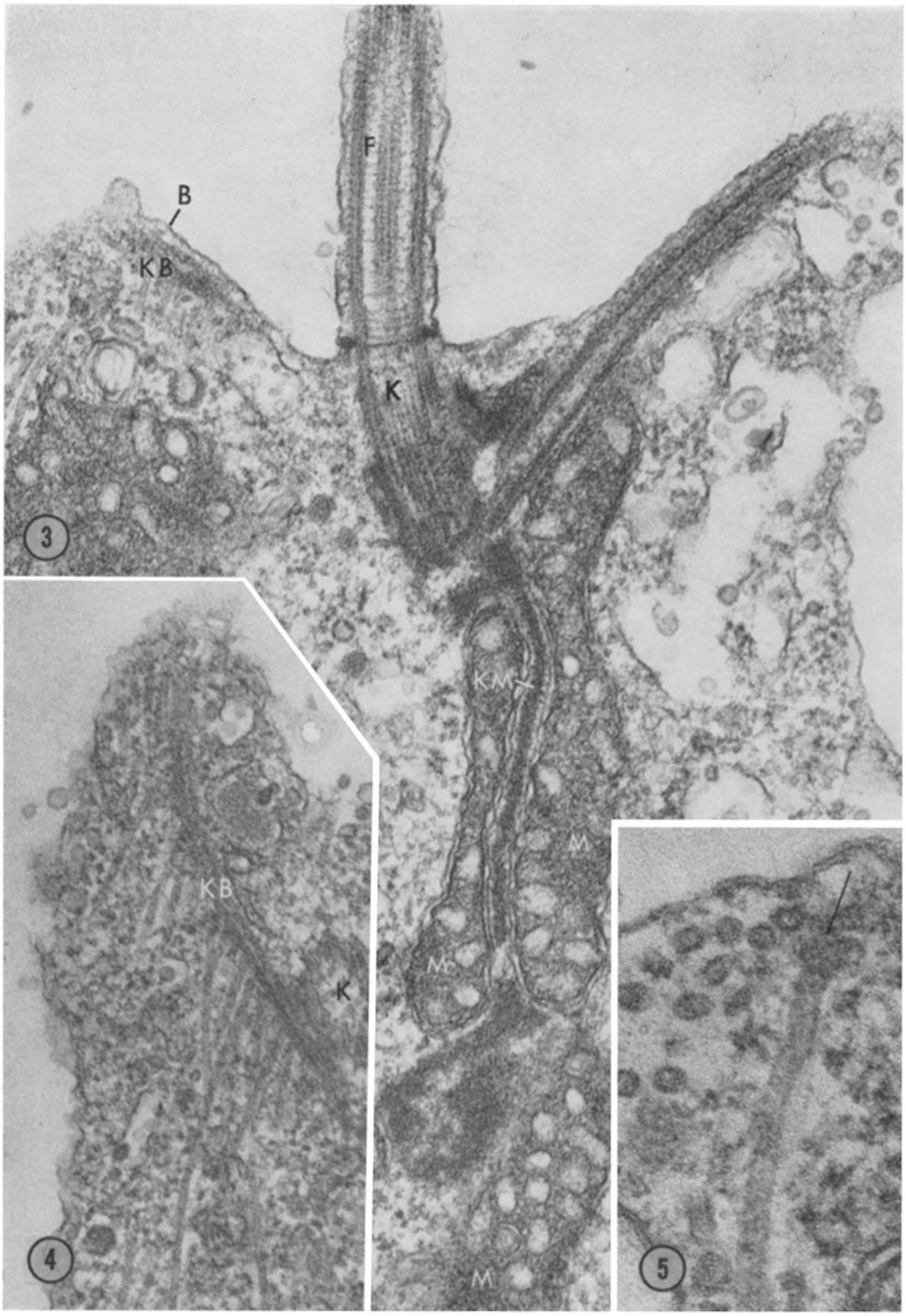
FIGURE 3 Longitudinal section cut in the dorsal-ventral plane through the kinetosome (*K*) of the long flagellum (*F*). The kineto-mitochondrial fiber (*KM*) extends from the distal end of the kinetosome and ends blindly in the cytoplasm. Characteristically mitochondria (*M*) are distributed along the margins of this fiber. Portions of the kineto-beak complex (*KB*) which underlies the beak (*B*) can also be seen in this micrograph. × 59,000.

FIGURE 4 The kineto-beak (*KB*) complex viewed from a more anterior position than in Fig. 3 illustrating the arrays of beak microtubules radiating in two directions. *K*, kinetosome. × 54,000.

