AN UNUSUAL MITOTIC MECHANISM IN THE PARASITIC PROTOZOAN *SYNDINIUM* SP.

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ABSTRACT

Syndinium and related organisms which parasitize a number of invertebrates have been classified with dinoflagellates on the basis of the morphology of their zoospores. We demonstrate here that with respect to chromosome structure and chemistry as well as nuclear division, they differ fundamentally from free-living dinoflagellates. Alkaline fast green staining indicates the presence of basic proteins in *Syndinium* chromosomes. Chromatin fibers are about 30 Å thick and do not show the arrangement characteristic of dinoflagellate chromosomes. The four V-shaped chromosomes are permanently attached at their apexes to a specific area of the nuclear membrane through a kinetochore-like trilaminar disk inserted into an opening of the membrane. Microtubules connect the outer dense layer of each kinetochore to the bases of the two centrioles located in a pocket-shaped invagination of the nuclear envelope. During division kinetochores duplicate, and each sister kinetochore becomes attached to a different centriole. As the centrioles move apart, apparently pushed by a bundle of elongating microtubules (central spindle), the daughter chromosomes are passively pulled apart. During the process of elongation of the central spindle, the cytoplasmic groove on the nuclear surface which contains the central spindle sinks into the nuclear space and is transformed into a cylindrical cytoplasmic channel. A constriction in the persisting nuclear envelope leads to the formation of two daughter nuclei.

INTRODUCTION

Prokaryotes and eukaryotes, the major cell types known today, differ not only in the molecular organization of their genetic material but also in the way daughter genophores are moved apart after replication. In bacteria there is evidence that the DNA is attached to the cell membrane and that the products of replication are separated by some form of membrane action (40). The dividing eukaryote cell, on the other hand, develops a complex mitotic apparatus consisting predominantly of microtubules. There are two main components to this structure: one, a mass of parallel microtubules, serves as the scaffolding on which the chromosomes move, its inherent bipolarity directing the separating daughter chromosomes to form equal groups at each spindle pole; the other component also consists of microtubules, but these are thought to be directly involved with chromosome motility by virtue of their attachment to specific chromosomal regions, the kinetochores or centromeres (2, 34).

The existence of such distinct cell types raises the question of the evolutionary origin of the more complex eukaryote cell from a prokaryotic ancestor. Perhaps cells showing intermediate properties still exist and can provide some insight into the origin of eukaryote organization. Some years ago, the unique structure of dinoflagellate

chromosomes led to the idea that the dinoflagellate, an otherwise typical eukaryote cell containing a prokaryote-like genetic system (9, 38), might represent such a living fossil.

Subsequently, detailed study of nuclear division in a free-living dinoflagellate indicated that these organisms retained prokaryote-like features, not only in their chromosome structure and chemistry but also in the mechanism of chromosome separation during nuclear division (28). It was found that dinoflagellate chromosomes never make contact with microtubules but appear to be attached to and move on the nuclear membrane. Microtubules are present in the cytoplasm of these cells, however; and in the dividing nucleus, an interesting system of parallel cytoplasmic channels, each containing a bundle of microtubules, becomes established in the nuclear space. It appears likely that this microtubular system provides direction or bipolarity to that portion of the nuclear membrane on which chromosomes move, namely the membrane surrounding the channels. Thus, even though it is quite different in structure, the function of this system is analogous to the mitotic spindle of the typical eukaryote cell.

In 1920, Chatton described the genus *Syndinium*, organisms which parasitize marine copepods; related forms are also found in several radiolarian species (5, 7, 18, 19, 21, 22). On the basis of the morphology of free-living swarmers released after sporogenesis, *Syndinium* has been considered to be a specialized dinoflagellate (7, 13). Because of its small number of chromosomes (four to six as contrasted with the approximately 20–300 chromosomes of free-living dinoflagellates), *Syndinium* is especially well suited to the light microscope study of nuclear division; and several interesting features have been revealed (3, 6, 18, 19). In interphase, the V-shaped chromosomes are arranged with their apexes all pointing towards the centrioles situated on the nuclear surface such that the chromosomes have a typical umbrella- or rosette-like configuration. In division, centrioles are connected by a central spindle or "centrodesmose," and the apexes of daughter chromosomes follow the centrioles as if they were attached to them. Because of the apparent clarity and simplicity of *Syndinium* division, the leading investigators of dinoflagellates have considered it as the model example for dinoflagellate mitosis (6, 12, 13, 17). A detailed electron microscope study to compare the division of these parasites with that of free-living dinoflagellates was certainly warranted.

We have investigated *Syndinium*-like parasites found in the colonial Radiolaria *Collozoum* sp. and *Sphaerozoum* sp. collected in 1970 from the Mediterranean. As it turned out, nuclear division in *Syndinium* differs significantly from that described in free-living dinoflagellates. There are certain similarities: the chromosomes are attached to the nuclear membrane surrounding a cytoplasmic channel which contains a bundle of microtubules. More significant, however, are the dissimilarities: microtubules develop between typical centrioles which are here closely associated with nuclear division. The sites of chromosome-membrane attachment are highly differentiated dense disks which are inserted into openings of the nuclear membrane and are connected to the centrioles by a bundle of microtubules. The chromosome-membrane attachments and the microtubules joining them to the centrioles persist through the entire cell cycle and the many nuclear divisions during sporogenesis of the parasite. Thus, our electron microscope study confirms Chatton's conclusion based on light microscopy (6) that a previously unknown type of mitotic mechanism is operating in *Syndinium*. It was left to the higher resolution of the electron microscope to reveal the details of this unique type of mitosis.

MATERIALS AND METHODS

Colonial Radiolaria (Polycyttaria) such as *Collozoum* and *Sphaerozoum* may be infected by a number of different *Syndinium*-like parasites, some developing inside the central capsule, others extracapsular (7, 18, 19, 21, 22). *Collozoum* sp. and *Sphaerozoum* sp. which we collected at the Station Zoologique in Villefranche-sur-Mer (France) in September 1970, June 1971, and April 1972 were frequently infected by an intracapsular *Syndinium*. Two such intracapsular parasites have been described in the literature, *Syndinium Brandti* (21) and *Syndinium globiforme* (18). Since the taxonomy of these parasites is based mainly on the structure of spores and is still in a primitive state, we are not certain of the species of *Syndinium* with which we are dealing.

The extent of infection varied greatly from one collection to another. Since *Syndinium* nuclei are easily identified in acetocarmine squashes, the degree of infection and stage of development of the parasite was determined in a small piece snipped off from a

colony. Infected colonies in early or late stages of parasite sporogenesis could thus be selected for fixation.

Light Microscopy

Infected *Collozoum* or *Sphaerozoum* were fixed in ethanol-acetic acid (3:1) and squashed in 45% acetic acid. After removal of the cover slip by soaking in 90% ethanol, the squashes were stained by the Feulgen reaction. As a test for the presence of basic proteins, 5-μm sections of infected *Collozoum* and *Sphaerozoum* (fixed in 4% paraformaldehyde and embedded in paraffin) were stained with alkaline fast green according to Alfert and Geschwind (1). In order to prevent the loss of sections during the hydrolysis used in this procedure, they were affixed to slides with celloidin according to Hawes (16).

Electron Microscopy

Infected *Collozoum* and *Sphaerozoum* colonies were fixed at room temperature for 1 h in a modified Karnovsky (25) fixative (2% paraformaldehyde, 1.25% glutaraldehyde, 0.1 M phosphate buffer, pH 7.0). After washing overnight in 0.1 M phosphate buffer, the material was postfixed for 1 h in 2% OsO_4 in 0.1 M phosphate buffer, washed several times in distilled water, and stained for 2 h in 0.5% uranyl acetate in Ryter-Kellenberger buffer (41). Some material was kept in the Karnovsky fixative up to 2 wk before further processing without undue effects. After dehydration in ethanol, the material was embedded in Epon-Araldite (33) and polymerized at 37°C. Serial sections were collected on 0.5% Formvar films on wire loops and transferred to single hole (1 × 2 mm) grids. After staining for 2 h with uranyl magnesium acetate (10) and for 15 min with lead citrate (37), a thin layer of carbon was evaporated over the sections.

In order to explore the effect of DNase on chromosome structure, central capsules of *Collozoum* were fixed in Formalin and treated with DNase before dehydration and embedding in plastic. 0.5 mg/ml DNase I from a bovine pancreas (Worthington Biochemical Corp., Freehold, N. J.) was dissolved in 0.05 M acetate-Veronal buffer, pH 7.0 and the material incubated at 37°C for 3 h. Controls were treated with buffer only. Removal of DNA was checked by staining with the Feulgen reaction.

Micrographs were taken with a Siemens Elmiskop I with double condenser illumination, 200-μm condenser aperture, and 50-μm objective aperture on Kodak Electron Image Plates or Kodalith LR Estar-Base roll film, and development was in Kodak HRP or D-19 (Eastman Kodak Co., Rochester, N. Y.).

