

FINE STRUCTURE OF DIVISION IN CILIATE PROTOZOA

I. Micronuclear Mitosis in *Blepharisma*

R. A. JENKINS

From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010. The author's present address is the Department of Zoology and Physiology, University of Wyoming, Laramie, Wyoming 82070

ABSTRACT

The mitotic, micronuclear division of the heterotrichous genus *Blepharisma* has been studied by electron microscopy. Dividing ciliates were selected from clone-derived mass cultures and fixed for electron microscopy by exposure to the vapor of 2% osmium tetroxide; individual *Blepharisma* were encapsulated and sectioned. Distinctive features of the mitosis are the presence of an intact nuclear envelope during the entire process and the absence of centrioles at the polar ends of the micronuclear figures. Spindle microtubules (SMT) first appear in advance of chromosome alignment, become more numerous and precisely aligned by metaphase, lengthen greatly in anaphase, and persist through telophase. Distinct chromosomal and continuous SMT are present. At telophase, daughter nuclei are separated by a spindle elongation of more than 40 μ , and a new nuclear envelope is formed in close apposition to the chromatin mass of each daughter nucleus and excludes the great amount of spindle material formed during division. The original nuclear envelope which has remained structurally intact then becomes discontinuous and releases the newly formed nucleus into the cytoplasm. The micronuclear envelope seems to lack the conspicuous pores that are typical of nuclear envelopes. The morphology, size, formation, and function of SMT and the nature of micronuclear division are discussed.

INTRODUCTION

To date electron microscopy of protozoan nuclei has resulted in the description of numerous and diverse structures which are not always reconcilable in terms of a unifying story of nuclear morphology and division. Information on the micronucleus in division is particularly lacking and may be due, as Nanney and Rudzinska (26) suggest, to its small size although other factors, such as problems in fixation and identification of appropriate stages, have made an electron microscope study more difficult. A lack of ultrastructural information is emphasized by the fact that Pitelka's generally complete work (27)

includes almost no description of micronuclei and by the recent suggestion that a true mitosis does not occur in ciliate micronuclei (9).

The ciliate micronucleus may offer certain advantages in attempts to analyze the dynamic processes involved in nuclear division. Among current needs are a chemical dissection of essentially clean mitotic apparatus microtubules after the approach utilized by Gibbons for ciliary shafts (10) and a more complete knowledge of the unstructured, internal environment which immediately surrounds the kinetic elements (49). Since micronuclear mitosis is completed within the

confines of an intact nuclear envelope, the unstructured environment of the achromatic apparatus is more likely to be peculiar to the division process than in the case of nuclear envelope breakdown and the formation of the so-called mixoplasm (34) as occurs in most mitoses. Also, the isolation of the mitotic apparatus free of cytoplasmic contamination may be accomplished by the in vitro disruption of isolated, intact, dividing micronuclei. Small numbers of dividing micronuclei are now routinely isolated by the author and are found, by phase microscopy, to be free of cytoplasmic contamination. Assuming that the SMT of these nuclei can be stabilized through the isolation procedure (electron microscopy has not yet been done), it would seem a simpler task to isolate micronuclei, then disrupt the envelope, and collect clean spindle material in a controlled environment than to isolate the fibrous mitotic apparatus from the cytoplasmic material in which it is bathed, as is usually done, and then attempt to isolate SMT free of contaminating material. Synchronous cultures of ciliates could easily provide ample numbers of dividing micronuclei for most types of experimentation.

Microtubules have been described previously for dividing ciliate nuclei (4, 30, 36, 37), but all micronuclear stages have been studied only in situations where the elements of the achromatic apparatus were not preserved because of the fixation procedures employed (6-8). Spindle microtubules (SMT) are well preserved by the fixation technique utilized in this work, and all mitotic stages are reported. The major aim of this paper is to give a more complete description than had been presented of the fine structure of micronuclear division and to provide further information on the general involvement of microtubules in mitosis. The work has served the author as an introduction to further investigations such as the isolation of dividing micronuclei and the microtubules contained therein. The observations reported are restricted to the genus *Blepharisma* and then only to the micronucleus; fine structure studies of the micronuclear mitosis and macronuclear division in *Didinium* and the macronuclear division of *Blepharisma* will be subjects of subsequent reports (in preparation).

MATERIALS AND METHODS

The majority of the observations reported here were made on either *Blepharisma undulans americanum* Suzuki

or an organism (obtained from General Biological Supply House, Chicago, Ill.) which is similar in size but distinct from *B. undulans americanum*; this organism will be referred to as *Blepharisma* sp. Turtox strain. The other species (subspecies, strains; see Hirshfield et al., reference 14 and Suzuki, reference 45) utilized were *Blepharisma intermedium*, obtained from Dr. Henry Hirshfield (New York University, New York) and *Blepharisma undulans japonicus* Suzuki which, along with *B. undulans americanum*, was obtained from Dr. A. C. Giese (Stanford University, Palo Alto, Calif.). Mass cultures were derived from clones initiated from the original stock and were maintained under conditions of reduced light at 23°C in a 0.1% Cerophyl-lettuce infusion buffered with 5 mM phosphate at pH 6.8 and inoculated with *Bacillus subtilis* at least 24 hr before the introduction of ciliates.

