ULTRASTRUCTURE OF NUCLEAR DIVISION IN *PARAMECIUM AURELIA*

I. MITOSIS IN THE MICRONUCLEUS

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Abstract

Ultrastructural details of micronuclear mitosis in the ciliate *P. aurelia* are described. Throughout mitosis the nuclear membrane remains intact and centrioles are not associated with the dividing nucleus. At late interphase the micronucleus has a peripheral layer of microtubules, a fibrous zone, and a core of condensed chromatin. This core fragments at prophase, the peripheral microtubules disappear, and microtubules extend from the fibrous zone. At metaphase, over 100 chromosomes could be seen, each attached to a bundle of microtubules though kinetochores were indistinct. Anaphase was accompanied by a 10-fold increase in length of the micronucleus, accompanied by growth of continuous microtubules, resulting in the formation of a separation spindle over 30 μm long. Daughter nuclei were pinched off the separation spindle at telophase, leaving a projecting spindle scar on the nucleus. No new membrane formed around daughter nuclei. Both the spindle scar and the separation spindle disappeared as the nucleus returned to interphase. After mitosis is complete the peripheral layer of microtubules is not present. It must appear sometime during interphase. Several aspects of micronuclear mitosis are discussed in relation to mitosis in various other organisms.

I. INTRODUCTION

Many aspects of the genetics and biology of *Paramecium aurelia* have been studied intensively over many years. Its fine structure has been investigated extensively and recently Jurand and Selman (1969) have published an ultrastructural atlas of the organism. One important process which is not described in detail, however, is nuclear division, either of the micronucleus—the diploid "genetic" nucleus—or of the macronucleus, the polyploid "cellular control" nucleus. Indeed, there is a dearth of information on nuclear division in holotrichous ciliates generally. This is largely because they are difficult subjects for the study of nuclear division by light microscopy. The nuclear membranes do not break down, the spindle apparatus is very small in the micronucleus (usually only a few microns across), and is atypical and usually extremely difficult to observe in the macronucleus. In many ciliates including *Paramecium* no structure resembling a spindle is visible in the macronucleus without the aid of a polarizing microscope (Schwartz 1957).

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Until very recently the only accounts of nuclear division available dated from the 19th century. For *P. aurelia* Hertwig (1889) gives a very detailed account (summarized in English by Sonneborn 1947). In recent years, electron microscopy has resulted in more detailed accounts of nuclear division in several ciliates becoming available (Raikov 1962, 1966; Flickinger 1965; Jenkins 1967; Tucker 1967; Inaba and Sotokawa 1968; Jurand and Selman 1970). The last of these deals with *P. aurelia*, but it is incomplete and lacks descriptions and illustrations of many of the stages of nuclear division. In view of the importance of *P. aurelia* and ciliates generally in present-day cell biology, a good understanding of their nuclear division processes is essential. The present paper describes the fine structure of micronuclear mitosis in *P. aurelia*, while Part II of the series (Stevenson and Lloyd 1971) will describe the division of the macronucleus. Our observations greatly extend those of Jurand and Selman (1970) and enable us to describe all stages of micronuclear mitosis.

II. Materials and Methods

Stock 540 of syngen 1 of *P. aurelia*, supplied by Professor G. H. Beale, Institute of Animal Genetics, University of Edinburgh, was used throughout. Cells were cultured monoxenically in 0·1% (w/v) Vegemite (a commercial yeast extract manufactured by Kraft Foods Ltd., Australia) inoculated with *Aerobacter aerogenes* strain SYD as food organism. Samples of actively growing small mass cultures (75 ml) were examined in depression slides and dividing animals selected and fixed individually. Cells which are about to divide can often be recognized, and we have termed them predividers. They are larger and rounder, slower, and roll rather more in swimming, and have a less obvious oral groove as a result of the formation of a new gullet and surface structures. Such cells were picked out with a micropipette and fixed. Cells undergoing cytokinesis were also selected.

Cells were removed with a micropipette and fixed in 2·5-cm watchglasses containing 4% glutaraldehyde buffered with Tris–HCl (1 g/l) at pH 7·4 and 0·003M $CaCl_2$, for 1–1·5 hr at room temperature. Cells were washed twice with Tris–$CaCl_2$ buffer in the watchglass by removing 90% of the liquid and then adding buffer back to the original volume, taking care to ensure that the cells did not dry out. After the second wash the cells were embedded in 2% Bacto agar (Difco). The agar was examined under a dissecting microscope, and plugs containing cells were removed. These were transferred to a stoppered vial and washed again with buffer. Cells were then post-fixed overnight in either 1 or 2% OsO_4 (w/v) in the same buffer, washed twice, and block-stained with 0·5% (w/v) uranyl acetate for a similar period of time. These times were used to ensure thorough penetration of solutions into the agar plugs. After two washes in buffer, cells were dehydrated through an ethanol series over 8 hr and, using three changes of 100% ethanol, passed through two changes of propylene oxide over 2 hr. They were impregnated overnight in Spurr's resin (Spurr 1969) after passing through mixtures of propylene oxide : Spurr's resin (2:1) and propylene oxide : Spurr's resin (1:2) over a 2-hr period. All processing steps were carried out at room temperature. The resin was polymerized at 70°C overnight using flat embedding boats.