RESULTS

Chromatin Structure and Chemistry

Light Microscopy

In Feulgen-stained squash preparations of infected *Collozoum* or *Sphaerozoum*, one finds the characteristic *Syndinium* nuclei in addition to the nuclei of the Radiolarian host and of an extracapsular symbiotic dinoflagellate (Xanthellae). These three nuclear types are easily differentiated: (*a*) The Radiolarian nuclei are dense and evenly filled with chromatin. (*b*) Extracapsular dinoflagellates contain a large number of rodlike chromosomes of even thickness and in this respect do not differ from typical free-living dinoflagellates (Fig. 1). (*c*) The parasite *Syndinium* has only four chromosomes which are individually distinct throughout the cell cycle and which, compared with typical dinoflagellate chromosomes, are more irregular in outline and unevenly thick (Figs. 2 and 3).

In interphase all four *Syndinium* chromosomes are V-shaped with the apexes pointing inward in a characteristic umbrella-like configuration (Figs. 2 and 3). As shown by electron microscopy (see below) this appearance is due to the attachment of the chromosome apexes to a restricted region of the nuclear envelope. During division, the daughter chromosomes separate first at their apexes with the arms trailing behind like the ribs of two umbrellas pointing away from each other (Figs. 5 and 6).

Typical dinoflagellate chromosomes lack basic proteins (9, 26, 38). To test whether this is true also for *Syndinium* chromosomes, infected central capsules were stained with alkaline fast green (1). To our surprise, *Syndinium* chromosomes are clearly stained with this technique, and, as in typical eukaryote chromosomes, the density of the fast green stain is similar to the density of the Feulgen staining (Fig. 4). The extracapsular dinoflagellate symbiotes present in the same sections serve as an internal control: their chromosomes remain unstained by alkaline fast green as is characteristic of typical dinoflagellates. *Syndinium* chromosomes, therefore, differ from typical dinoflagellate chromosomes in having appreciable amounts of basic protein associated with their DNA.

Electron Microscopy

In thin sections, dinoflagellate chromosomes have a very characteristic appearance. Fibers 20–

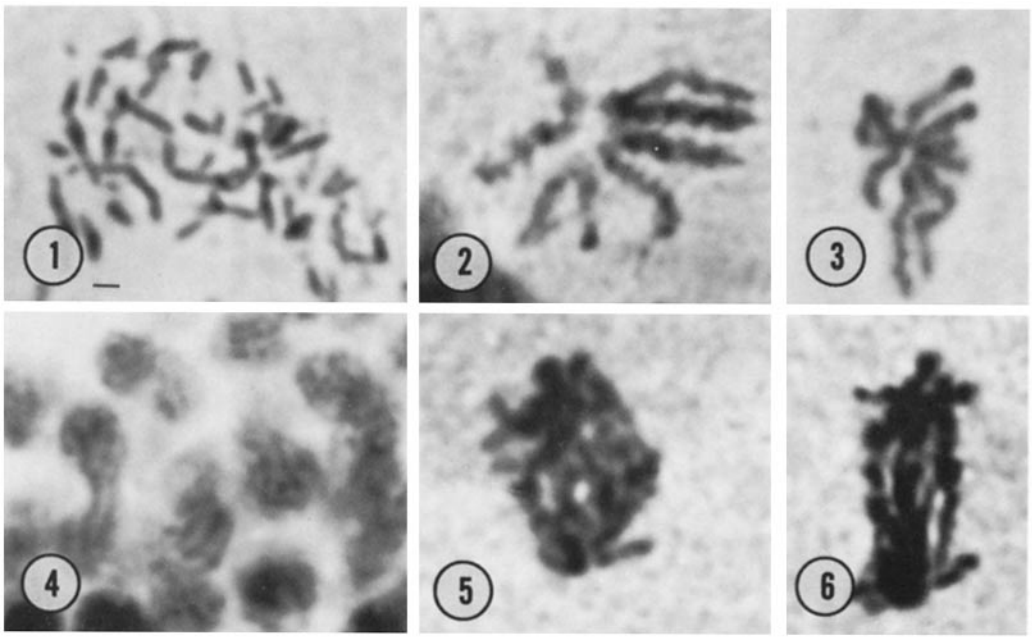


FIGURE 1 Chromosomes of Zooxanthella (extracapsular symbiotic dinoflagellate) from *Collozoum* sp. Feulgen squash. × 3,700.

FIGURES 2 and 3 Interphase chromosomes of *Syndinium* sp., intracapsular parasite of *Collozoum* sp. and *Sphaerozoum* sp. Feulgen squash. × 3,700.

FIGURE 4 *Syndinium* sp. in various stages of division. 5-μm paraffin section of infected central capsule of *Collozoum* fixed in 4% formaldehyde and stained with alkaline fast green. *Syndinium* chromosomes are positively stained in this test for basic proteins. × 3,700.

FIGURES 5 and 6 Nuclear division in *Syndinium* sp. Feulgen squash. × 3,700.

30 Å thick are arranged in a regular fashion so that in longitudinal sections chromosomes have a banded appearance with the fibers running alternately in the plane of the section and at right angles to it (11). Such chromosomes are present in the extracapsular dinoflagellate symbiotes of *Collozoum* or *Sphaerozoum*. The chromosomes of *Syndinium*, however, have a very different ultrastructure. The basic fiber is also very thin, approximately 30 Å, but there is nothing regular in its arrangement. Throughout the cell cycle and stages of sporogenesis, chromosomes have a mottled appearance since the packing of the jumble of fibers varies irregularly throughout the chromosome mass (Figs. 10 and 28). An attempt was made to determine the number and shape of the chromosomes by means of reconstruction from serial sections. This turned out to be impossible not only because the chromosome outline is very irregular, but also because chromosomes appear to be contiguous so that in interphase only one continuous chromatin mass is found in each nucleus.

The presence of protein in *Syndinium* chromosomes and its absence in the dinoflagellate symbiote is confirmed by their different reaction to DNase treatment after formaldehyde fixation. In the extracapsular dinoflagellate all structure disappears, and chromosomal regions appear as empty spaces. In *Syndinium*, on the other hand, chromosome structure is unchanged, and the only effect of DNase digestion is a decreased staining with uranyl magnesium acetate.

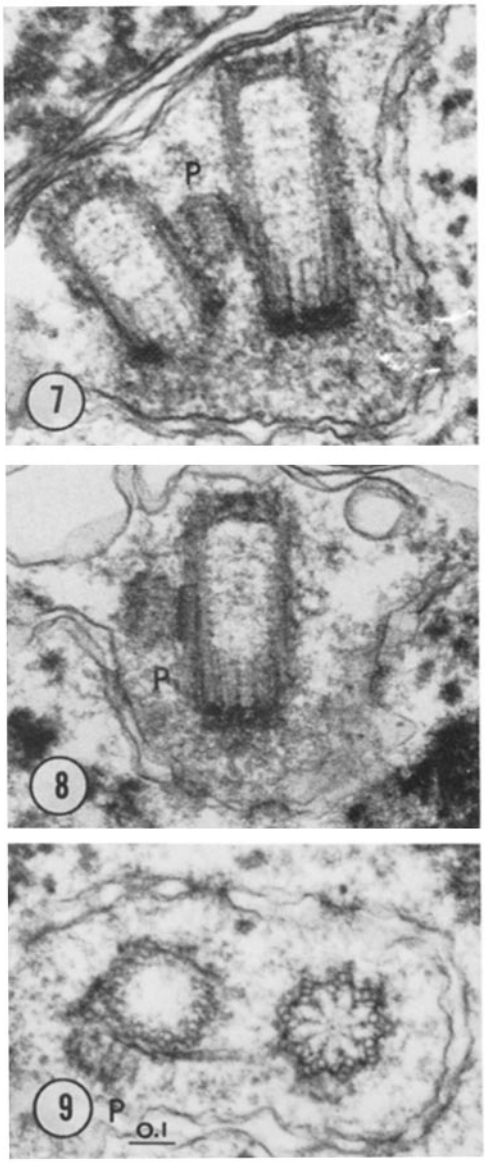
Chromosome-Nuclear Membrane-Centriole Relationships and the Mechanism of Chromosome Segregation

In many ways *Syndinium* mitosis resembles that previously described for dinoflagellates. The nuclear membrane persists through the cell cycle, chromosomes are attached to specific areas of

the nuclear membrane, and extranuclear microtubules are present in a cytoplasmic channel through the nucleus, determining the direction of chromosome segregation. Cersain features of *Syndinium* division, however, set it apart from that of free-living dinoflagellates and establish it as a new type of mitosis. The site of attachment of chromosomes to the nuclear membrane is a well-defined structure inserted into an opening of the nuclear membrane. Since these structures have all the relevant characteristics, we shall refer to them as kinetochores. Basal body replication is coordinated with nuclear division, perhaps in connection with the absence of flagella during sporogenesis. Basal bodies act as focal points for the extranuclear microtubules; in other words, they play the role of centrioles. Perhaps the most significant difference, however, is the presence of microtubules which connect kinetochores to centrioles. These microtubules and differentiated kinetochores both persist through the cell cycle and through the many divisions of sporogenesis. Whether they are also present in the mature free-swimming spore is as yet unknown. We shall now describe this chromosome-membrane-centriole relationship in more detail, first in the nondividing nucleus and then during the process of chromosome segregation.