Dividing *Blepharisma* were easily selected from concentrated cultures which were transferred in mass to a fresh culture medium 1 day before their intended use. Organisms with the typical division morphology (46) were selected individually by micropipet until 20-30 were collected. A small drop of culture fluid containing the ciliates was placed at the center of a microscope slide coverslip which was then inverted and placed over the mouth of a container of aqueous 2% osmium tetroxide. A fixation time of 4 min at 23°C was used routinely. Dehydration was accomplished by rapid changes of acetone or ethanol, usually two 5-min changes of 50%, one 10-min change of 75%, one 5-min change of 95%, two 5-min changes of absolute, and two 5-min changes of propylene oxide for epoxy embedding. Organisms were embedded either in methacrylate (a Na₂SO₄-dried mixture consisting of 57 ml *n*-butyl methacrylate, 43 ml ethyl methacrylate, 1.5 ml divinylbenzene (DVB) and 1 g benzoyl peroxide) or in an Epon-Araldite mixture (2) containing 5% D. E. R. 732; micrographs made from epoxy-embedded organisms will be identified in the figure legends. In all cases, organisms were embedded singly in capsules or, with epoxy, in a flat matrix so that specific orientations could be achieved more easily. As a final step in the preparation of blocks for sectioning, each organism was staged according to the general scheme used by Suzuki (46); then drawings were made and used as aids in fixing orientation of structures in electron micrographs.

Thin sections were cut with an LKB or Reichert ultramicrotome, were mounted on grids with thin Parlodion or Formvar supporting films and were stained with calcium permanganate or doubly stained with uranyl acetate and lead citrate (31). Sections were examined with an RCA EMU 3F electron microscope operated at 100 kv; a 150 μ condenser aperture and a 35 μ objective aperture were employed.

The work reported here results from the study of some 40 organisms in known stages of division.

OBSERVATIONS

The ultrastructure of *Blepharisma undulans* has been reported recently by Kennedy (18) and by Dembitzer and Hirshfield (5). Since the present study has revealed interphase features generally consistent with those described by these workers, a detailed description of the general fine structure of the nondividing organism will not be presented here.

Also, since observations of micronuclear behavior for each of the four strains utilized in this study are essentially the same and since most mitotic stages have been studied in at least two strains, no distinction as to strain will be made in the text; however, figure legends will include identification of specific organisms.

Interphase

The interphase micronucleus is a nearly spherical body 1.5–2 μ in diameter (Fig. 1, *Mi*) and is limited by a two-membrane envelope in which pores are not visible (Fig. 2, *NE*). That the envelopes of the macronucleus and micronucleus are very different with respect to the presence of pores is clearly shown in Fig. 2 where the envelope of each nucleus is cut tangentially. Pores with dense annuli are obvious in the macronuclear envelope (*NP*) and absent in the micronuclear envelope (Fig. 2, *NP*, *Ma*, and *NE*). The micronucleoplasm has a cortex consisting of a fine matrix surrounding an electron-opaque, reticular, central region which is a densely packed fibrillar structure (Fig. 1, *CR*). The structure of the interphase nucleus is essentially identical with that described by Seshachar (42) and similar to that described for other ciliates (7, 8, 17, 43). Occasionally, micronuclei similar to those described by Kennedy (18) are observed which seemingly lack the less dense cortex but are thought not to represent the interphase condition. The several micronuclei (from 6 to 20) are found throughout the cytoplasm, but most are located near the macronucleus.

Several cytoplasmic features of *Blepharisma* are shown to good advantage in Fig. 1. Mitochondria (*M*), vesicles (*V*), and paraglycogen granules (*P*) are distributed throughout the cytoplasm. Pigment granules (*PG*) are confined primarily to the region just beneath the pellicle, but some

are found deeper in the cell. Golgi bodies (*G*), often paired, are numerous and are present during the entire division cycle.

Prophase

At least two of the micronuclei (*PMi*) near the macronucleus in Fig. 1 are taken to be in prophase while one other is quite obviously in interphase (*Mi*). Although the micronuclear division is correlated with macronuclear changes, e.g. metaphase and the condensed stage, all micronuclei do not divide in synchrony, and it appears that some may forego division completely in both cell division and in regeneration following merotomy.

In most descriptions of mitosis, the beginning of nuclear division (prophase) is marked by cytological changes observable at the light microscope level, usually a visible condensation of the chromosomes. In the earliest known division stages studied here, organisms with very early oral primordia but no noticeable macronuclear change, micronuclei are usually enlarged to a diameter of 3–4 μ and lack dense central chromatin; instead several small, dense chromosomes are distributed throughout the nucleoplasm (Fig. 1, *CH*). Other authors (8, 29, 41, 44, 46) report similar prophase volume changes for *Tetrahymena*, *Nassulopsis*, *Paramecium*, *Frontonia*, and *Blepharisma*, respectively.

At a slightly later stage, possibly equivalent to prometaphase as described in other cells, spindle microtubules (SMT) are present in small numbers but without precise array, and chromosomes are clearly visible but not yet aligned at a metaphase plate (Fig. 3, *SMT* and *CH*). There is no indication of well-defined association of these early SMT with a specific center of formation; that is, neither centrioles nor kinetochores are identifiable. As is the case for all stages, SMT appear to terminate at or very near the inner membrane of the nuclear envelope (Fig. 3, arrow). Structural changes (porosity) in the nuclear envelope have not been detected either during earlier swelling or at this stage.