Sections were cut with the cells in longtitudinal orientation, mounted on naked 200-mesh or 150-mesh hexagon grids, stained with lead tartrate (Millonig 1961), and examined in a Hitachi HU11E electron microscope at 75 kV.

Adjacent sections with blue or green interference colours were also cut, dried down on glass slides, and stained with 1% (w/v) toluidine blue in 1% borax solution. Photomicrographs were taken using a Zeiss Universal microscope and either 4 by 5 in. Polaroid film or 35 mm film.

III. Results

As remarked earlier, micronuclear mitosis is difficult to observe with the light microscope owing to the small size of the nucleus (3–4 μm). It is complicated further by the fact that, at least in the initial stages in *P. aurelia*, orientation of the micro-

nucleus may be random. Mitosis may occur at any angle, even 90°, to the long axis of the cell. This means, of course, that one cannot guarantee obtaining sections of a given orientation of mitosis. Further, although the micronucleus is often found close to the macronucleus, this is not necessarily the case, as it may be carried by the powerful cyclosis of the *Paramecium* cytoplasm to any area of the cell except the cortical zone just below the pellicle.

Previous descriptions of mitosis in ciliates have used the terminology of mitosis in Metazoa. In most cases the appearance of the dividing nucleus is close enough for the meaning of these terms to be self-evident, but there may be occasions on which this terminology is not entirely satisfactory. Figure 1 represents the stages of mitosis in *P. aurelia* diagrammatically, and is based on Sonneborn's (1947) summary of Hertwig's (1889) account and our own observations.

Each *P. aurelia* cell has two micronuclei, so that the process is duplicated. Strict synchrony is not necessarily observed, as Jurand and Selman (1970) also report.

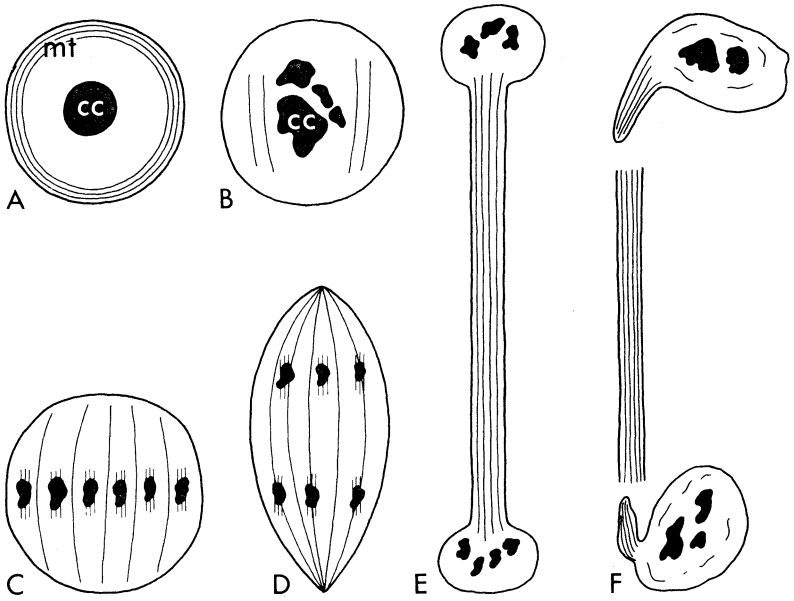

Fig. 1.—Mitosis of the micronucleus (diagrammatic—not all to same scale). *A*, interphase: *cc*, core of condensed chromatin; *mt*, microtubules; outer heavy line is nuclear membrane. *B*, prophase; *C*, metaphase; *D*, early anaphase; *E*, late anaphase; *F*, telophase.

(*a*) *Interphase*

The structure of the interphase micronucleus has been described by several authors (Sonneborn 1947, 1953; Jurand and Selman 1969). In the light microscope the micronucleus appears as a weakly staining sphere about 3–4 μm in diameter with a densely staining core (Fig. 2). In the electron microscope the appearance is similar to that seen in the light microscope (Fig. 3). The core is of variable size and shape, and often contains small areas of low density. Surrounding the core is a granular zone again of variable size. The outer area of the nucleus contains fibrous material of lower electron density. The nuclear membrane has pores at intervals of 50–100 nm.

There is a layer of microtubules of diameter 23 nm, which may be several tubules thick, running immediately beneath the nuclear membrane. We have not always been able to observe these microtubules in interphase nuclei, as fixation is very variable; osmium tetroxide alone often gives equivalent or superior results to glutaraldehyde followed by osmium tetroxide.

(*b*) *Prophase*

At prophase the intranuclear spindle begins to form. Polymerization of microtubules occurs initially in the fibrous region of the nucleus (Figs. 4 and 5). The microtubules appear in an amorphous matrix, the core of the nucleus begins to fragment, and the microtubules near the membrane disappear. At a slightly later stage of prophase than that shown in Figure 4, the nucleus usually becomes more nearly circular in section; frequently it is somewhat irregular in outline during interphase.