Nondividing Nucleus

In early stages of infection, when relatively few parasites are present, one finds characteristic binucleate cells in which the nuclei are separated by a vacuole (Figs. 32 and 33). Presumably these represent interphase after the division of a uninucleate cell. We have prepared serial sections through several such nuclei. On the distal surface of each nucleus, one finds a cup-shaped invagination of the nuclear envelope about 0.7 μm deep and 0.8 μm in diameter (Figs. 10 and 32, arrows). These indentations contain two centrioles, one about 0.6 μm long and a shorter one approximately 0.5 μm long. They are arranged radially with regard to the nucleus and subtend an angle of approximately 25° with each other. Each centriole has a short procentriole attached near its middle (*P*, Figs. 7–9). The nuclear envelope surrounding this invagination is rich in nuclear pores and associated annuli. In contrast, the remainder of the nuclear envelope shows rather few such structures. Distributed on the bottom third of this cup are four kinetochores,



FIGURES 7–9 Longitudinal and cross sections of the centrioles situated in a cup-shaped invagination (centriolar pocket) of the interphase nucleus. *P*, procentriole. × 60,000.

one for each chromosome. This number has been determined from complete serial sections through seven such invaginations of nondividing nuclei.

We shall now look at the kinetochores in more detail. In cross sections they are seen to consist of two dense disks, each about 250 A thick, separated by a less dense space (Figs. 11–14, 17 and 18). From tangential sections it follows that the

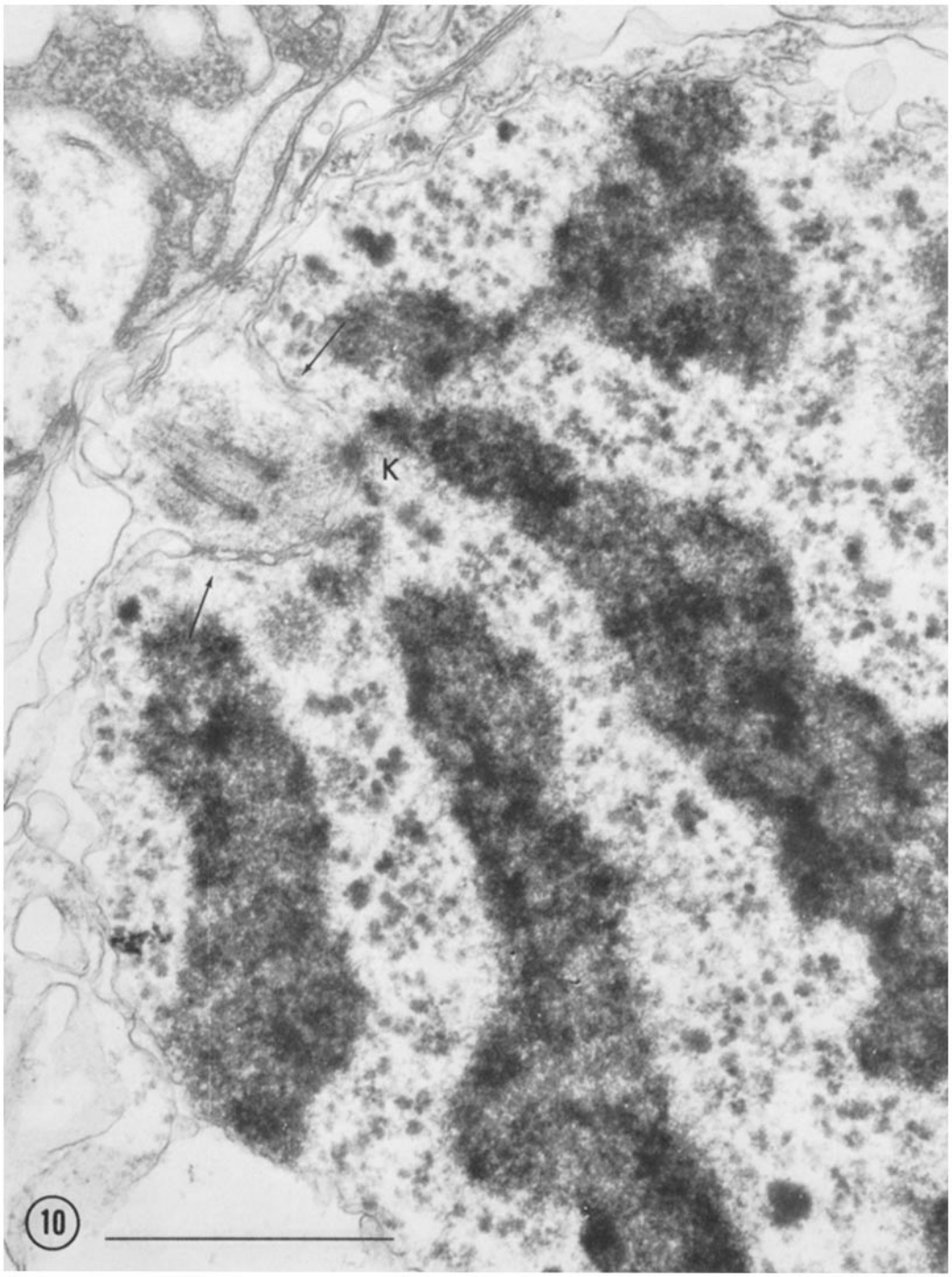
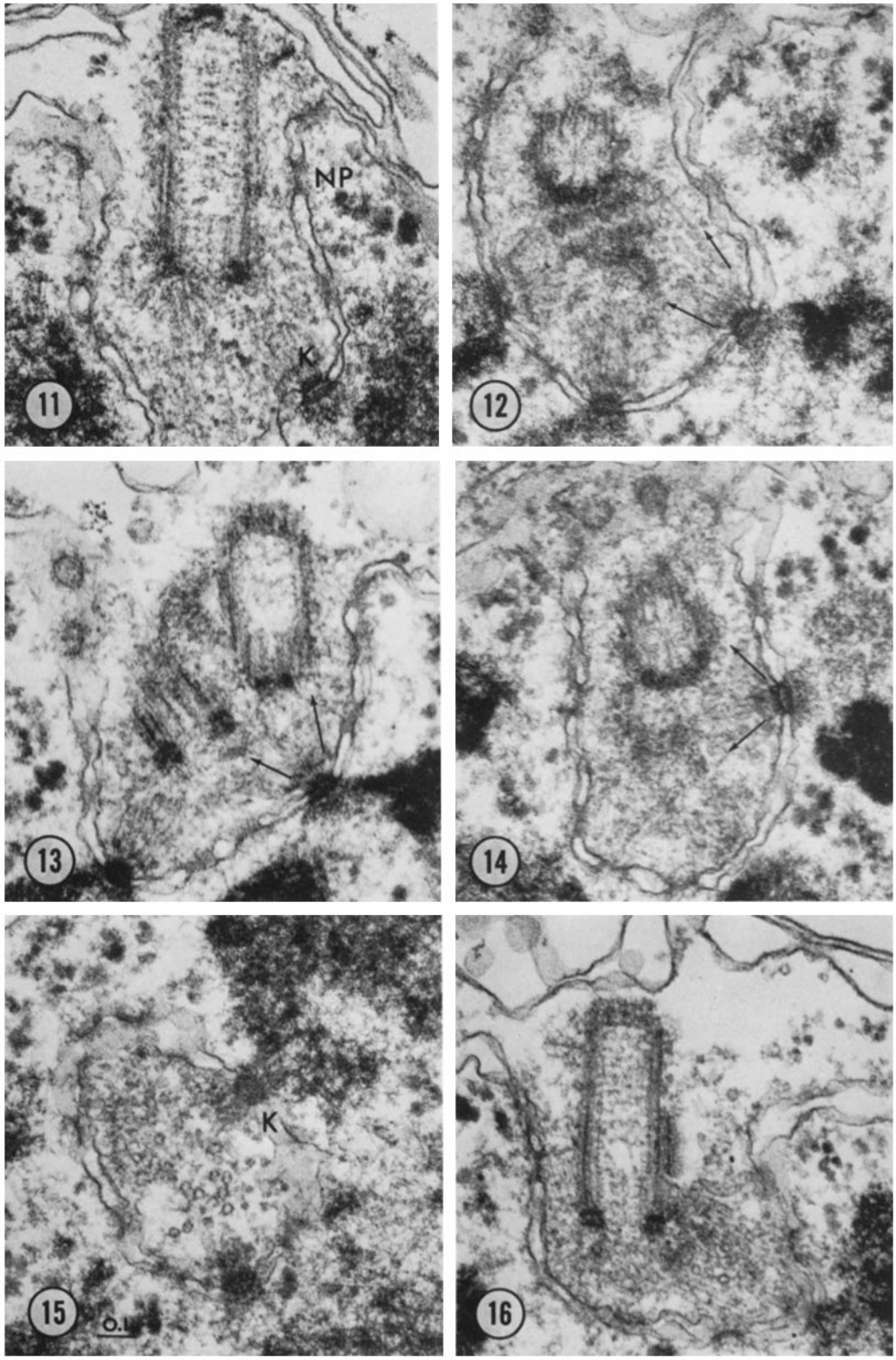
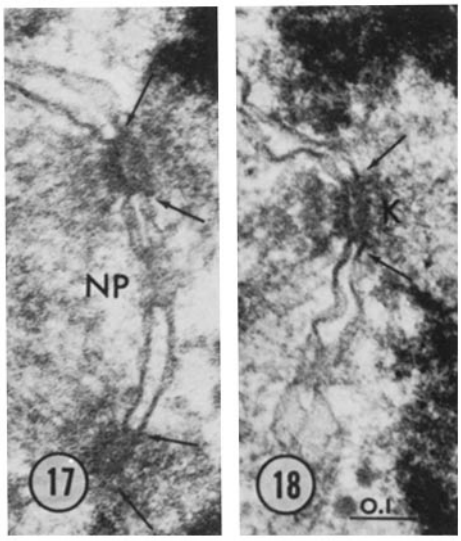