FIGURE 5 Section in the right-left longitudinal plane through the kineto-beak complex showing apparent attachment of microtubule to amorphous materials (arrow). Other microtubules are seen in cross-section. This view of the beak is approximately at right angles to that of Fig. 3. ×180,000.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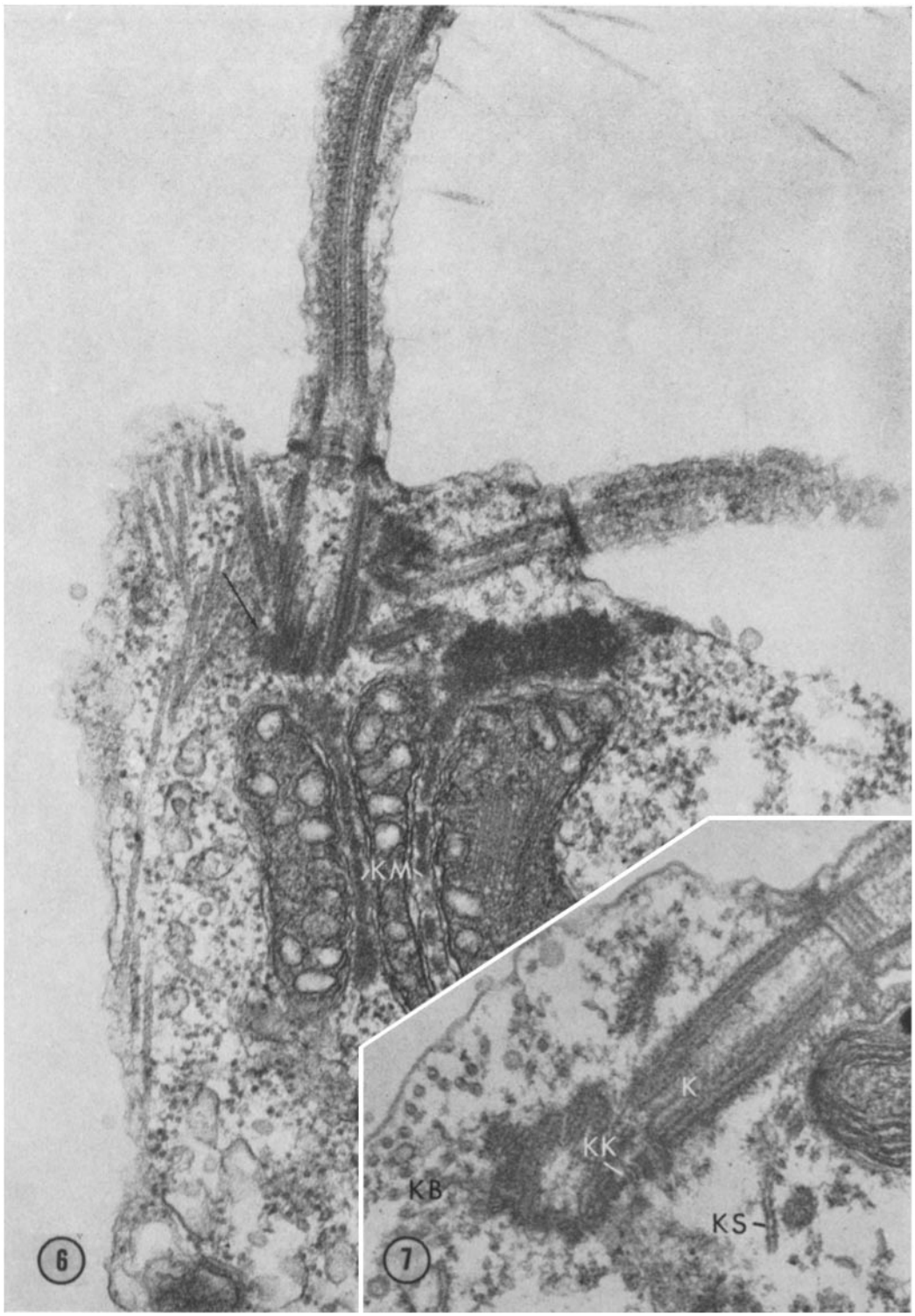
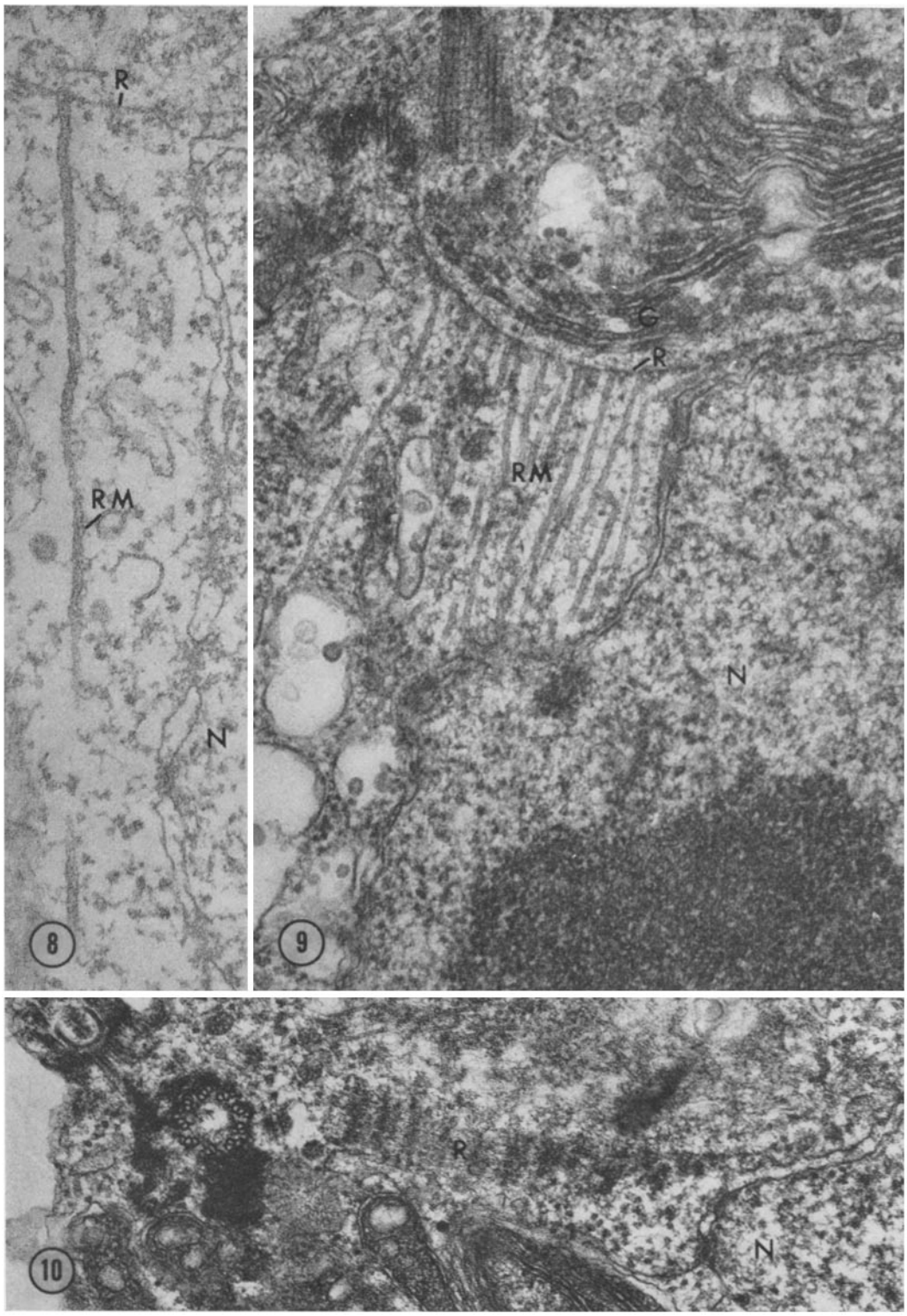