(*c*) *Metaphase*

The core has completely fragmented; the condensed chromatin of the region has either fragmented to become the chromatin bodies or coalesced with these bodies as they condense from the fibrous region. Microtubules are now evident in much of the nucleus, with the chromosomes attached to them at indistinct kinetochores (Figs. 6 and 7). With so many chromosomes (over 100 can be seen in some sections) the nucleus appears to have more chromosomal microtubules than continuous spindle microtubules, but the latter are certainly present.

Centrioles are never associated with the dividing micronucleus and there is no marked convergence of microtubules towards "poles", so we shall use the word "pole" to mean the regions of the nucleus furthest from the metaphase plate. No evidence was found for any structure resembling the polar vesicle described by Tucker (1967) in *Nassula*. However, it is possible that there may be specialized areas of the nuclear membrane able to serve as poles. Usually the microtubules appear less well organized near the poles of the nucleus.

(*d*) *Anaphase*

Separation of the chromosomes at anaphase is accompanied by an approximately 10-fold elongation of the spindle. As the chromosomes start to separate the nucleus becomes markedly elliptical (Fig. 8), then sharply pointed at the poles (Fig. 9). It is difficult to ascertain how far chromatid separation is due to nuclear lengthening or to traction of the chromatids due to shortening of chromosomal

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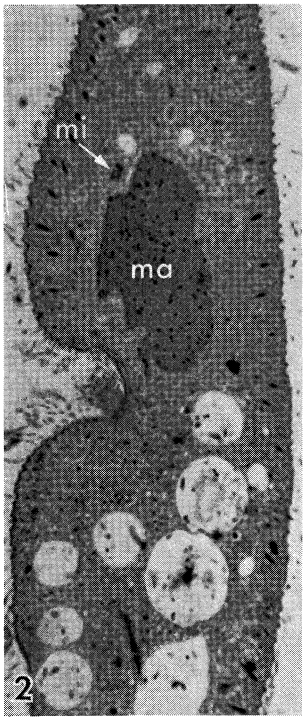
Fig. 2.—Section of *P. aurelia* stained with toluidine blue. Macronucleus (*ma*) with interphase micronucleus (*mi*) in a pocket. This is a typical position for one or both micronuclei in interphase. ×800.

Fig. 3.—Interphase micronucleus. Core of condensed chromatin (*cc*), surrounded by a granular zone (*gz*) and outer fibrous zone (*fz*). Microtubules (*mt*) running beneath the nuclear membrane can be seen. This nucleus was fixed with osmium tetroxide only. ×26,000.