FIGURE 10 Section of interphase nucleus showing the centriolar pocket (arrows) with centriole and a kinetochore (*K*) inserted in the nuclear envelope. Microtubules connect the kinetochore to the base of the centriole. Note the characteristic arrangement of the chromosome arms pointing away from the centrioles. × 42,000.



FIGURES 11–16 Sections through the centriolar pocket (interphase) showing kinetochores (*K*) inserted into openings of the nuclear envelope. *NP*, nuclear pore. Microtubules connect each kinetochore to the bases of both centrioles (Figs. 12–14, arrows). × 60,000.



FIGURES 17 and 18 Kinetochores at interphase. Kinetochores are inserted in openings of the nuclear envelope (arrows) having the same dimension as nuclear pores (*NP*). × 88,000.

disks are circular in outline (Fig. 15). Each double disk is inserted into an opening of the nuclear membrane (Figs. 17 and 18, arrows). The opening is approximately 700 Å in diameter, just the size of the nuclear pores (*NP*, Fig. 11), and this raises the possibility that the sites of kinetochores are actually modified nuclear pores. The inner kinetochore disk is continuous with a rather loose mass of chromatin fibers which connects it to the main chromosome mass. On the cytoplasmic side, a bundle of microtubules extends from the vicinity of the outer dense disk to the bases of the two centrioles (Figs. 11–18). Whether these microtubules make direct contact with the dense disk cannot be determined in our material. Where a section contains the bases of both centrioles together with kinetochores, one gains the impression that microtubules extend to both centrioles from any one kinetochore (Figs. 12–14). The characteristic arrangement of the V-shaped chromosomes that we noticed in Feulgen squashes is now quite understandable. The four V-shaped chromosomes are attached at the apex of the V to the nuclear membrane through specialized structures, the kinetochores, which are furthermore anchored by a bundle of microtubules to the pair of centrioles situated on the nuclear surface.

CHROMOSOME SEGREGATION AND NUCLEAR DIVISION

The process of nuclear division in *Syndinium* as we understand it on the basis of the examination of a large number of dividing cells is summarized in Fig. 19. Briefly, the centrioles separate, apparently pushed apart by a bundle of elongating microtubules. The cup-shaped nuclear indentation which contains these centrioles elongates and eventually sinks into the nucleus to become a cylindrical cytoplasmic channel traversing the nucleus. During this channel formation, centrioles have duplicated so that a pair of centrioles is found at each end of the completed channel. One daughter chromosome set follows each pair of centrioles, apparently pulled along passively by means of the chromosome-microtubule-centriole attachment system. After fission of the nucleus, the channel disappears, leaving only the cup-shaped, centriole-containing depressions at opposite poles of the daughter nuclei.

We shall now look in more detail at the various aspects of this unusual nuclear division.

DUPLICATION OF KINETOCHORES: As described earlier, in interphase kinetochores are disk-shaped dense structures inserted into openings of the nuclear membrane, and each of them is apparently connected by microtubules to the bases of both centrioles. The first visible change from interphase, even before the centrioles separate or duplicate, is the appearance of double kinetochores in which two interphase-like disks lie side-by-side (*KK*, Figs. 20–22). A separate bundle of microtubules originates from each unit of these pairs, each bundle connecting to a separate centriole (Fig. 22, arrows). The doubleness of attachments at this early division stage is quite obvious where they lie within the plane of section. However, it is also evident when they are oriented at right angles to the plane of section, since then they occupy two to three consecutive sections; single interphase attachments are usually contained within a single section. In the complete series through a centriolar invagination from which Fig. 21 is taken, all four attachments had this double aspect.

CENTRIOLE SEPARATION AND FORMATION OF THE CYTOPLASMIC CHANNEL: Early stages of centriole movement are illustrated in Figs. 23–25. As division begins, the centrioles, which during interphase had lain at an angle of 25°, come to