Metaphase

In metaphase, when the chromosomes are aligned at the equatorial plate, well-defined SMT are present in great numbers (Figs. 4 and 5, *SMT*); SMT diameters are apparently subject to differences in embedment, since they measure approximately 16 $m\mu$ in methacrylate and 22 $m\mu$

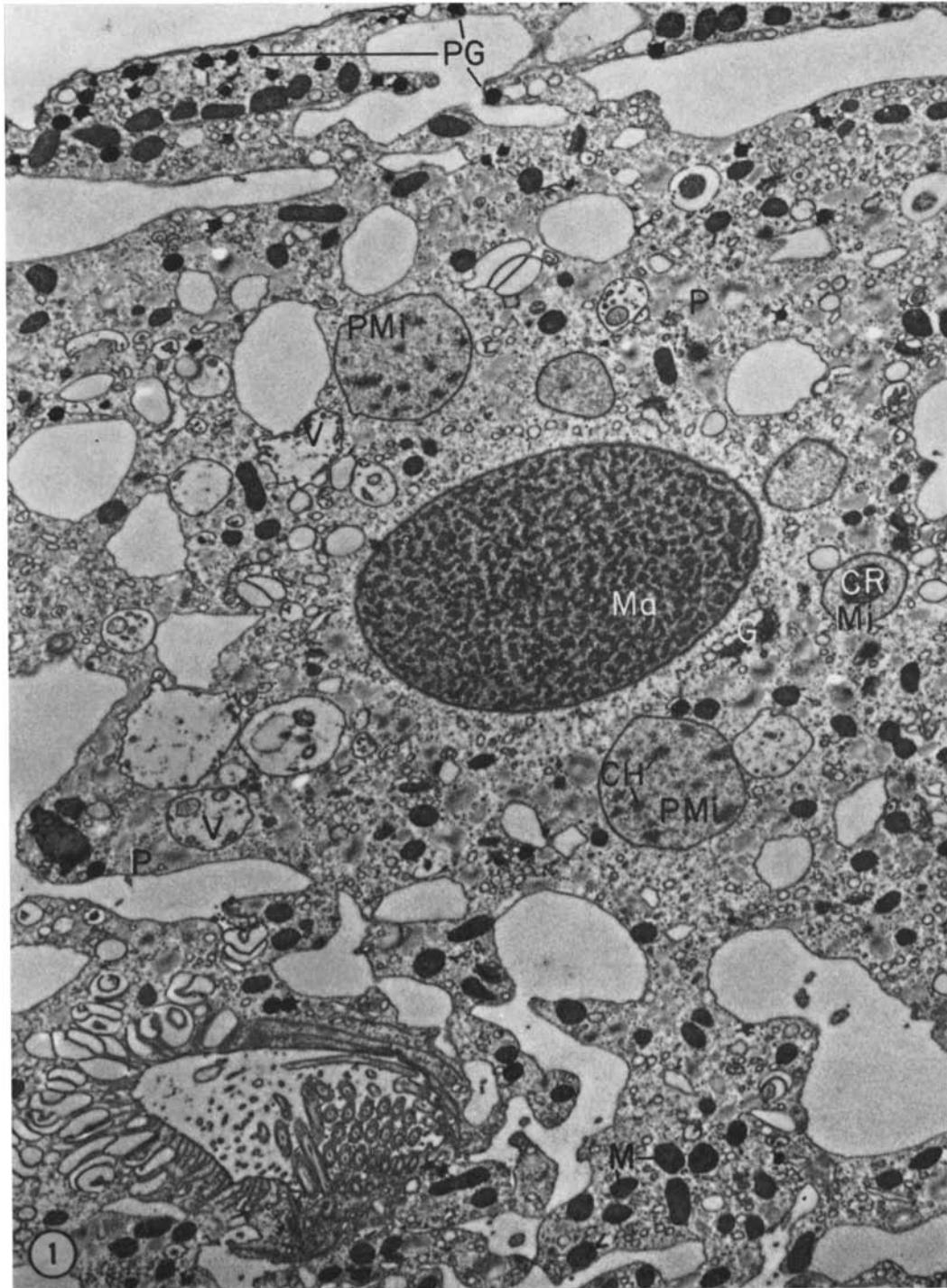


FIGURE 1 Survey micrograph of the mid-region of a longitudinal section in *Blepharisma undulans americanum*. A single lobe of the moniliform macronucleus (*Ma*) is centrally located and is surrounded by several micronuclei, two of which (*PMi*) are in prophase and show condensed chromosomes (*Ch*), while at least one (*Mi*) is in interphase and shows a dense, centrally located chromatin mass (*CR*). Mitochondria (*M*), paralygocogen granules (*P*), pigment granules (*PG*), a variety of vesicles (*V*), and Golgi bodies (*G*) are easily identified. A region of the cytostome is shown at the bottom left. Lead citrate stain. $\times 7,400$.