Fig. 4.—Early prophase micronucleus. Microtubules (*mt*) are appearing in the fibrous zone, in an amorphous matrix. The peripheral microtubules have almost disappeared, but a few are still evident (small arrow). Pores are also evident (large arrowheads). ×28,000.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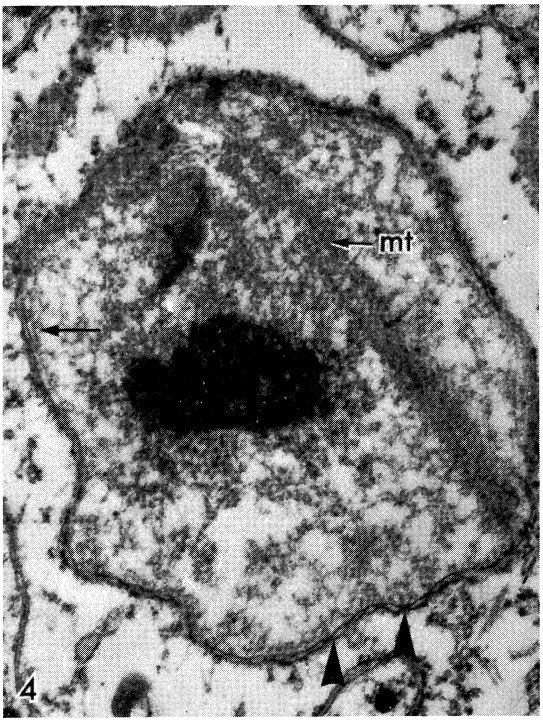
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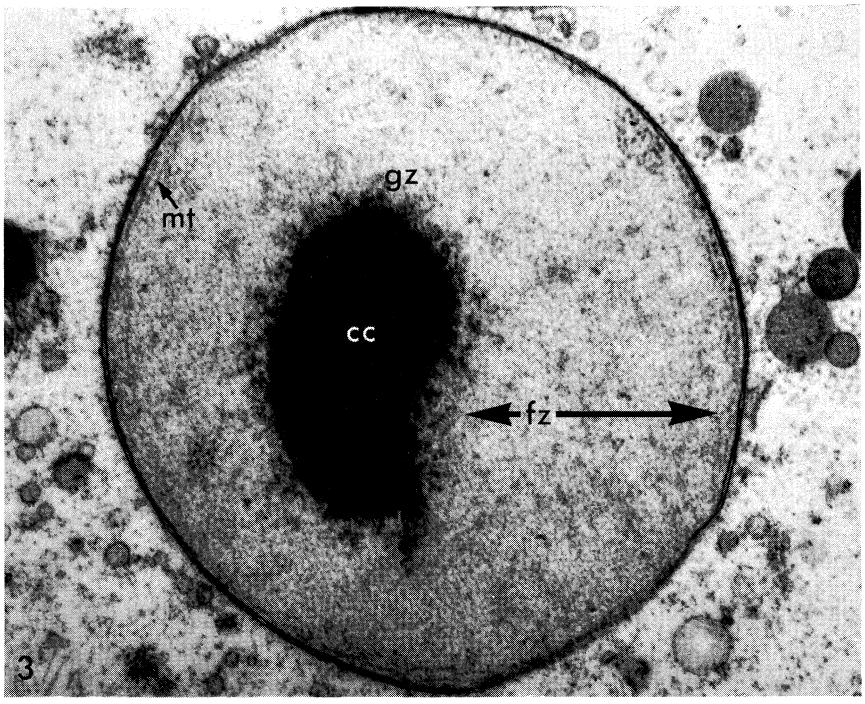
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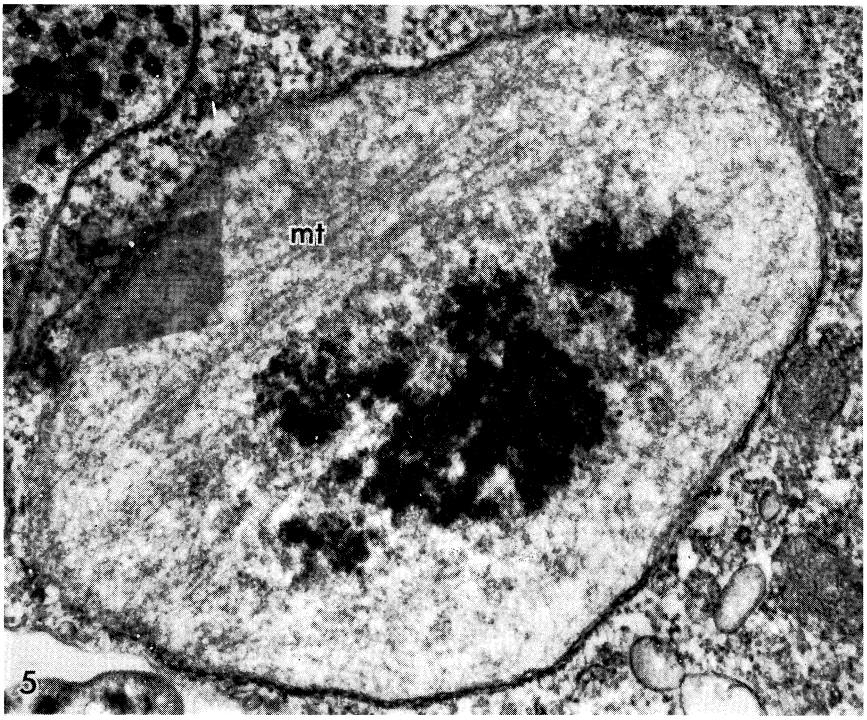


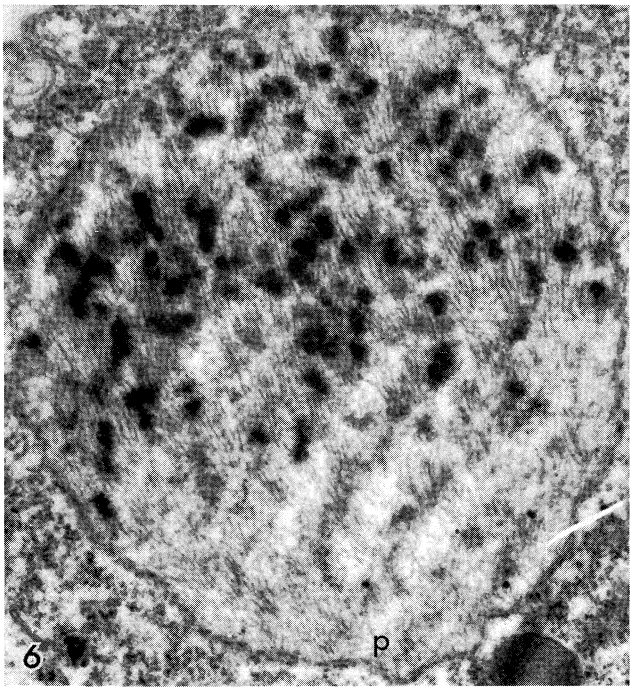


Fig. 5.—Later prophase micronucleus. The core is fragmenting and more microtubules (*mt*) are evident in the fibrous zone. ×29,400.

Fig. 6.—Metaphase micronucleus. Over 80 separate chromosomes can be seen. The spindle microtubules are less well organized at the pole (*p*). ×19,600.

Fig. 7.—Metaphase micronucleus. Chromosome attached to bundle of microtubules. ×56,000.