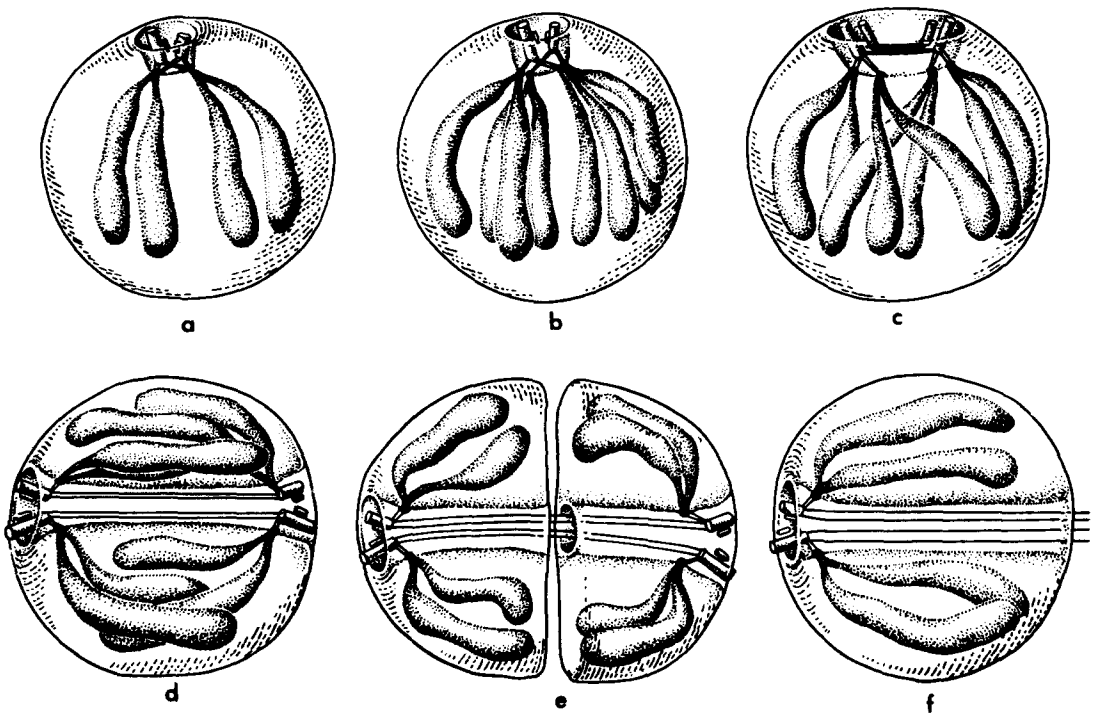
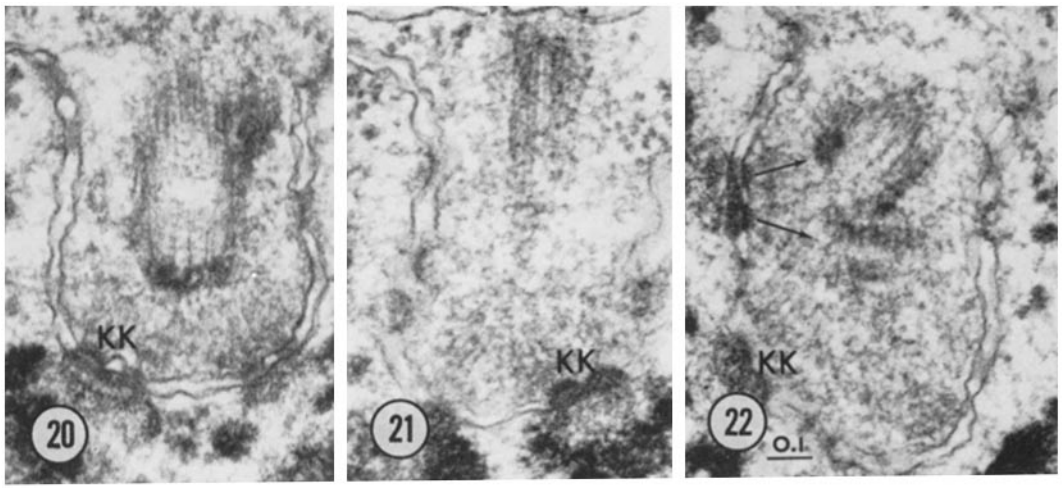
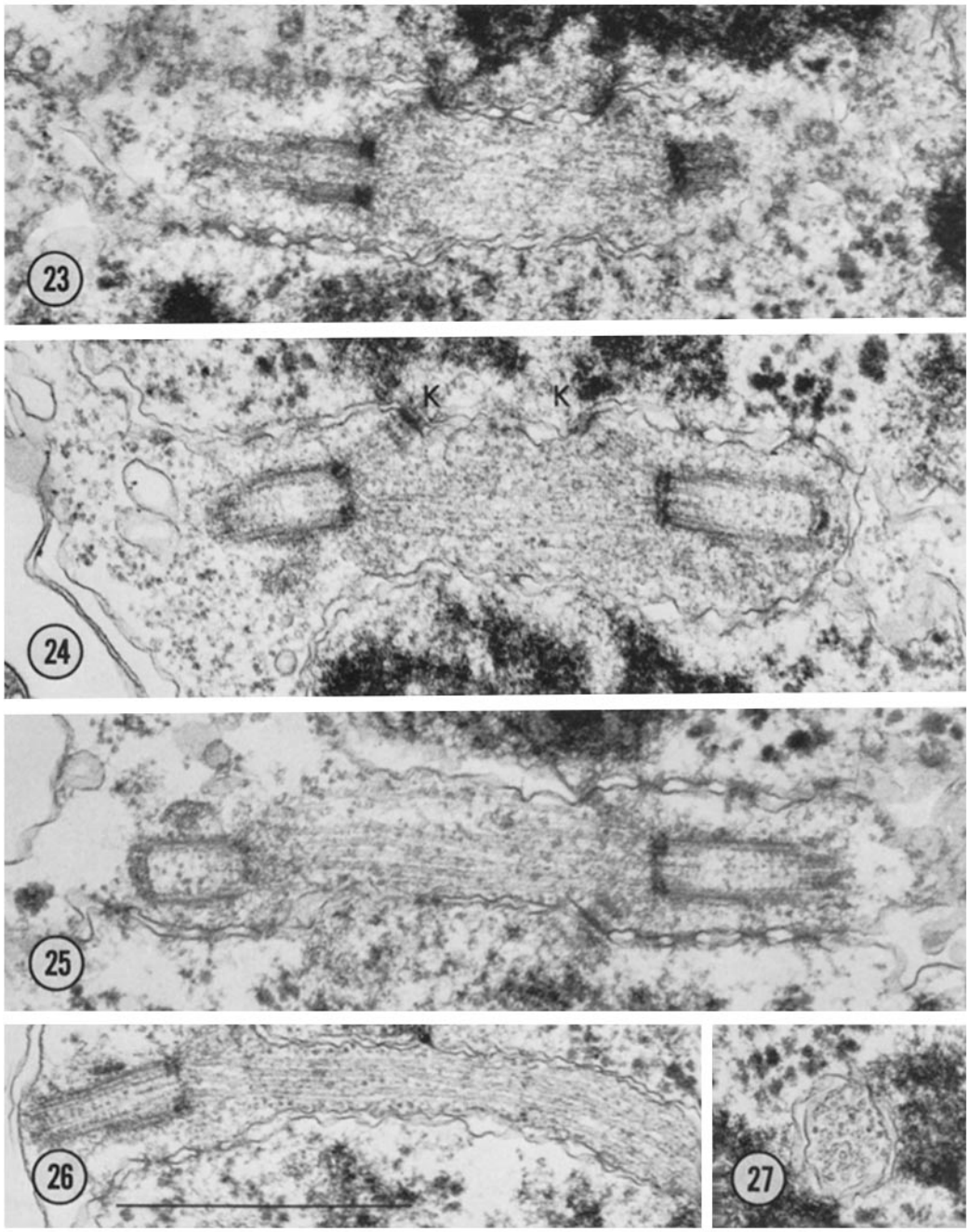


FIGURE 19 Diagrammatic representation of nuclear division in *Syndinium* sp. (*a*) Interphase. (*b*) Early division, kinetochores and chromosomes have duplicated. (*c*) Early stage of chromosome segregation. Central spindle between separating centrioles. (*d*) Late stage of chromosome segregation. Central spindle in cytoplasmic channel through nucleus. Note that length of microtubules attaching kinetochores to centrioles has remained unchanged. (*e*) Division of nucleus. (*f*) Early daughter nucleus with persisting channel and microtubules. Note that on one side the microtubules end freely in cytoplasm.



FIGURES 20–22 Double kinetochores (*KK*) are interpreted as stage in kinetochore duplication before chromosome segregation. In views such as that in Fig. 22 each unit kinetochore now appears to be connected to a separate centriole (arrow). × 60,000.



FIGURES 23–27 Early (23–25) and late (26, 27) stages in chromosome segregation. Microtubules (central spindle) are seen to connect the baseplates of the two separating spindles. As in interphase (cf. Fig. 7) one centriole is shorter than the other. The microtubules connecting kinetochores (*K*) to centrioles remain constant in length throughout chromosome segregation. The centriolar pocket elongates into a trough and then sinks into the nucleus forming a cylindrical cytoplasmic channel (longitudinal section in Fig. 26, cross section in Fig. 27). × 42,000.

lie more or less in a straight line with their base plates facing each other. Microtubules appear between them and extend from one base plate to the other. As we noticed in interphase, one centriole is shorter than the other (Figs. 24 and 25). A later stage is illustrated in Fig. 28. The centrioles are 3.8 μm apart and must have duplicated since now two pairs of centrioles are present. The larger centriole on the right in Fig. 28 already seems to have developed a small procentriole (arrow).

Concomitant with disjunction of the centrioles, the depression in which they lie elongates, forming a groove on one side of the nucleus. Analysis of the complete series of sections from which Fig. 28 was taken revealed that at this stage the centriolar groove had penetrated deep into the nucleus, forming a narrow canyon on one side of it. The nuclear membrane lining this invagination is much folded and characterized by many pores. This is in contrast to the remainder of the nuclear surface which seems to have only few, scattered nuclear pores.

The two pairs of centrioles finally come to lie at opposite poles of the nucleus, and the nuclear membrane fuses around the bundle of microtubules to form a cylindrical cytoplasmic channel through the center of the nucleus. The openings of this channel are wider than the channel itself, giving them a funnel-like appearance (Fig. 26). In cross sections through the channel (Fig. 27) one finds approximately 20 microtubules which abut on the base plates of the centrioles (Fig. 26). Whether these microtubules are still continuous from one centriole to the other has not been determined.

MECHANISMS OF CHROMOSOME SEGREGATION: Two examples of an early stage in daughter chromosome segregation are shown in Figs. 23 and 24. In each, the bases of the centrioles have separated and are now about 1 μm apart. In both pictures two kinetochores are visible. These, from the symmetry of their arrangement, appear to be sisters which have separated about 0.5 μm. The bundle of microtubules connecting each kinetochore to its respective centriole is clearly visible and is approximately 0.24 μm long, identical to its length in interphase (Figs. 11–13). A later stage is shown in Fig. 28 which is from a complete series of sections through a dividing nucleus. Here, the centrioles are 3.8 μm apart, while the kinetochores maintain a 0.24-μm separation from the bases of the centrioles. From an analysis of this series, we know that there are four single kinetochores symmetrically arranged around each of the two pairs of centrioles.

The constant length of the microtubule bundles joining kinetochores to centrioles in interphase and throughout division suggests that chromosome movement is essentially passive, daughter chromosomes being dragged along as the centrioles separate (see Discussion). In support of this idea is the orientation of kinetochores on the nuclear membrane of dividing nuclei (Figs. 23 and 24). In all cases they make a sharp angle with the surrounding membrane, the face of the disk turned toward the centrioles as if tension were being exerted on them in this direction.