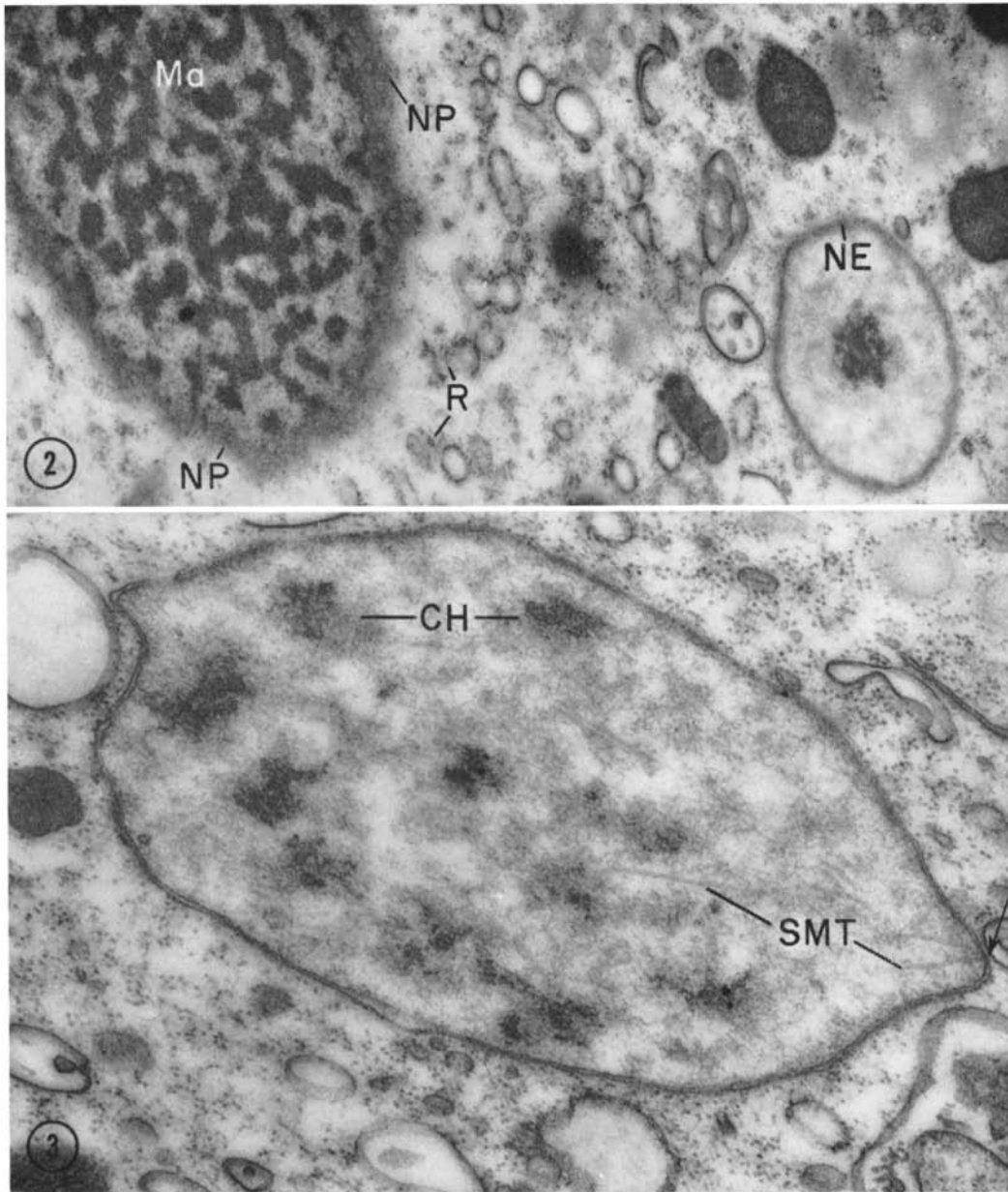


FIGURE 2 The envelope of the macronucleus (*Ma*) has numerous pores (*NP*); however, the micronuclear envelope (*NE*) lacks such pores. Both nuclear envelopes are sectioned tangentially. Numerous ribosome-like particles (*R*) fill the ground substance, and many are associated with membranous vesicles. Lead citrate stain. *B. undulans americanum*. $\times 23,500$.

FIGURE 3 At prometaphase, few spindle microtubules (*SMT*) are present in the micronucleus, and the chromosomes (*CH*) are distributed throughout the nucleoplasm. Termination of *SMT* at inner membrane of nuclear envelope is indicated by arrow. Lead citrate stain. *B. undulans americanum*. $\times 40,000$.

in the epoxy mixture. Some SMT terminate at chromosomes while others pass between them and extend from pole to pole (Fig. 4, *SMT* and Fig. 5, *SMT* and *C*). Early metaphase nuclei are barrel-shaped with slightly depressed polar regions. Later, the mitotic figure is more elongate, typically fusiform, and in Fig. 4 shows some evidence of a very early separation of chromosomes (*CH*). When sectioned across their long dimension, late metaphase micronuclei reveal numerous SMT evenly distributed throughout the nucleus, except within the body of the chromosomes, and completely contained by the nuclear envelope (Fig. 7, *SMT* and *CH*); SMT cut in exact cross-section present a circular profile with a dense cortex and a less dense center. Spindle microtubules are surrounded by a fine, fibrous material which is possibly an integral part of the tubule (Figs. 4, 5, and 6, *FM*); differential concentrations of this material are not observed at the chromosome plate or at the polar region. Nor is there a concentration layered just inside the nuclear envelope as was found in *Diplodinium* (37). Metaphase chromosomes are 250–300 $m\mu$ masses with poorly defined substructure except for a generally fibrous appearance (Figs. 4–7, and 9, *CH*). Longitudinal sections (Fig. 5, *CH*) and cross-sections (Fig. 7, *CH*) reveal the same morphology and indicate that the chromosome has the form of a tightly coiled, spherical mass. Well-defined structures (kinetochores) marking chromosome connections with SMT have not been observed (Fig. 6, arrows).