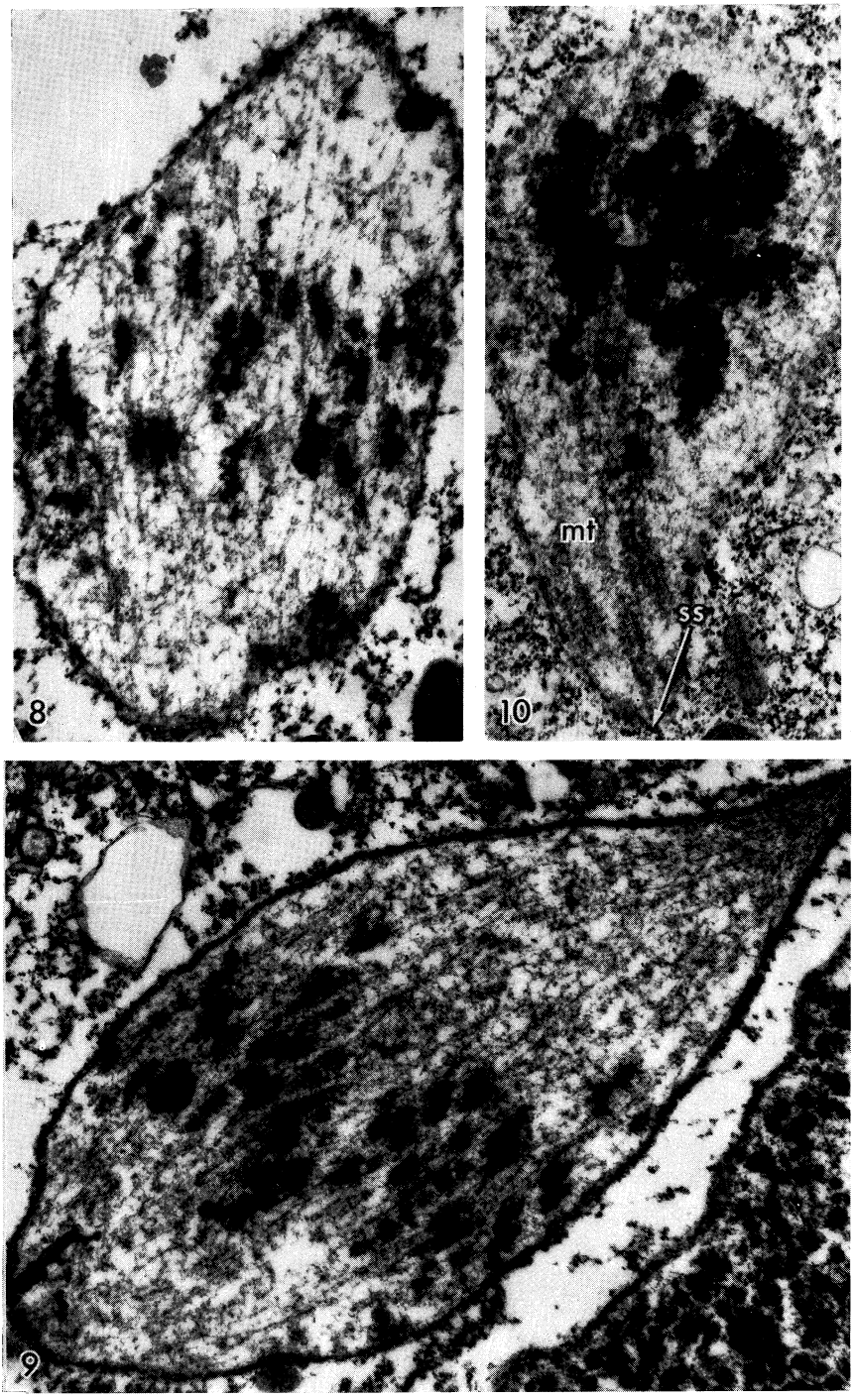
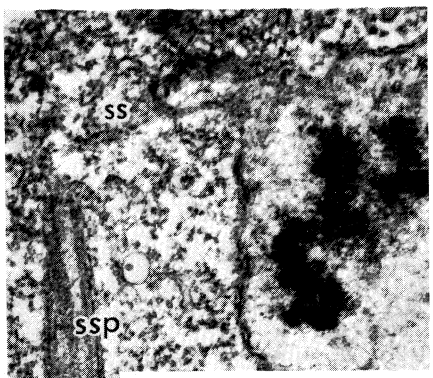


Fig. 8.—Early anaphase micronucleus. Chromosomes beginning to separate. ×15,200.

Fig. 9.—Later anaphase micronucleus. Nucleus lengthening, with sharply pointed poles. ×16,000.