NUCLEAR DIVISION: In *Syndinium*, the nuclear envelope remains intact at all stages of division. As the centrioles move apart and the cytoplasmic channel is established, the nucleus elongates in the direction of the channel. Eventually a fold of the nuclear envelope invades the nucleus perpendicular to the channel and cleaves the nucleus in two (Fig. 29). It is of interest that only the inner leaflet is involved at this stage. We have not investigated the further progress of this cleavage.

After nuclear cleavage, the cytoplasmic channel persists for some time in newly formed daughter nuclei. However, these nuclei are easily distinguished from those still in the process of division: centrioles are found only at one, funnel-shaped opening of the channel, while at the opposite narrow opening microtubules end freely in the cytoplasm a short distance beyond the nuclear surface (Figs. 30 and 31, cf. Fig. 19*f*). Generally, serial sections are required to make this distinction. In other similar young daughter cells, channels were found without microtubules. This suggests that microtubules vanish first, and only then does the channel disappear, leaving the wider portion as a cup-shaped depression containing centrioles. In the section illustrated in Fig. 32, centrioles, as would be expected, are found at opposite poles of the daughter nuclei. In all serially sectioned pairs of daughter nuclei such as that shown in Fig. 33, centrioles are found in similar disposition.

In early stages of infection, cell division is delayed, and multinucleate plasmodia are formed Figs. 32 and 33 are from central capsules in which binucleate cells of the parasite predominated.

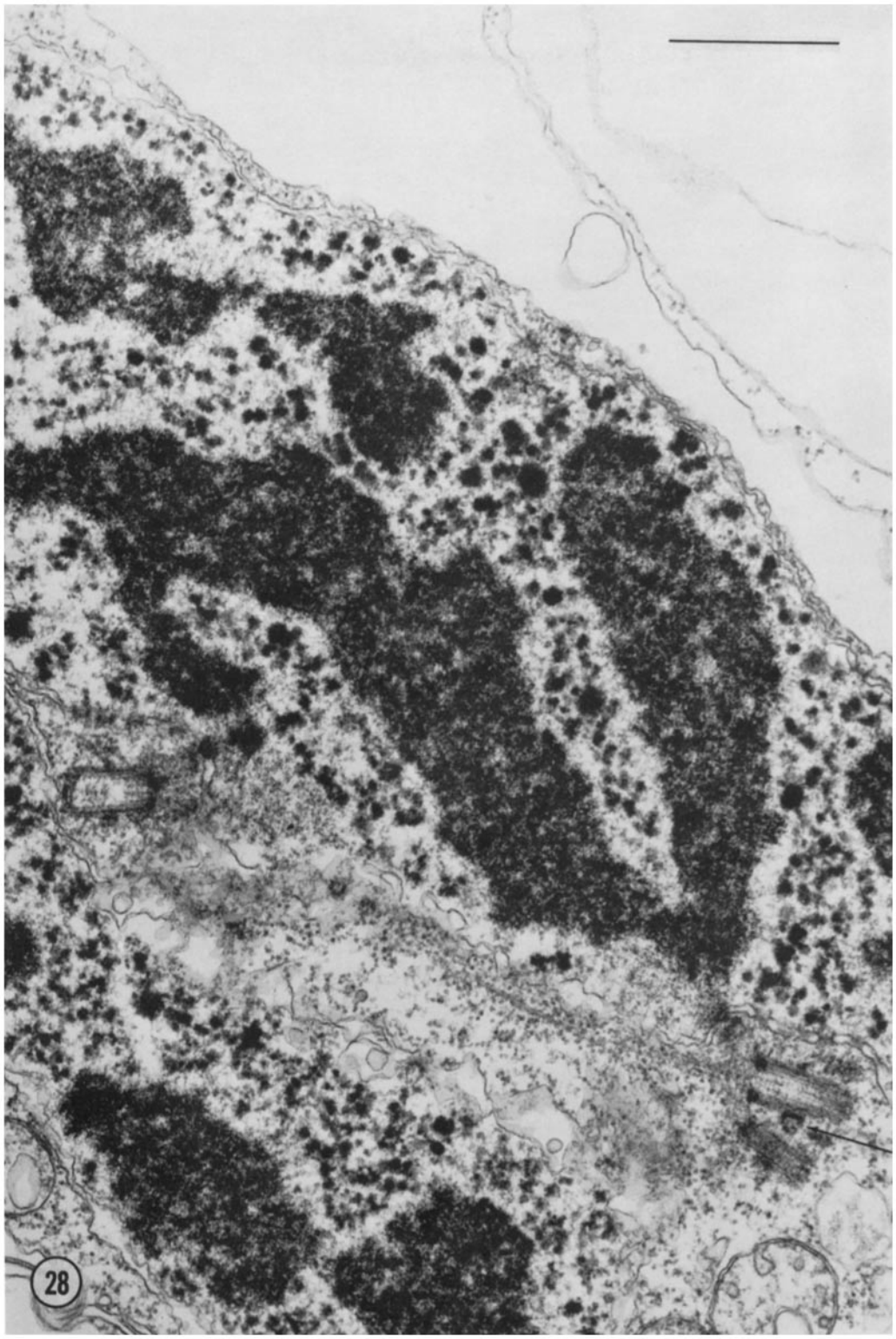


FIGURE 28 One of a complete series of sections through a nucleus in an intermediate stage of division. From the serial sections we know that the central spindle lies in a deep and narrow cytoplasmic trough with a pair of centrioles at each end and that four single kinetochores are located in the nuclear membrane near each centriolar pair, each connected by a bundle of microtubules to the centriolar bases. The V shape of one of the chromosomes is clearly visible in this section. Procentriole, arrow. × 26,000.

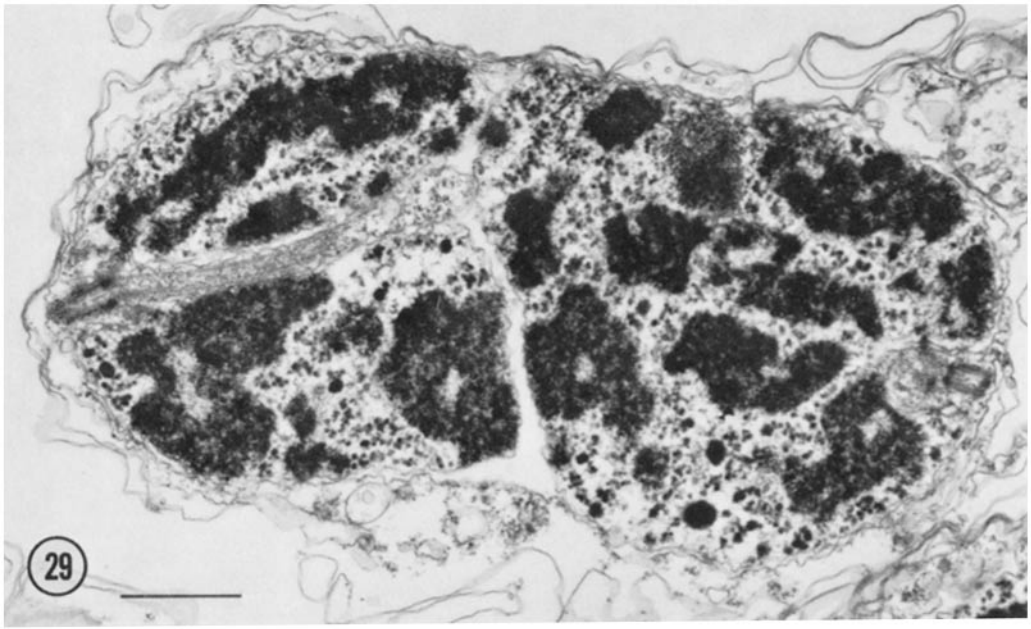


FIGURE 29 An invagination of the nuclear membrane divides the nucleus in two. Note portion of the channel at left and centrioles at opposite ends of nucleus. × 16,000.