The macronucleus shows a more homogeneous nucleoplasm at micronuclear metaphase, and, as its elongation begins, microtubules are observed for the first time at an extranuclear location, situated adjacent to the nuclear envelope and directed parallel with the axis of the elongation (Figs. 4, and cross-section Fig. 8, *E*). These microtubules have the same morphology as the SMT of the micronucleus and are similar in location to those described by others (36, 37). A complete description of this “extranuclear division apparatus” will be the subject of a subsequent report.

Anaphase

Early anaphase micronuclei provide the clearest demonstration that both continuous and chromosomal fibers (composed of SMT) are present in the micronuclear mitotic apparatus (Fig. 6, *SMT*). The interzonal space previously occupied

by the now separated chromosomes contains fine fibrous material; this indicates that at least a portion of this material is apparently an unstructured environment which surrounds the SMT (Fig. 6, *FM*). In mid-anaphase, when a chromosome separation of 4–5 μ has been effected, SMT are present at the poleward side in greatest numbers, but they are also present in the interzone (Fig. 9, *I*, *SMT*). There is some evidence for a slight torsion of the entire nuclear apparatus at this stage; unfortunately this is shown to better advantage in sections adjacent to that shown in Fig. 9. Suzuki (47) shows drawings which have such a feature; Schwartz (41) cites twisting of the anaphase figure as a typical feature in isolated micronuclei of *P. bursaria*; a similar phenomenon has been described in dividing amoebae nuclei (21). The nuclear envelope remains continuous, but on occasion a less regular separation between component membranes occurs (Fig. 9, arrows).

Measurement of several metaphase nuclei reveals a plate-to-pole distance of approximately 2.25 μ . There appears to be a slight shortening of this distance as the interzonal distance increases during anaphase. Based on limited measurements of anaphase figures, a plate-to-pole distance of about 1.75 μ is found, while distal ends of the same nuclei are separated by several times this distance. Chromosome separation is primarily by elongation of continuous SMT.

In very late anaphase micronuclei, the chromosomes of the daughter nuclei are fused as single masses which resemble the interphase form and are located at each end of the mitotic figure (Figs. 10 and 11, *CR*). Chromosome-to-pole SMT are no longer detectable, and an abundance of fine fibrous material surrounds the SMT at this time (Fig. 11, *FM*). A short time later, there is indication that the chromatin mass has “rolled to the side” and continuous SMT extend beyond it and distend the nuclear envelope (Fig. 14, *SMT*).

Telophase

During anaphase the spatial separation of daughter nuclei is completed, and the chromatin mass fully regains its spherical form and the dense reticulum typical of interphase (compare *CR*, Figs. 1 and 10). The two presumptive daughter nuclei are enclosed, during the early part of telophase, within a common milieu as described by the intact nuclear envelope. When an early