FIGURE 6 Section similar to the of Fig. 3 but showing the origin of the kineto-beak complex (arrow) at distal end of the kinetosome of the long flagellum. Portion of two kineto-mitochondrial fibers (*KM*) are also visible in this micrograph. × 53,000.

FIGURE 7 The kineto-beak complex (*KB*), kineto-kinetosome fiber (*KK*), and kineto-stigmatic fiber (*KS*) as seen from the anterior left view of *Ochromonas*. Note the kineto-stigmatic fibers (*KS*) appear as two appressed plates, open at the margins but connected by periodic bridges. *K*, kinetosome of short flagellum. × 75,000.



FIGURES 8–10 Section through the kineto-nuclear (rhizoplast) fibers seen in longitudinal (Figs. 8 and 9) and surface view (Fig. 10). Note rhizoplast (*R*) extends over the nuclear surface but does not fuse with it. The rhizoplast is polarized with a Golgi complex (*G*) along its upper surface (Fig. 9) and rhizoplast microtubules (*RM*) attached to its lower surface. *N*, nucleus. Fig. 8, × 70,000; Fig. 9, × 56,000; Fig. 10, × 54,000.

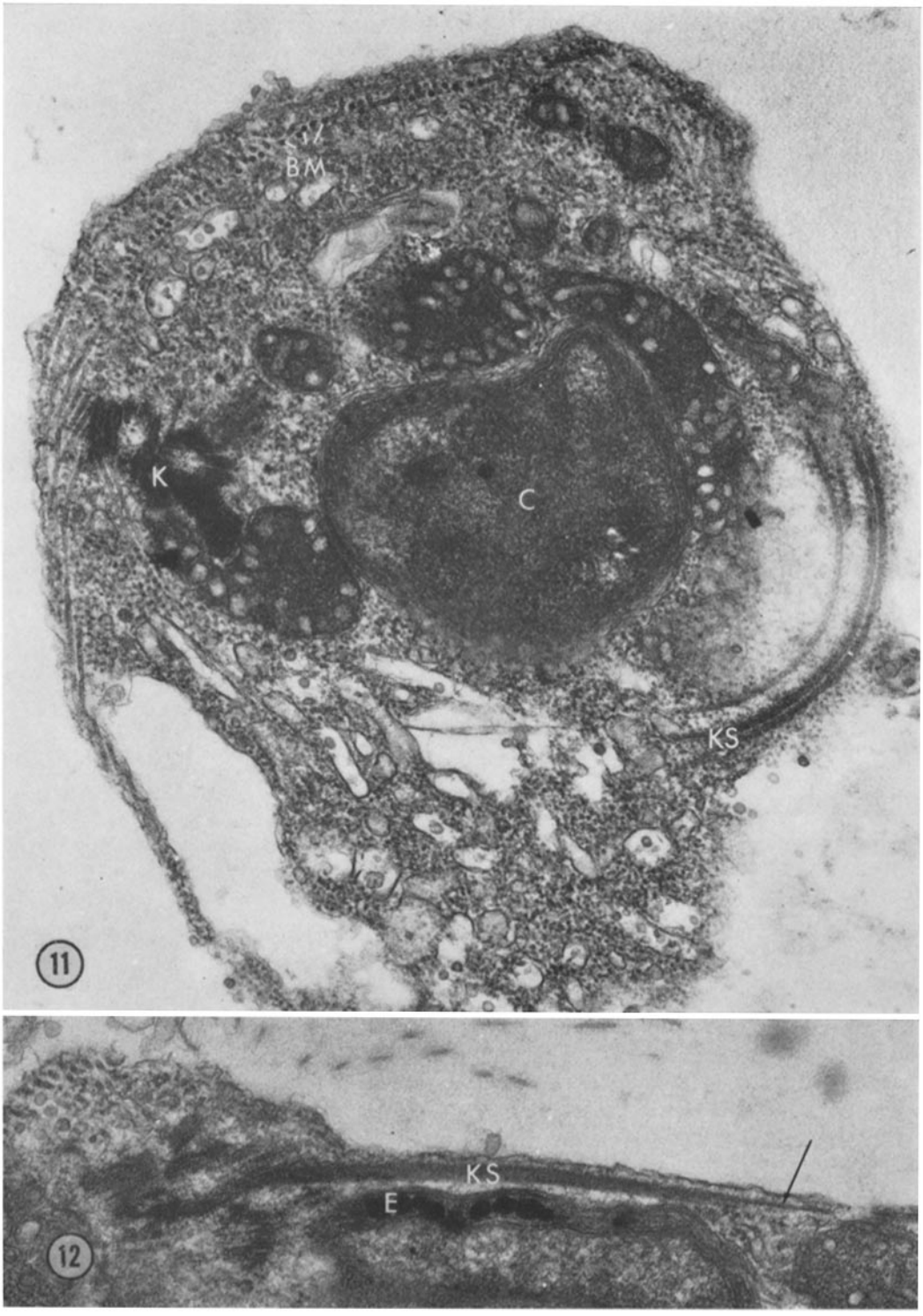


FIGURE 11 Anterior view of beak microtubules mostly in cross-section (*BM*), the two kinetosomes in glancing section, and portions of the kineto-stigmatic fibers (*KS*) in surface view. A portion of the chloroplast (*C*) cut below the eyespot is also visible. *K*, kinetosome. × 28,000.

FIGURE 12 Section cut at nearly right angles to Fig. 11. Note kineto-stigmatic fibers (*KS*) with membrane-like appearance (arrow) and compare with cross-section in Fig. 7. *E*, eyespot. × 36,000.

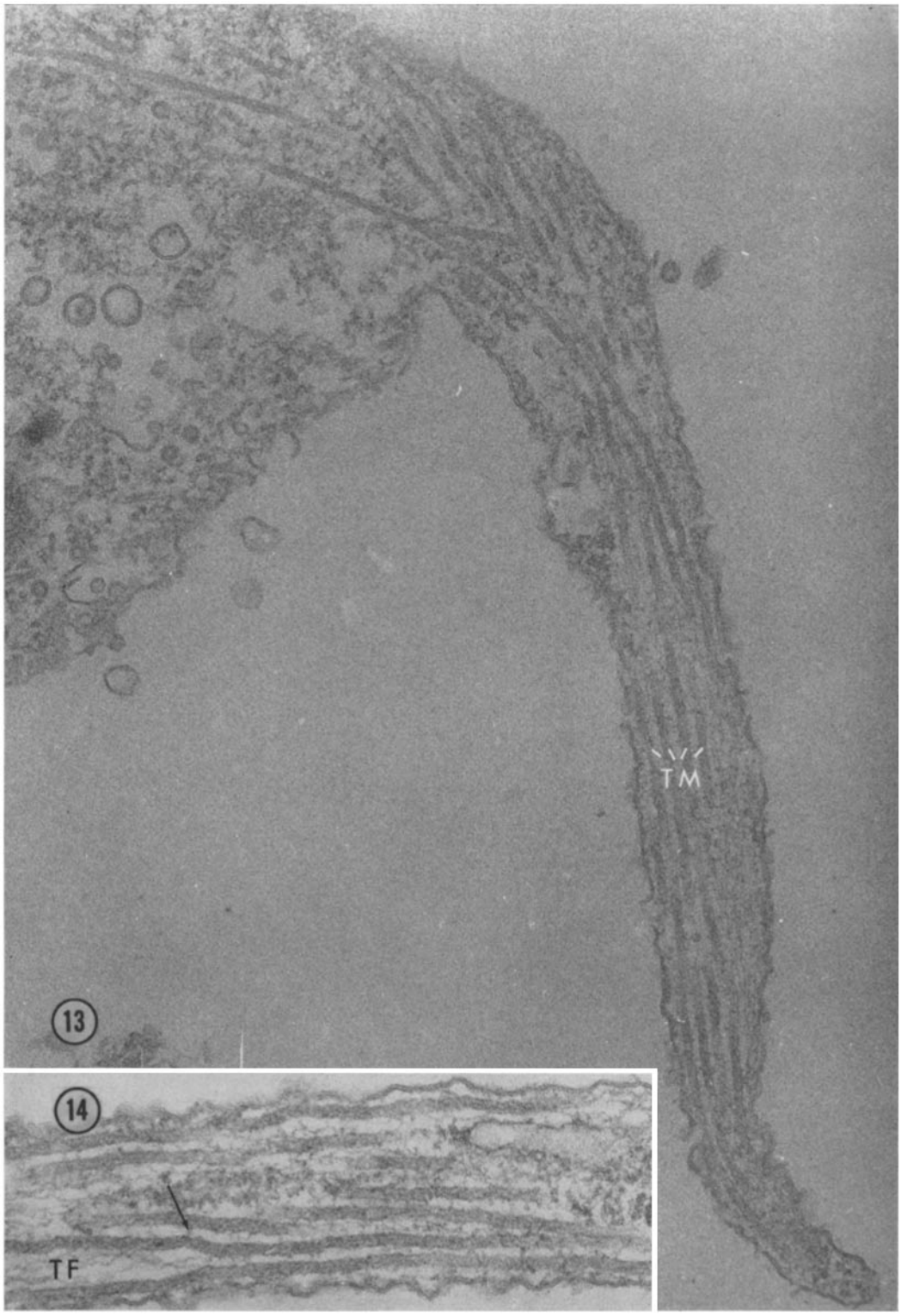


FIGURE 13 Longitudinal section of a flexed tail. Note microtubules (*TM*) are the dominant structural component. × 60,000.

FIGURE 14 Distal portion of the tail illustrating fine filaments (*TF*) and possible cross-bridges between microtubules at arrow (seen most convincingly if micrograph is viewed obliquely from left margin). × 72,000.