Fig. 10.—Early telophase micronucleus, with spindle scar (*ss*), containing microtubules (*mt*). Chromatin beginning to condense. ×19,600.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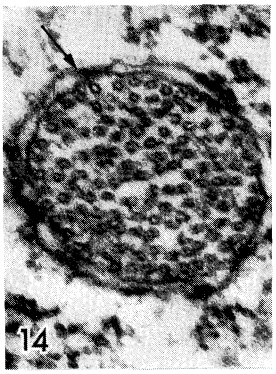

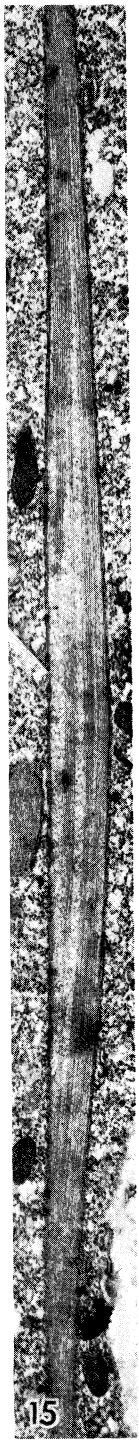

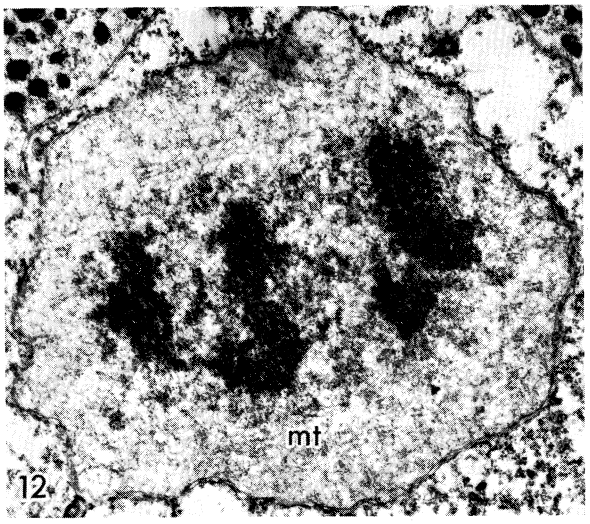
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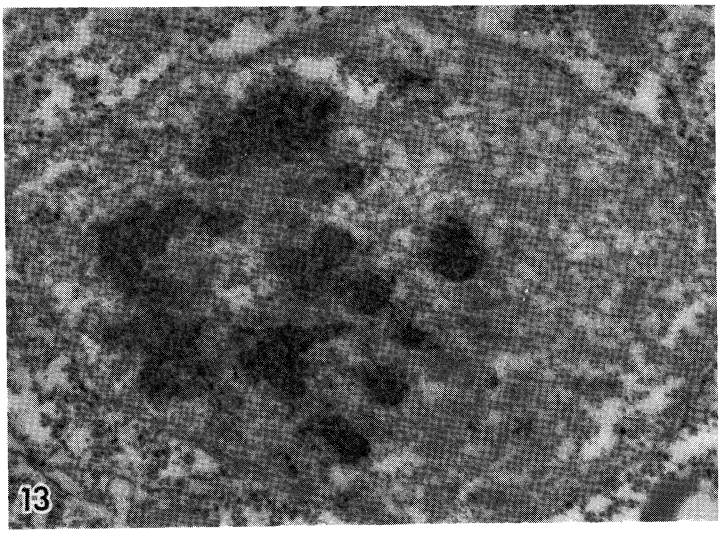

microtubules, but with such a large and rapid increase of length it is likely the increase in length is the more important factor. The nuclear membrane remains intact and by the end of anaphase the nucleus is a dumbbell-shaped structure, usually about 35 μm long, with the chromosomes tightly packed in the terminal knobs.

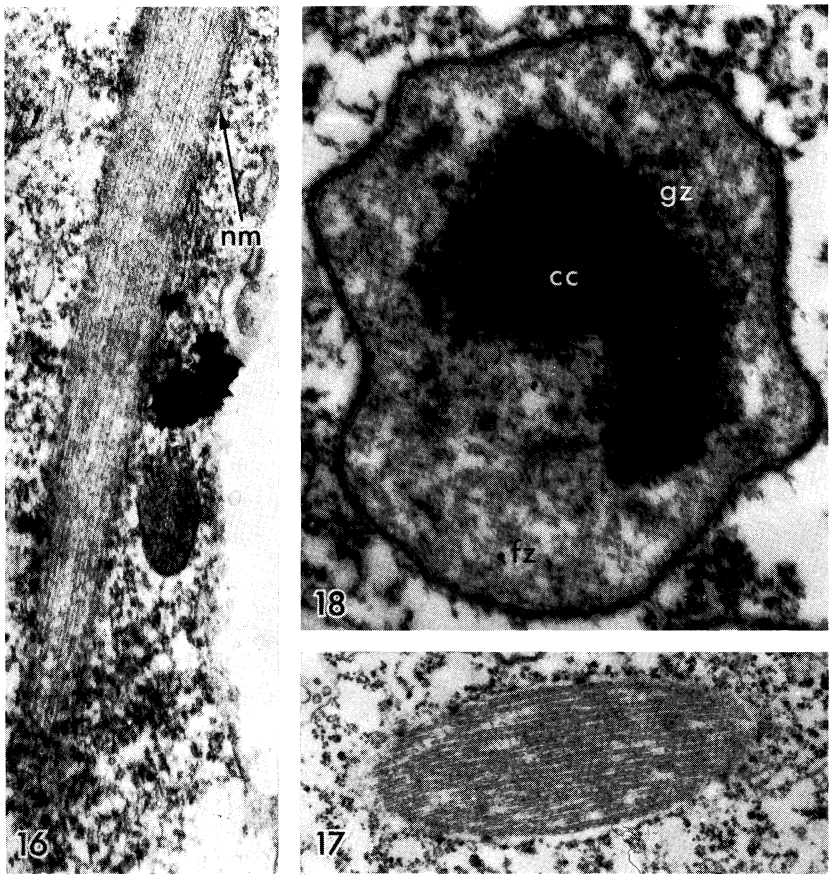

Fig. 16.—Separation spindle. Detail of one end of the spindle illustrated in Figure 14. The sleeve of nuclear membrane (*nm*) does not cover the ends of the separation spindle. ×22,400.