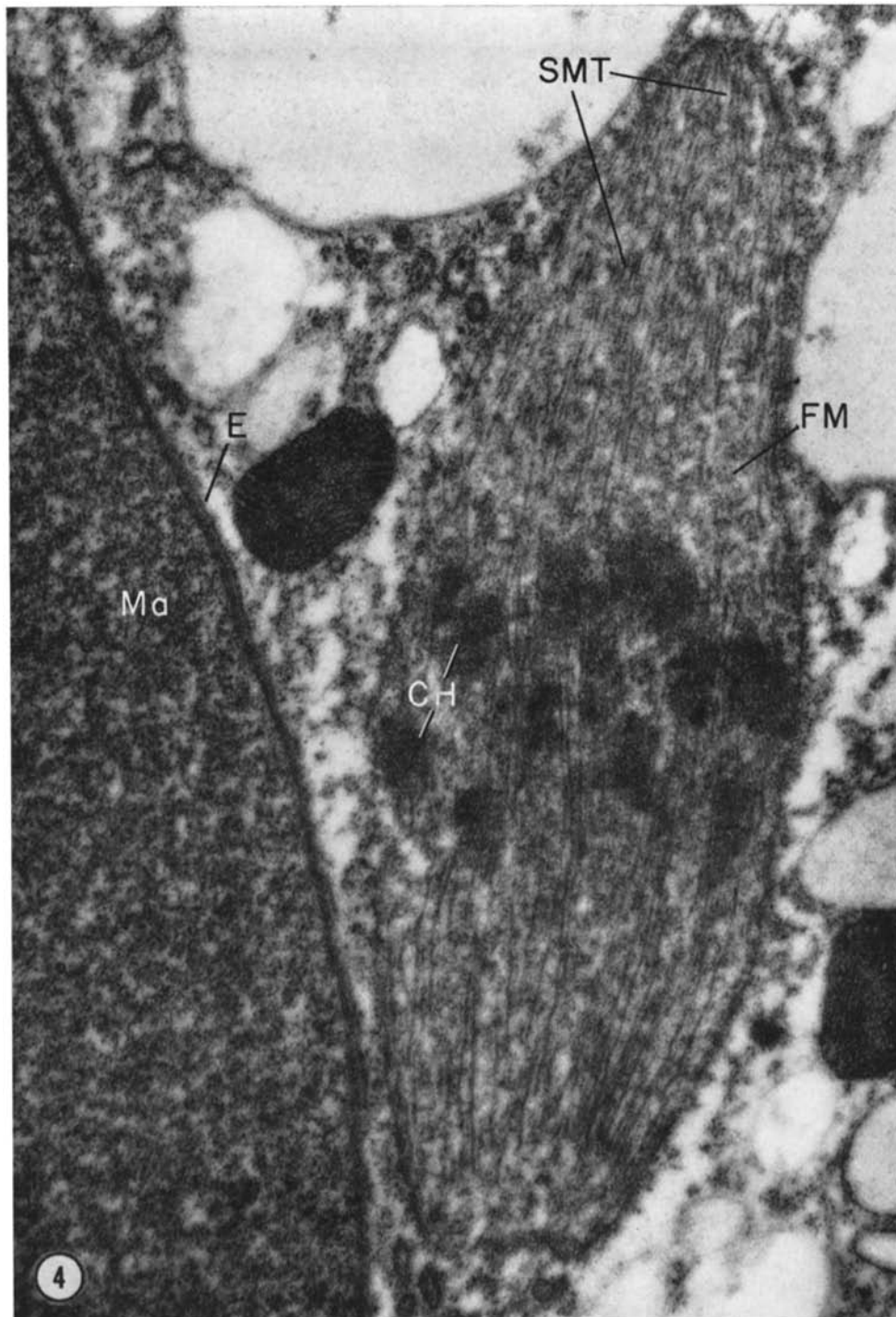


FIGURE 4 The late metaphase micronucleus in *Blepharisma* sp. Turtox strain is a fusiform body 4–5 μ in length. Spindle microtubules (SMT) surrounded by fine material (FM) extend continuously from pole to pole or show apparent termination at the fibrous chromosomes (CH). Ribosomes are not present within the achromatic apparatus, nor are centrioles present at the poles. The macronucleus (MA) is in the late condensed stage, and a few extranuclear microtubules (E) are found closely appressed to the cytoplasmic surface of the macronuclear envelope. Calcium permanganate stain. $\times 40,000$.

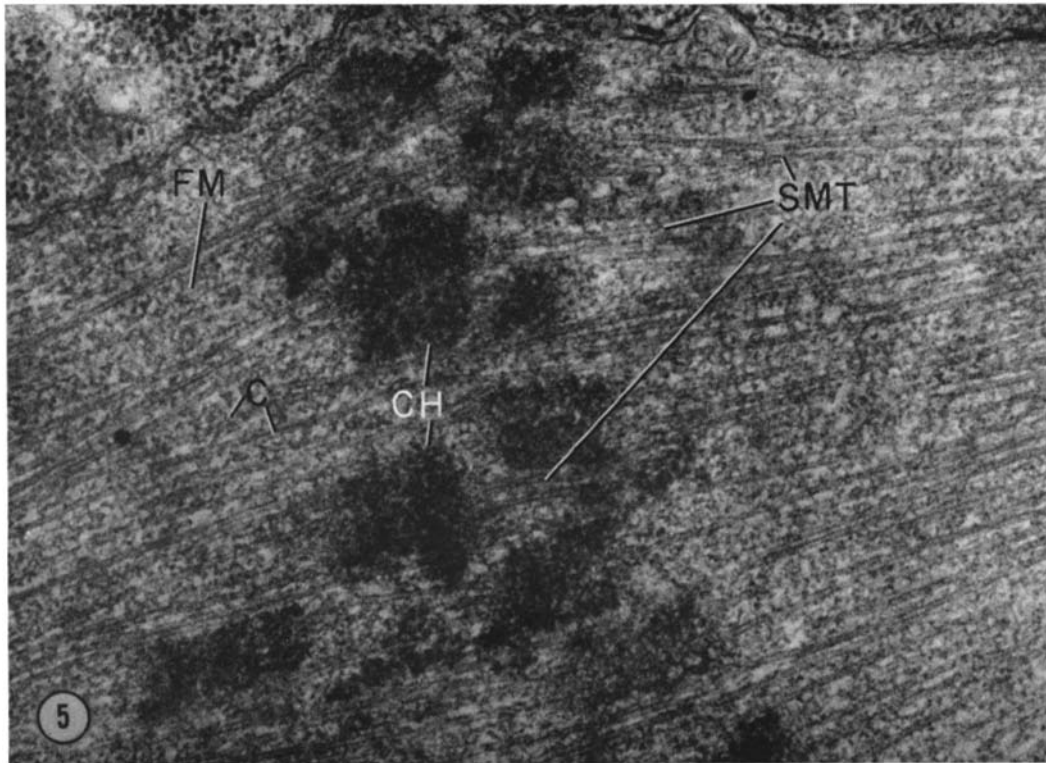


FIGURE 5 Higher magnification of the equatorial plate in *Blepharisma* sp. Turtox strain. Chromosomes (CH) are aligned, and spindle microtubules (SMT) are present in precise array, in great number, and are surrounded by fine fibrous material (FM); both continuous (C) and chromosomal spindle microtubules (SMT) can be identified. Epoxy-embedded; uranyl acetate-lead citrate stain. $\times 50,000$.

telophase organism is sectioned at a right angle to the long axis, the separation spindles of micronuclei (*S*) and the macronucleus (*Ma*) are cross-sectioned, and SMT with a circular profile are present within the confines of the nuclear envelope (Fig. 8, *NE*). These structures represent cross-sections through separation spindles (*S*) at a location near the site at which cleavage would have occurred. A propensity of telophase SMT to associate in pairs has been observed in fibroblasts (20). A similar condition seems to exist in early telophase micronuclei; also, little, fine material is found surrounding SMT in regions distant to the daughter nuclei (Figs. 8 and 13, *SMT*).