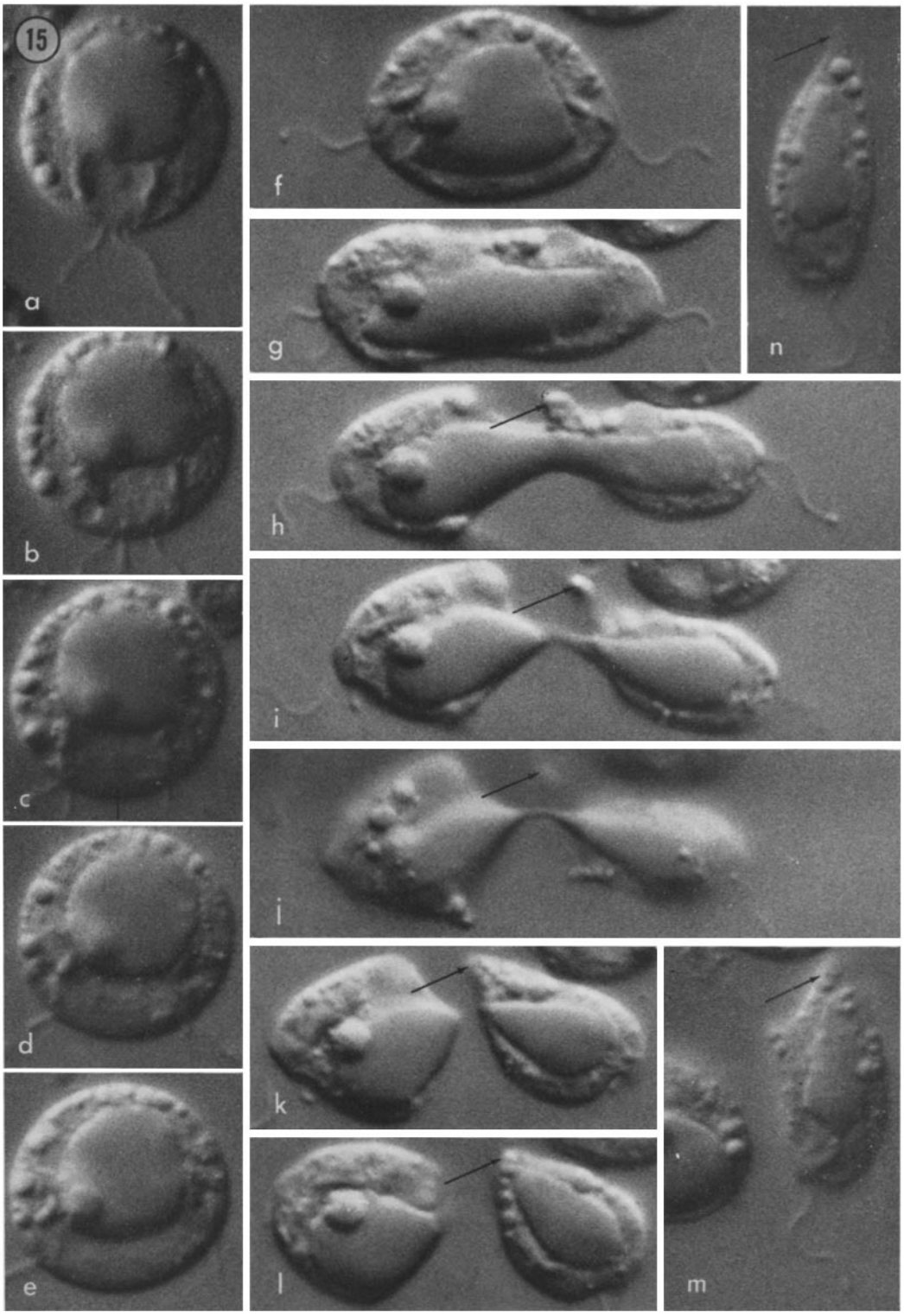
when the chromosomes reach the poles, the flagella move rapidly apart, until they are separated by nearly 180° (Figs. 15 f–15 i). The dividing cell assumes an increasingly oblong shape concomitantly with the migration of the two sets of flagella and with the increasing separation of the spindle poles. This stretching of the cell is especially apparent in the change of shape of the vacuole (Figs. 15 f–15 j). The actively beating flagella now on opposite ends of the cell and the two daughter cells are ultimately held together by only a slender connection (Fig. 15 j). Eventually this connection parts, but remnants of the severed strand are often visible on the daughter cells for some time after separation. The reformation of the tails of the daughter cells is not directly related to the position of the final separation strand. Before the final stages of separation several lobes appear from each daughter cell (Figs. 15 h), and one of these lobes eventually becomes the new tail (upper lobe Figs. 15 h–15 n). After separation there is some repositioning of the vacuole and other components, the additional lobes retract, and the tail becomes fully extended (Fig. 15 n).

Electron microscopy of dividing cells has clarified some details of spindle microtuble attachment and the behavior of other cell organelles Perhaps most surprising is that spindles examined at metaphase (Fig. 16) or telophase (Figs. 17 and 18) were found not to converge on the kinetosomes, but appear to be attached to a structure similar to the rhizoplast of vegetative cells. The identity of the "mitotic rhizoplast" is further confirmed by its connection to the kinetosomes (Fig. 16), and by the presence of a Golgi complex on its outer (nonmicrotubular) surface. However, the regular periodicity of the vegetative rhizoplast has not yet been seen in the mitotic rhizoplast. By metaphase the principal cell organelles (excepting the vacuole) have duplicated, and have become associated with one or the other of the spindle poles, so that there is a Golgi complex, a chloroplast, and a rhizoplast near each spindle pole. The small chromosomes do not appear to have a well defined kinetochore (Fig. 16). Continuous spindle microtubules are evident particularly at telophase where they can be seen traversing the chromosome mass and converging on the rhizoplast (Figs 17 and 18).