Fig. 17.—Separation spindle at last recognizable stage. No nuclear membrane is now apparent. ×39,000.

Fig. 18.—Micronucleus of cell undergoing cytokinesis. The nucleus has returned to an interphase condition, but does not have the peripheral layer of microtubules. ×26,000.

Fig. 11.—Telophase micronucleus, with spindle scar (*ss*) in close apposition to the separation spindle (*ssp*). ×19,600.

Fig. 12.—Telophase micronucleus. Spindle scar has now nearly disappeared and chromatin is more condensed. Degenerating microtubules (*mt*) in fibrous region. ×19,600.

Fig. 13.—Late telophase. Spindle scar is now barely evident and the nucleus is largely back to an interphase condition. ×28,000.

Fig. 14.—Separation spindle in transverse section, showing sleeve of nuclear membrane (arrow). ×65,000.

Fig. 15.—Separation spindle. Low-power view of whole spindle at early telophase. ×8,400.

(e) *Telophase*

At telophase the daughter nuclei become detached from the separation spindle. The separation spindle is the name given by Wenrich (1926) to the region of spindle between the terminal knobs. The spindle and membrane appear to constrict about 1 μm from the terminal knob and leave a projection on the nucleus, the spindle scar, containing microtubules (Figs. 10 and 11). The spindle scar is evident on newly divided micronuclei for some time after division. The chromatin now begins to coalesce; usually at the stage of splitting off it has condensed into a few masses (Figs. 12 and 13). The area of lower density of the nucleus contains degenerating microtubules (Figs. 12 and 13). During the separation process the daughter nuclei may be pushed about by cyclosis and may end up at an angle to the separation spindle, which, however, stays oriented longitudinally in the cell, usually across the cell isthmus which is now beginning to form across the cell. There appear to be no special cytoskeletal elements in *P. aurelia* to ensure that each daughter nucleus is deposited in the correct half of the cell. It seems rather that the spindle may be pushed into a suitable position by cyclosis. We shall discuss this point later.

The separation spindle disappears after the daughter nuclei have detached from it. It becomes shorter and shorter and finally vanishes by the time cytokinesis is concluded. As the separation spindle becomes smaller it may be carried away from the cell isthmus. Initially, however, it is visible as a bundle of microtubules enclosed by a sleeve of nuclear membrane (Figs. 11, 14, 15, 16). Figure 17 shows the final stage of dissolution of the separation spindle. The ends, but not the sides, of the cylindrical separation spindle are open to the cytoplasm (Fig. 16) in contrast to the spindle scar on the nucleus, which stays closed throughout. The daughter nucleus returns to interphase following the conclusion of cytokinesis, though the band of microtubules under the membrane is absent initially (Fig. 18). The band appears later in interphase, although the precise time of reappearance has not been established.

IV. Discussion

It has become evident that mitosis in ciliate protozoa has several distinctive features. The principal ones are the persistence of the nuclear membrane and the non-involvement of centrioles. In *P. aurelia* the mode of separation of the daughter nuclei at late anaphase and telophase is also interesting, because of the great increase in length of the whole nucleus and the formation of separation spindles.

Closed spindles occur in some protozoa, algae, and fungi (see Pickett-Heaps 1969 for review). Among cells with closed spindles there is great variability in organization of nuclear division. In all the ciliate protozoa which have been studied, mitosis is what Jenkins (1967) has called closed-acentric. In contrast to nuclear division in dinoflagellates (Leadbetter and Dodge 1967; Kubai and Ris 1969), trypanosomes (Vickerman and Preston 1970), and *Euglena* (Leedale 1968) mitosis in the ciliate micronucleus resembles, in many respects, that of higher animals and plants. Certainly the names of most of the mitotic stages are applicable, whereas in some other protists they may not be. It is only at anaphase and telophase that mitosis in the ciliate micronucleus is appreciably different from that of other organisms. In

dinoflagellates and trypanosomes the differences are greater, and the nuclear membrane appears to have a kinetic role, while in *Euglena* segregation mechanisms are still very ill-understood.

The persistence of the nuclear membrane means that polymerization of the microtubules of the spindle must take place entirely within the nucleus, though precursors might enter through nuclear pores. In contrast to this, in many algae—*Chlamydomonas* (Johnson and Porter 1968), *Spirogyra* (Fowke and Pickett-Heaps 1969), *Hydrodictyon* (Marchant and Pickett-Heaps 1970)—and in higher plants (Bajer and Molé-Bajer 1969) and animals (Threadgold 1967) polymerized microtubules invade the nucleus or the nuclear area after breakage of the membrane. This invasion is a marked feature of algae which have partially closed spindles. However, nuclei with closed spindles possess an ability to polymerize microtubules. This raises questions concerning microtubule-organizing centres. Pickett-Heaps (1969) has argued forcefully for the existence of such centres. In early prophase nuclei of *Paramecium* the microtubules (still short and indistinct) are surrounded by amorphous material. Clearly these microtubules are polymerized and it seems possible that any area of the nucleus could serve as "centre". Such a centre might only be a site within the nucleus at which suitable conditions for orientated polymerization occurred. Polymerization could then proceed to contribute to the formation of the spindle.