Formation of a new nuclear envelope is typically a telophase phenomenon. In micronuclei of *Blepharisma* the envelope has not been visibly disrupted; nevertheless, a new envelope is formed within the confines of the old and is closely juxtaposed to the chromatin mass; thereby the new envelope

separates and excludes essentially all of the achromatic material from the chromatic substance of the daughter nucleus. The formation of a new nuclear envelope begins with the appearance of small membranous vesicles and flattened cisternae applied to the distal side of the chromatin mass (Fig. 11, *CI* and Fig. 12, arrows). Formation continues by the fusion of the small vesicles possibly derived as elaborations from the old nuclear envelope until finally an intact nuclear envelope is formed (Figs. 11, 12, 14, 16, and 17, for sequence); it appears that there is membrane formed in excess of that required for the new envelope since flattened cisternae are invariably present in the vicinity after the envelope is completed (Figs. 16 and 17, *CI*). Fig. 13 shows a cross-section through a separation spindle (*S*) at some distance from the chromatin mass and through a telophase daughter nucleus of a second mitotic figure. The chromatin mass (*CR*) has new nuclear

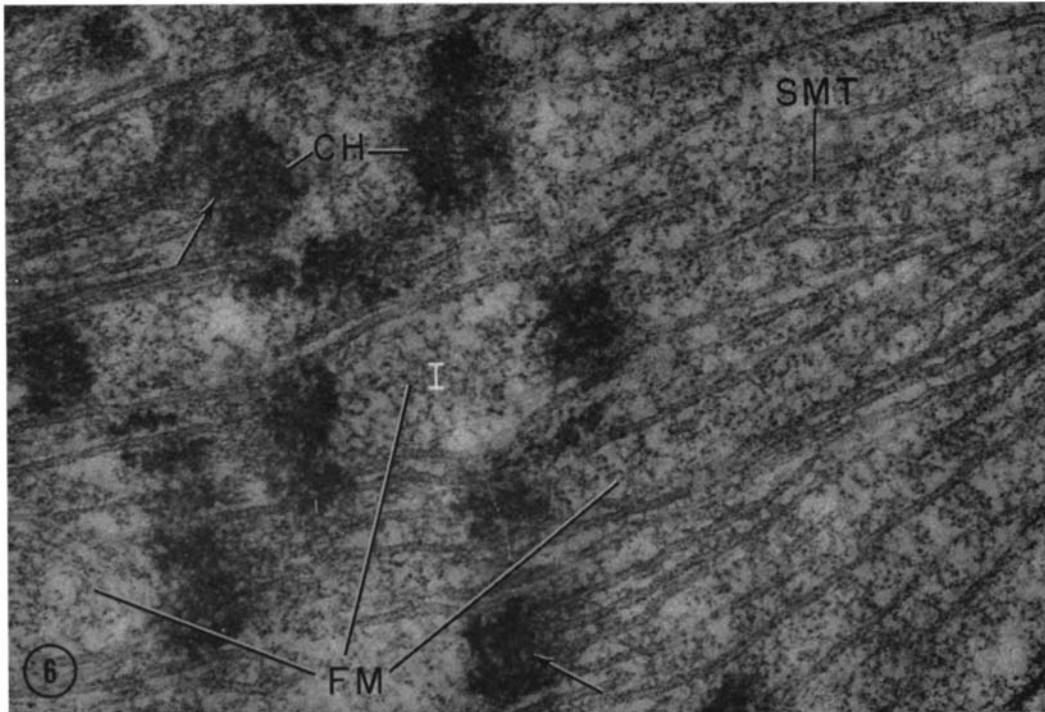


FIGURE 6 A higher magnification micrograph of a portion of the early anaphase micronucleus in *Blepharisma* sp. Turtox strain. Sister chromosomes (*CH*) are separated by less than 300 m μ . Fine material (*FM*) occupies the brief interzone (*I*) and surrounds as well the SMT of the achromatic apparatus. Structures identifiable as kinetochores are not observed at the SMT insertion into the chromosome (arrows). Epoxy-embedded; uranyl acetate-lead citrate stain. $\times 60,000$.

envelope in the form of flattened cisternae (*CI*) closely applied; much fine material (*FM*) is present as are cross-sections of SMT. Spindle microtubules are present during formation of the new nuclear envelope, but begin to disperse during the process and are eventually replaced by a great amount of fine, fibrous material (Figs. 12 and 15, *FM*).

The final process of micronuclear division in *Blepharisma* is the disruption of the original nuclear envelope (Fig. 17, *ONE*) with the release of the newly limited nucleus into the cytoplasmic environment and, finally, the complete dispersal of the SMT material into the cytoplasm. Although all details of this later process have not yet been observed, late division stages show linear masses of finely fibrous material arranged parallel with the long direction of the previously existing separation spindles (Fig. 15, *FM*).