DISCUSSION

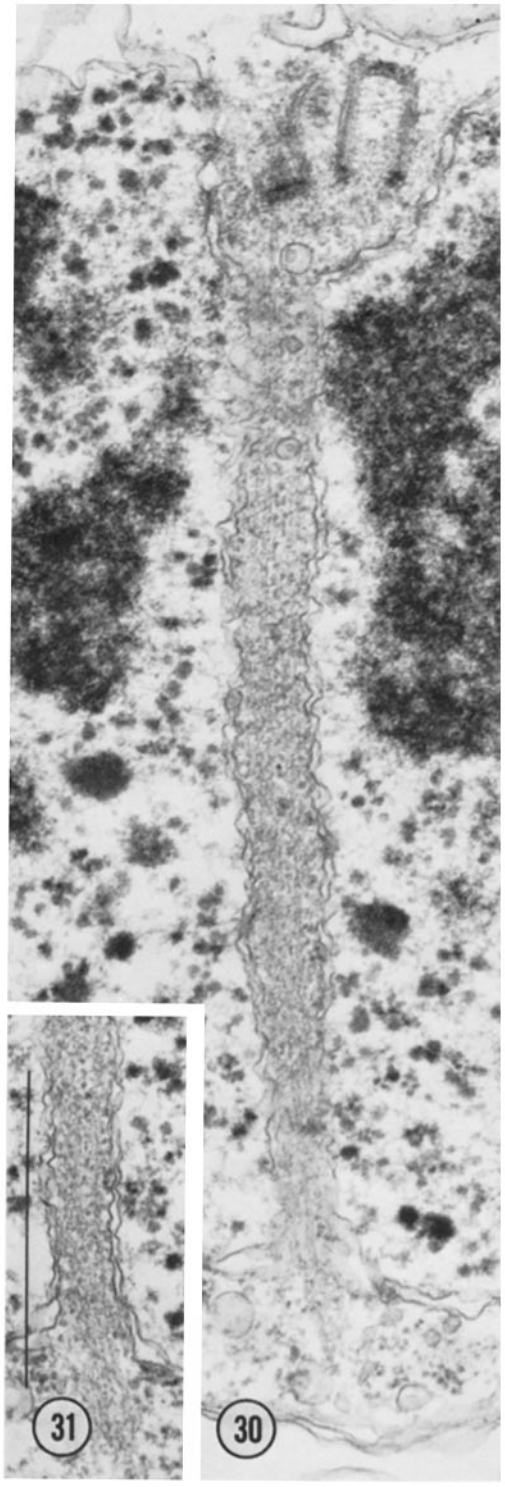
Syndinium Mitosis: A Unique Type of Chromosome Segregation

Several features of nuclear division in *Syndinium* which are revealed by our electron microscope study have not been seen in other organisms:

PERSISTENT INSERTION OF KINETOCHORES IN OPENINGS IN THE NUCLEAR MEMBRANE: At all stages in the cell cycle in the many divisions of *Syndinium* sporogenesis, chromosomes are associated with the nuclear membrane at highly specialized regions. These regions have all the characteristics of kinetochores, being trilaminar disks consisting of an outer dense layer from which microtubules originate, an inner dense layer continuous with the chromatin, and a less dense space separating these two components (cf. kinetochores of Radiolaria, reference 20; rat, reference 24; *Oedogonium*, reference 36; rat kangaroo, reference 39). At all stages these kinetochores are inserted in porelike openings of the nuclear membrane.

PERSISTENT ASSOCIATION BETWEEN MICROTUBULES AND KINETOCHORES: Microtubules are always found to be associated with each kinetochore, i.e., there is no stage in the cell cycle during which chromosome-associated microtubules are not present. These microtubules form a direct connection between kinetochores and centrioles, and therefore, the chromosomes are at all times attached to the centrioles. In interphase nuclei, each kinetochore is attached to the bases of *both* centrioles. Later, after kinetochores have duplicated and are seen as pairs of sister kinetochores, each is connected to a *separate* centriole by a bundle of microtubules.

EXTRANUCLEAR CENTRAL SPINDLE FORMED BETWEEN CENTRIOLES AND LOCATED IN CYTOPLASMIC CHANNEL THROUGH NUCLEUS: Early in division a bundle of microtubules forms between the bases of the centrioles, and, apparently as a result of elongation of these microtubules, the centrioles move apart. Microtubules are frequently associated with centrioles in other organisms and seem to be involved in centriole movements (35). It is, however, rare that microtubules attach at the bases of centrioles; to our knowledge, the only example of a similar situation was described for the second spermatocyte division of the diatom *Lithodesmium* (31). The centriole-spindle complex is at first in a groove on the nuclear surface but eventually becomes enclosed in a cytoplasmic channel through the center of the nucleus.

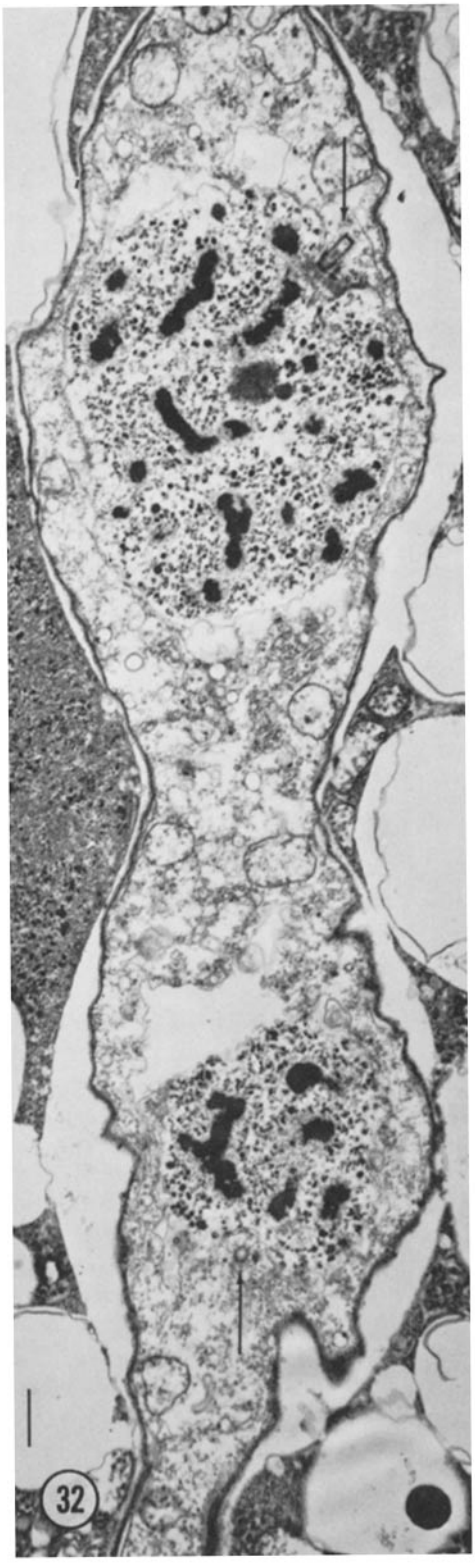


FIGURES 30 and 31 Two adjacent sections through an early daughter nucleus (cf. Fig. 19 *f*). The channel and microtubules have persisted, with centriolar pocket at one end of the channel and microtubules ending freely in the cytoplasm at the other end. × 42,000.

CONSTANT LENGTH OF KINETOCHORE MICROTUBULES DURING DIVISION: The microtubules connecting kinetochores to the bases of centrioles remain constant in length in interphase and throughout centriole movement and chromosome segregation.

These electron microscope observations of the structure of mitotic organelles in *Syndinium* suggest a rather unusual mechanism of chromosome segregation. After kinetochores have duplicated, centrioles separate. Because each kinetochore of a sister pair is connected through microtubules to a separate centriole, sister chromosomes are pulled in opposite directions, each following the centriole to which it is anchored. That this is essentially a passive mechanism of chromosome segregation is indicated by the fact that the distance between kinetochores and centrioles remains constant throughout division. In other words, there is no relative poleward movement of chromosomes. Thus, central spindle elongation provides the motile force not only for centriole movement, but also for the movement of chromosomes which are anchored to the centrioles. In this connection it is worth noting that the kinetochore microtubules and central spindle (interpolar) microtubules are completely separated from each other; according to the sliding-tubule hypothesis (32, 34), intermingling of interpolar and kinetochore microtubules would be necessary for chromosome movements relative to the spindle poles.

The pulling apart of sister kinetochores which are embedded in openings of the nuclear membrane raises questions as to the behavior of the membrane at regions of kinetochore insertion. Either kinetochores glide along in the plane of the membrane, or their positions are fixed in the membrane, and their relative separation is the result of the addition of new membrane between them through membrane flow or synthesis. We hold the second alternative to be more likely, since the sharp angle in the nuclear membrane at the site of kinetochores (Figs. 23, 24, and 28) indicates tension on the membrane and speaks against any facile gliding in the plane of the nuclear envelope. Such tension exerted on the nuclear membrane at the kinetochores during elongation of the central spindle might also be



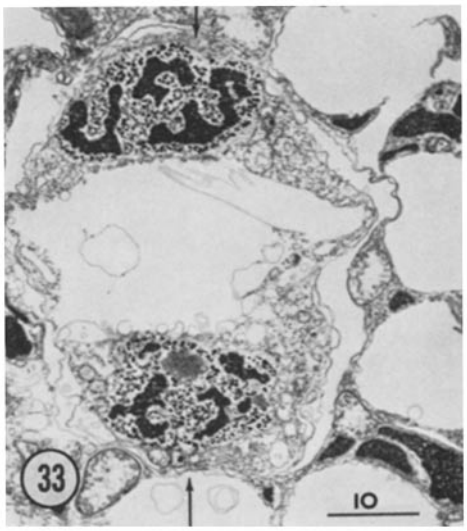


FIGURE 32 Daughter nuclei in undivided cell (young plasmodium). The centriolar pockets of the daughter nuclei are, as expected, always situated at far sides of the nuclei (arrows). × 7,700.