Centrioles are never associated with the dividing nucleus in *P. aurelia*, or in other ciliates which have been studied (Raikov 1966; Jenkins 1967; Tucker 1967; Inaba and Sotokawa 1968). Since a ciliate has hundreds of centrioles distributed beneath the pellicle, it clearly has no need to associate centrioles with the mitotic apparatus to ensure their even distribution to daughter cells. Cells which are not ciliated, but which retain the ability to produce cilia, clearly must ensure such a distribution and do so by associating the centrioles [which Pickett-Heaps (1969) regards as highly specialized microtubule-organizing centres] with the mitotic apparatus.

In the micronucleus at late anaphase (maximal extension) it can be calculated that there is about five times as much surface area as in an interphase nucleus. Hence there is a very extensive synthesis of new membrane which correlates with the frequent presence in the vicinity of dividing nuclei of the orthotubular system (Ehret and DeHaller 1963) and the flattened membrane stacks, which are the closest approximation to a Golgi apparatus in *Paramecium*, and many mitochondria and ribosomes. The function of the orthotubular system is unknown (Jurand and Selman 1969) but seems to be related to membrane and microtubule formation as it is commonly found in areas of the cell where active membrane and microtubule synthesis is occuring—for example, near the contractile vacuoles.

The long spindles observed in late anaphase were named separation spindles by Wenrich (1926) in a light microscope study of mitosis in *P. trichium*. Raikov (1966) calls the similar (and even longer) structures in *Nassula* "Verbindungstränge"—connecting strands. Elongation of the spindle proceeds very rapidly, accompanied by growth of the continuous microtubules, which may enter the nucleus in a precursor form from the cytoplasm. Hence it is possible the nucleus is being "invaded",

not by polymerized microtubules as in some other organisms, but by precursors. Individual microtubules have been followed for lengths of over 10 μm, so it seems likely that at the end of extension the microtubules run continuously the whole length of the separation spindle. A length of 35 μm is shorter than that given by Hertwig (1889) but in stock 540 we have never observed it to be larger, nor have we observed a "mid-piece" wider that the rest of the spindle. Of course the stock and syngen of his material is unknown. It is quite possible that other stocks or syngens may have longer separation spindles and mid-pieces.

As remarked earlier, the initial stages of mitosis do not necessarily occur parallel to the long axis of the cell. As extension proceeds, however, the spindle becomes more and more longtitudinally oriented. The cyclosis of the cytoplasm may move the spindle apparatus into position, as no cytoskeletal elements have been seen, in contrast to the situation in *Nassula* (Tucker 1967) in which bridges form between the membrane of the anaphase micronucleus and that of the elongating macronucleus. Certainly in *Paramecium* as the spindle enlarges the possible orientations must become fewer. A predivider is forming a new gullet for its future opisthe, so that the cell effectively has two gullets. Also by the time of elongation of the micronucleus the macronucleus has also elongated and fills most of the side of the cell away from the gullets. The route of cyclosis (Mast 1947) would suggest that the long and inflexible spindle could be carried towards the centre of the cell. It is possible that other factors, for example the commencement of cytokinesis or macronuclear karyokinesis, might also exert some influence.

We have added a number of significant details to earlier accounts of mitosis in *P. aurelia*. Observations on telophase are amongst the most interesting. The daughter nuclei are pinched off from the separation spindle. How this happens is unclear but a definite projection, the spindle scar, is formed on each daughter nucleus. This scar initially has microtubules lying within it but these very quickly disappear. The projection itself is resorbed more slowly and it is evident for some time. Concurrent with the resorption of the spindle scar, the central area of chromatin reforms and the nucleus resumes its interphase appearance, except for the lack of the peripheral microtubules. The spindle persists longer than the spindle scar on the nucleus. It is still bounded by the nuclear membrane, but ends are open to the cytoplasm and it seems that the microtubules depolymerize from these ends. The sleeve of nuclear membrane disappears before the microtubules, although by the time the membrane has been resorbed, the separation spindle is reduced to a small vestige (Fig. 16). The whole structure has vanished by the time of cell separation. It is clear also that no new envelope is formed around the daughter micronuclei, in contrast to *Nassula ornata* (Raikov 1966) and *Blepharisma* (Jenkins 1967; Inaba and Sotokawa 1968).

Evidently there is much variation in the mechanisms of the closed-acentric mitoses of ciliates, as all the described examples show significant differences. However, these differences appear to be idiosyncrasies of individual species, rather than basic differences of mechanism.

V. Acknowledgments

We would like to thank Professor D. J. Carr and Mr. H. J. Marchant for helpful discussions and comments.

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