DISCUSSION

The three apparently unrelated sets of microtubles each associated with a separate attachment site which have been identified in *Ochromonas* are (*a*) those microtubules associated with the beak, (*b*) those associated with the rhizoplast, tail, and spindle, and (*c*) those associated with the flagellar axonemes. The first two of these sets contribute to the characteristic species-specific form of this organism. That is microtubule distribution closely parallels the external form of the cell, suggesting that these sets of microtubules must polymerize in a manner dictated by the genetic constitution of these cells. However, microtubules themselves as self-assembling polymers (Stephens and Kane, 1968) consisting of a few polypeptides (c.f. introduction) would not appear to possess the specificity to determine the different distributional patterns found in different regions of the cell. Nor would it seem likely that bridges or linkers alone could provide the mechanism for the precise pattern of *Ochromonas* microtubules. Alternatively it has been suggested by previous workers that the number and/or orientation of microtubules is mediated by some kind of nucleating site. Thus it is nucleating sites which likely provide the seeds or initial template for the total display of microtubules, and it may therefore be through these nucleating sites that the genetically-specified cell form in this case of *Ochromonas* is expressed. Therefore it is an analysis of these complexes which should provide important clues to development in these unicellular forms as well as in other organisms.

FIGURE 15 Mitosis in *Ochromonas*. Before nuclear breakdown (nucleus can be seen as a small sphere between the flagella and the large spherical vacuole) two sets of flagella are formed and the cell has assumed a spherical configuration (Fig. 15 a). By prophase the two sets of flagella have separated somewhat (Fig. 15 b). A metaphase plate is faintly visible in Fig. 15 c (arrow) and chromosome separation is seen at anaphase in Fig. 15 d. Lateral separation of the flagella continues throughout these stages until the flagella are on opposite ends of the dividing cell (Fig. 15 f). The tail begins as a lobe from one daughter cell (Fig. 15 h, arrow) and is not formed at the site of cell constriction (arrows trace tail development in Figs. 15 h–15 n). Complete sequence from Fig. 15 a–Fig. 15 n takes about 30 min. × 2400.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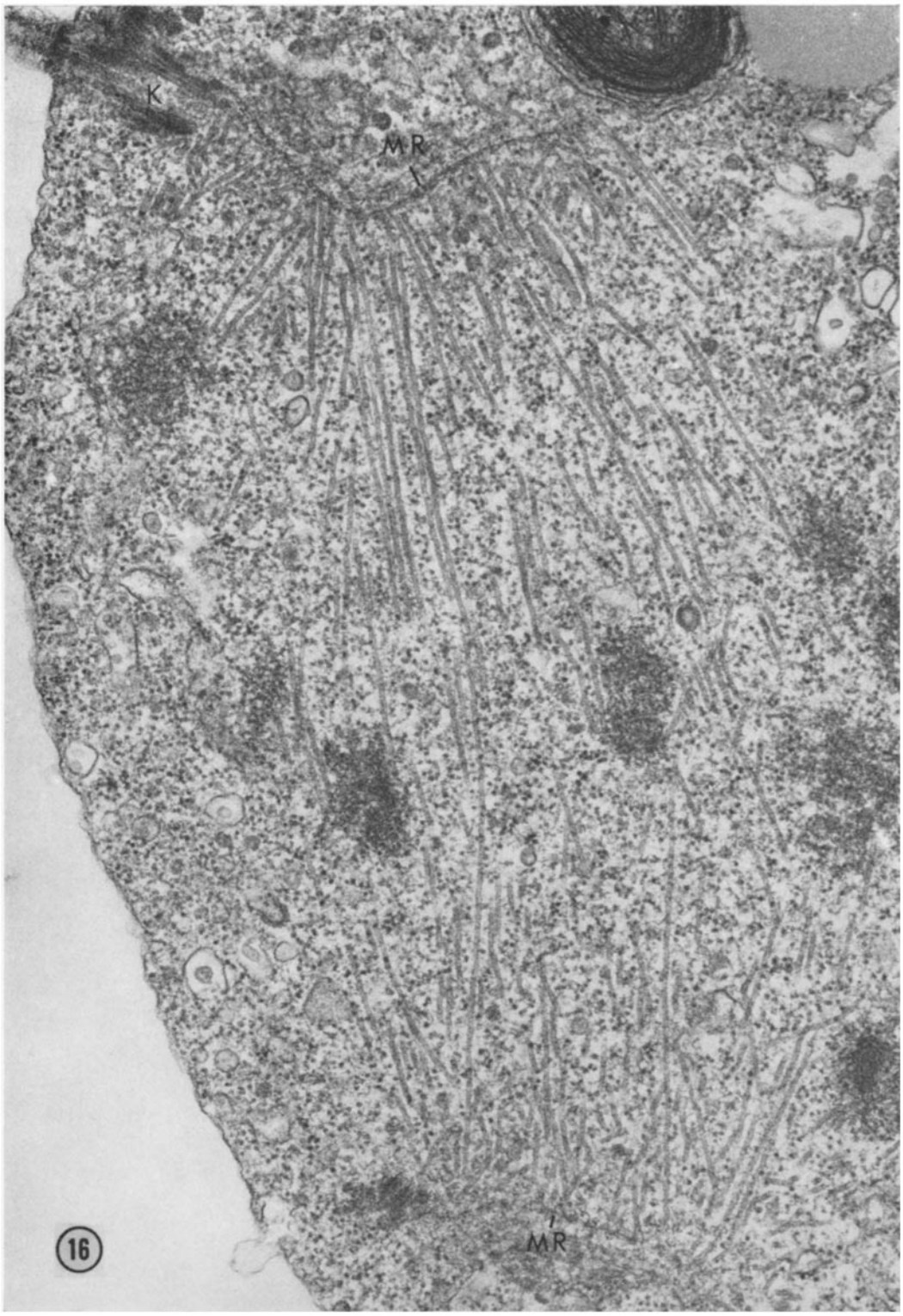
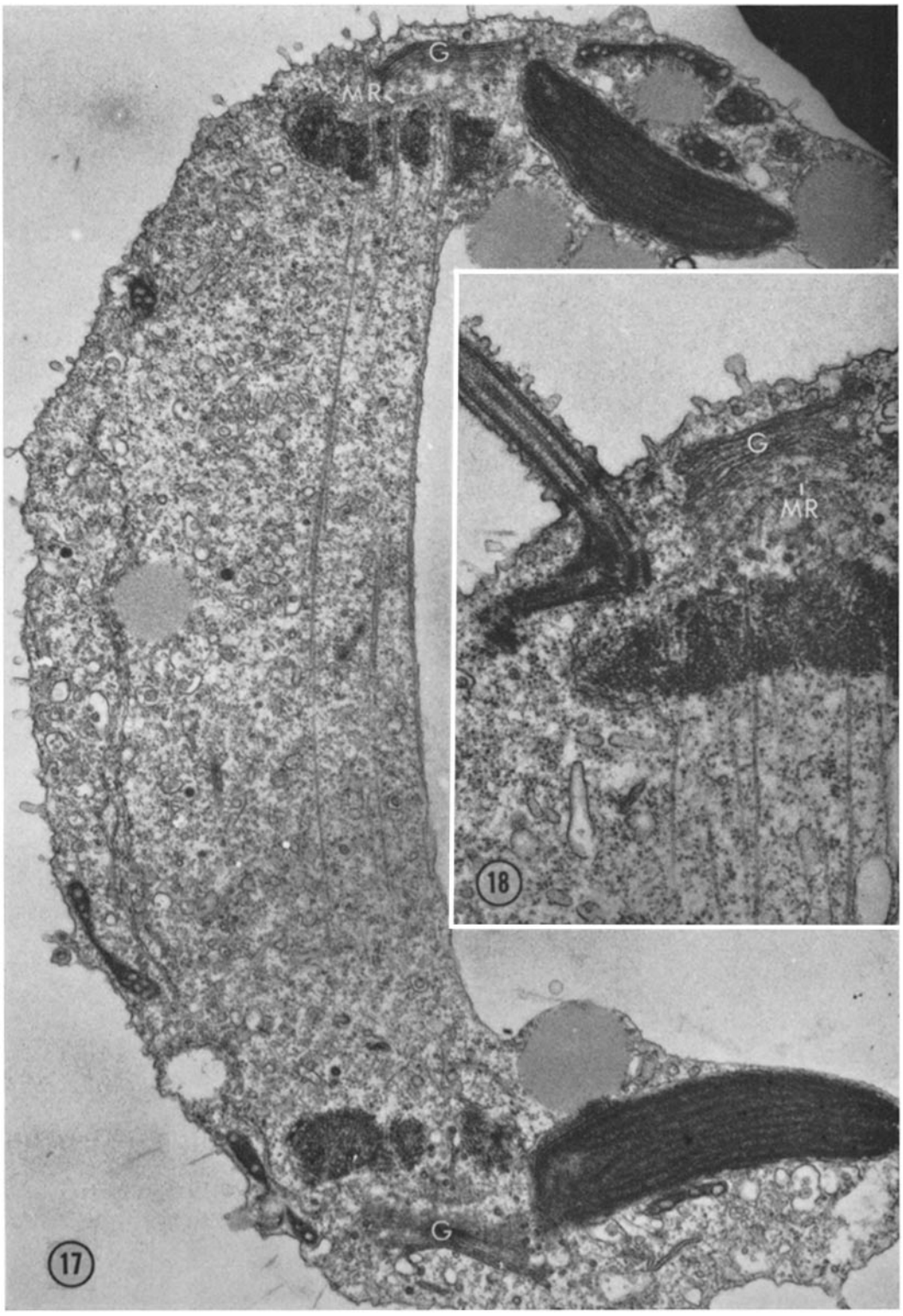