FIGURE 33 Daughter nuclei separated by vacuole in early plasmodium. Several such nuclei were serially sectioned. The centriolar pocket was always located at far sides of the nuclei in regions indicated by arrows. × 4,200.

responsible for the more extensive deformations of the nuclear surface which develop into the troughlike depression and the central cytoplasmic channel.

Syndinium Mitosis and Nuclear Division in Free-Living Dinoflagellates

Chatton, who first described the genus *Syndinium* (5), classified it with dinoflagellates largely on the basis of the general morphology of its spores which resemble certain free-living dinoflagellates. Our electron microscope study now shows that nuclear division in *Syndinium* is very different from that of previously studied dinoflagellates, either free-living (28, 29, 44) or parasitic (43). Best understood in terms of nuclear division is *Cryptothecodinium* (*Gyrodinium*) *cohnii* where division was described on the basis of complete serial sections (28). There are several basic differences: (*a*) During division, chromosomes in *Cryptothecodinium* become attached to to the nuclear membrane surrounding the channels, but specialized structures are never found at such attachment sites. Chromosome attachment to the membrane is thus specific but restricted to the time of nuclear division. (*b*) Never is there any connection between microtubules and chromosomes. Careful study of serial sections through dividing nuclei failed to show any contact between microtubules and the intact nuclear membrane. Leadbeater and Dodge (29) studying *Woloszynskia micra* and Soyer (42, 43) who examined *Blastodinium* concluded independently that in these species chromosomes are attached to the nuclear membrane without any visible differentiation. They also noted the complete absence of chromosome-microtubule interaction. Both Kubai and Ris (28) and Soyer (42, 43) suggested that in this absence of chromosome-microtubule contact the segregation of daughter chromosomes in dinoflagellates must be accomplished through some form of the membrane action as had been suggested in bacteria. (*c*) In *Cryptothecodinium* basal bodies are not involved in nuclear divisions, while in *Syndinium* basal body segregation is intimately involved in chromosome segregation.

In addition to the profound differences in its mechanism of mitosis, *Syndinium* is distinct from typical dinoflagellates also in the structure and chemistry of chromosomes. As we have shown here, *Syndinium* chromosomes do not have the typical structure of dinoflagellate chromosomes, and furthermore, their DNA is associated with basic proteins. We have not yet been able to study mature spores, but Hollande and Enjumet in a drawing of a mature spore (fig. XLIV, C2 of reference 18) show the chromosome rosette characteristic of *Syndinium* nuclei. In addition Manier et al. (30) recently published electron micrographs of spores of *Syndinium gammari* in which chromosomes have the structure we have described here, not the structure typical for dinoflagellates.

In view of the basic differences in their nuclear organization and mitotic mechanisms it is questionable whether *Syndinium* and related species should be classified with the dinoflagellates. Further, the terms dinokaryon, dinomitosis, and dinospore, therefore, must not be applied as has been done in the past to *Syndinium* and related forms. Future cytochemical and electron microscope studies might well show that many others of the fascinating parasites (see reference 4) or free-living forms like *Oxyrrhis* (14, Fig. 11, 15) now classified as dinoflagellates belong more appropriately with *Syndinium* in a new and separate group of protozoa.

Syndinium and Mitosis in Hypermastigote Flagellates

Grassé (12, 13), on the basis of light microscope studies, first called attention to the similarity of nuclear division in *Syndinium* and the hypermastigote flagellates. He observed, as had Cleveland (8), that kinetochores were attached to the nuclear membrane and concluded that, in the absence of an intranuclear spindle, kinetochores move on the nuclear membrane. These findings led Grassé to equate mitotic mechanisms in these large symbiotic flagellates and in *Syndinium*, and in agreement with Chatton, to extend this interpretation to the division of all dinoflagellates.

We have shown in the last chapter that the nuclear characteristics of *Syndinium* are totally different from those of typical dinoflagellates, and we shall show now that despite certain similarities, *Syndinium* mitosis is also quite distinct from mitosis in hypermastigotes such as *Trichonympha*. Hollande and Valentin have recently published several electron microscope investigations of division in hypermastigotes (for reference see 17). They showed that during division kineto-

chores are inserted into openings of the nuclear membrane and connected through kinetochore microtubules to the poles of the extranuclear central spindle. From this observation they concluded that hypermastigote chromosome segregation was accomplished in an orthodox manner with kinetochore microtubules pulling chromosomes towards spindle poles. This interpretation has recently been questioned by Kubai who has reinvestigated mitosis in *Trichonympha agilis* (27). Her analysis of chromosome segregation, based on complete serial sections of several stages of mitosis, shows that sister kinetochores have already separated and have begun to form two groups on the nuclear membrane facing the central spindle before the kinetochores become inserted into openings of the nuclear membrane and make contact with microtubules. It is clear then that a portion, if not all, of the disjoining motion of chromosomes is accomplished on the nuclear membrane without intervention of microtubules. Such results demonstrate that mitosis in *Trichonympha* is clearly distinct not only from the orthodox karyokinesis of higher organisms, but also from the unusual nuclear divisions of free-living dinoflagellates and *Syndinium*.

This is in disagreement with Hollande (17) who, in a recent review, has attempted to extend the interpretation of unusual divisions in terms of orthodox mechanisms not only to *Trichonympha* but also to *Syndinium* and all dinoflagellates. He draws the general conclusion that chromosome segregation in all eukaryotes is accomplished through action of kinetochore microtubules engaged in a mitotic spindle. To be meaningful, such sweeping generalizations must of course be founded on detailed electron microscope studies. Hollande, without producing any additional evidence, arbitrarily denies the observation of all investigators of dinoflagellate mitosis that microtubules are not attached to chromosomes. His interpretation furthermore is based on ignorance of the ultrastructure of *Syndinium* and on incomplete understanding of mitosis in the hypermastigote flagellates.

Thus Hollande has by no means invalidated Grassé's view (12, 13) that unusual mitotic mechanisms operate in a number of protozoans. However, Grassé's concept of "pleuromitosis" which suggests that these mechanisms are all closely related is no longer useful, since the electron microscope study of some of these protozoans has now made it clear that a number of distinct mitotic mechanisms are operating in these forms.

Syndinium and the Evolution of Mitotic Mechanisms

On the basis of present knowledge we see three types of mechanisms operating in chromosome segregation which may have originated sequentially during evolution:

(*a*) Attachment and movement of chromosomes on the membrane as in prokaryotes and dinoflagellates and in early chromosome segregation of *Trichonympha*.

(*b*) Differentiation of a microtubule-organizing center (kinetochore) at the chromosome-membrane attachment site. This kinetochore, inserted into the nuclear membrane, becomes linked to the poles of a central spindle by kinetochore microtubules. These kinetochore microtubules remain spatially separated from the central spindle microtubules and may not shorten during chromosome segregation. The motile force of spindle elongation is used to separate sister kinetochores inserted in the nuclear membrane (*Syndinium* and late division in *Trichonympha*).

(*c*) Incorporation of chromosomes within the mitotic spindle as in the orthodox mitosis of typical eukaryotic cells. In such mitoses, kinetochore microtubules are intermingled with the interpolar (central spindle) microtubules. These microtubules are involved in the orientation of chromosomes in a metaphase plate with sister kinetochores pointing to opposite spindle poles. Kinetochores are moved towards the spindle poles either by a polymerization-depolymerization equilibrium (23), or by a sliding-microtubule mechanism (32, 34). The presence or absence of differentiated structures at the spindle poles (for example centrioles, spindle pole bodies, spindle plaques), or the persistence or disappearance of the nuclear membrane are clearly secondary variations and not essential aspects of this type of mitotic mechanism.

Mitosis in *Syndinium* appears to resemble most closely nuclear division in hypermastigote flagellates. It is not far fetched to speculate that a *Syndinium*-like karyokinesis might have been derived from a *Trichonympha*-like division by the establishment of permanent kinetochore-spindle pole connections, thus obviating the need for membrane-mediated kinetochore movements. These observations might perhaps prove useful

for an understanding of the relationship of *Syndinium* to other protozoa.

We wish to dedicate this study to Sally Hughes-Schrader who for many decades with unerring instinct has demonstrated how to use exceptional behavior in exotic organisms as nature's experiments to illuminate cellular phenomena. We are grateful to Professor P. Bougis for the us of the facilities at the Station Zoologique, Villefranche-sur-Mer (France) on several occasions. Our special thanks go to Drs. Jean and Monique Cachon whose continued interest, help, and advice have greatly facilitated this investigation. The expert technical assistance of Renate Bromberg is gratefully acknowledged. We thank Cheryle Hughes who made the drawing for this paper.

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