FIGURE 16 Metaphase cell. Spindle microtubules do not converge on the kinetosome (*K*) but on the mitotic rhizoplast (*MR*). Note that the mitotic rhizoplast is still attached to the kinetosome. × 27,000.



FIGURES 17 and 18 Adjacent sections through same early telophase cell. Note that some spindle microtubules pass through the chromosome mass and converge on the mitotic rhizoplast (*MR*). A Golgi complex (*G*) is associated with the outer surface of both rhizoplasts. Fig. 17, × 15,000; Fig. 18, × 27,000.

The Rhizoplast as a Possible Microtubule-Nucleating Site in Nondividing and Mitotic Cells

The rhizoplast is a fibrous structure recognized in early light microscope studies as extending from the kinetosome to the surface of the nuclear envelope (Chatton, 1924). In the Chrysophycean group of organisms which includes *Ochromonas*, rhizoplasts were correctly illustrated by Rouiller and Fauré-Fremiet (1958) as a striated fiber which approached but did not fuse with the nuclear envelope. These and other studies of Chrysophytes (Manton, 1955; Hibberd, 1970) have also recognized that the Golgi apparatus may be positioned along the upper rhizoplast surface, but microtubules have not previously been demonstrated in association with the lower rhizoplast surface in any organism. The function of rhizoplasts (or whether all rhizoplasts are in fact homologous) is still largely conjecture (e.g. Simpson and Dingle, 1971), but in *Ochromonas danica* the rhizoplast clearly provides an attachment site, and experimental evidence strongly suggests that microtubules are initiated on the lower rhizoplast surface (Bouck and Brown, 1970; Brown and Bouck, 1971, Brown and Bouck, 1973, adjoining paper). In contrast to most other organisms in which sites thought to be associated with microtubule initiation are structurally amorphous (e.g. Tilney and Porter, 1967; Pickett-Heaps, 1969; Dippell, 1968), the *Ochromonas* rhizoplast is unique in its structural definitiveness and its apparent persistence throughout the vegetative and mitotic cycle. It remains yet to be determined whether the rhizoplast itself divides directly or is synthesized *de novo* during cell division.

Since rhizoplasts seem to assume a dual role in tail and spindle formation, it might be supposed that because tail microtubules and spindle microtubules are attached to the rhizoplast, the tail would reform precisely at the midpoint between the separating daughter cells during cell division without requiring further microtubule initiation. Such a situation might then be similar to midbody formation in dividing animal cells (Robbins and Gonatus, 1964) or to the "cord" of microtubules described in a diatom (Manton et al., 1970). However, this does not appear to be the basis of the mechanism for tail formation in *Ochromonas*. Rather the tail is formed independently of the division plane, which suggests that spindle microtubules and tail microtubules are not directly interchanged. In fact cell division is not required for tail formation since cells temporarily deprived of tails by depolymerizing microtubules with colchicine or pressure are fully capable of regeneration without cell division (c.f. Brown and Bouck, 1973, adjoining paper). It is of interest, however, that the tail retracts before spindle formation which may indicate that monomers of tail microtubules are available for spindle microtubules during mitosis (similar ideas that the cytoplasmic microtubules or their monomers may be used for spindle microtubules were originally proposed by Ledbetter and Porter, 1963, supported by the observation of Pickett-Heaps and Northcote, 1966, and supported by the experiments of Stubblefield and Brinkley, 1966). It might also be argued that rhizoplast microtubules found in apparently nondividing cells of *Ochromonas* are spindle microtubules formed before the onset of mitosis. However, this latter interpretation does not appear likely as cells deprived of biotin for 3 days (biotin starvation essentially eliminates cell division, Rosenbaum and Child, 1967), nonetheless retain the normal complement of microtubules on the rhizoplast.

The unique organization of the spindle pole into a more or less permanently visible structure (rhizoplast) is unparalleled in most other organisms. Excluding centrioles and kinetosomes whose direct participation in spindle organization is problematical, the spindle pole is generally characterized by an absence of structural organization. Notable exceptions are the spindle "plaques" attached to the inner portion of the nuclear envelope in dividing fungus cells (Robinow and Marak, 1966; Moens and Rapport, 1971), the less well defined intranuclear spindle poles characteristic of several other lower forms (c.f. Leedale, 1970), and especially the spindle ("centrosomal") plates formed initially outside the nuclear envelope in dividing spermatocytes of the diatom, *Lithodesmium* (Manton et al., 1969). The diatom spindle develops between a pair of parallel plates which continue to separate as the chromosomes insinuate themselves among the spindle elements. In these diatoms, however, microtubules are not associated with the spindle plates until plate separation is initiated, and the plates do not participate in microtubule formation in nondividing cells. The persistence of the *Ochromonas* rhizoplast would seem to be a condition intermediate between those more primitive organisms

which retain the nuclear envelope for spindle attachment and those organisms in which the spindle pole is transient and organized outside the nuclear envelope. It is significant that in both *Ochromonas* and the diatoms the spindle poles are clearly distinct from the kinetosomes. This condition lends further support to the concept that the kinetosomes may play no direct role in spindle initiation and chromosome separation (Pickett-Heaps, 1969; Friedlander and Wahrman, 1970), for in *Ochromonas* the kinetosomes as with the chloroplasts and Golgi apparatus would seem to be merely in the most advantageous position for distribution to the daughter cells after telophase.

Other Possible Microtubule-Organizing Centers in Ochromonas

In addition to the rhizoplast, among the other fibers associated with the kinetosome only one, the kineto-beak fiber, appears to operate as an attachment site for microtubules. The kineto-beak complex is not as well defined as the rhizoplast in *Ochromonas*, but it too might conceivably play a role during division. For example the flagella replicate and move apart somewhat before the breakdown of the nucleus during mitosis. No spindle is present at this time but kineto-beak fibers are maintained, and in fact must also be duplicated as the new beak forms in the daughter cells. No direct evidence is available for the participation of beak microtubules in the prespindle movements of the two sets of flagella of the mitosing cell, but such a possibility seems plausible from the distribution of the beak microtubules. Other kinds of flagellated cells also appear to have similar kinds of kineto-beak complexes (Reichle, 1969; Falk, 1967), which also suggests a more general function for this group of microtubules. The microtubule pattern common to all these complexes is a bidirectional orientation of microtubules whose presence apparently determines in part the shape of the anterior part of the cell. Originating near the proximal region of the kinetosome the kineto-beak fibers are thus positioned (as are the other kinetosome-associated fibers) to be distributed to the daughter cells during mitosis. All three microtubule attachment sites (the rhizoplast, kineto-beak fibers, and kinetosome) are apparently interconnected thereby ensuring the presence of each in the new daughter cells. The importance of this perpetuation of attachment sites is made apparent in the following paper in which the attachment sites are equated with the nucleating sites for microtubules in *Ochromonas*.

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Note Added in Proof: Self assembly of microtubules in vitro has recently been reported by Weisenberg (1972. *Science* (*Wash. D. C.*). **177**:1104) and Borisy and Olmsted (1972. *Science* (*Wash. D. C.*). **177**:1196). Slankis and Gibbs (1972. *J. Phycol.* **8**:243) have published micrographs confirming the participation of the rhizoplast as an attachment site for spindle microtubules in *Ochromonas*.

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