DISCUSSION

Micronuclear Mitosis

The nuclear apparatus of ciliate protozoa represents a striking example of intracellular differentiation. The presence, in a single cytoplasmic milieu, of two distinct nuclear types that are different in size, morphology, chemical composition, mode of division, and function, is of great interest (13) and will undoubtedly attract an increasing number of workers who conduct investigations of nuclear processes. One of the problems central to the completion of such studies has been the lack of good structural information, particularly on divisional changes. Early electron microscope studies of dividing *Tetrahymena* indicated the existence of filamentous structures associated with the amitotic macronucleus (36). However, a micronuclear spindle apparatus in *Tetrahymena* escaped detection in other studies

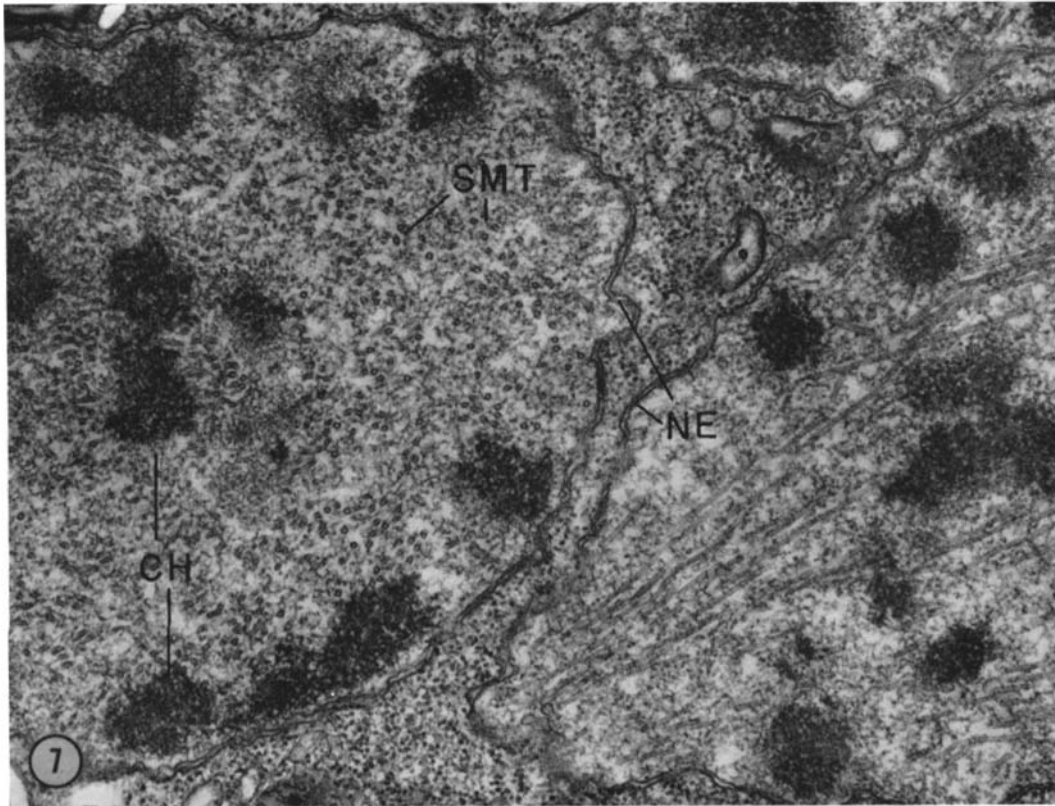


FIGURE 7 Portions of three dividing micronuclei are included in this micrograph. At the left, the section is approximately parallel with the chromosome plate and shows a metaphase micronucleus which contains numerous spindle microtubules (SMT) in cross-section. SMT are not seen within the fibrous chromosomes (CH). The double-membrane nuclear envelope (NE) is clearly shown. Epoxy-embedded; uranyl acetate-lead citrate stain. *Blepharisma* sp. Turtox strain. $\times 35,000$.

(6-8), most probably because of the failure to utilize specific conditions of osmium fixation necessary for the preservation of SMT integrity (35). Spindle microtubules have been described recently for the dividing nuclei in *Diplodinium* (37) and *Nassula* (30), the micronucleus in *Epistylis*, and the macronucleus in *Campanella* (4). Also, Grain (12) reports the presence of 20- μ nontubular filaments in dividing micronuclei of rumen ciliates. These reports document the presence of microtubular elements within and about dividing ciliate nuclei but do not elucidate the full mitotic process.

In *Blepharisma*, micronuclear divisions are mitotic, closed-acentric and asynchronous. Each of these features will be discussed in turn. The general succession of events which typify mitotic division can be summarized: (a) duplication of

chromosomes (longitudinal splitting), (b) alignment of doubled chromosomes at an equatorial plane of the spindle, (c) separation and movement of sister chromosomes to opposite poles, and (d) reconstruction of daughter nuclei. Mazia (24) defines a similar schedule and adds only the cyclic behavior of nucleoli. Observations reported here indicate that the micronucleus undergoes a mitotic division as classically described. The suggestion (9) that ciliate micronuclear divisions are not mitotic is untenable. The first consideration raised by these authors, that the nuclear envelope remains intact, is not pertinent to the mitotic versus nonmitotic division; the second, that chromosomal fibers or typical metaphases have not been observed, loses meaning in light of the observations made on *Blepharisma*. Micronuclear division is mitotic, and such variations as its

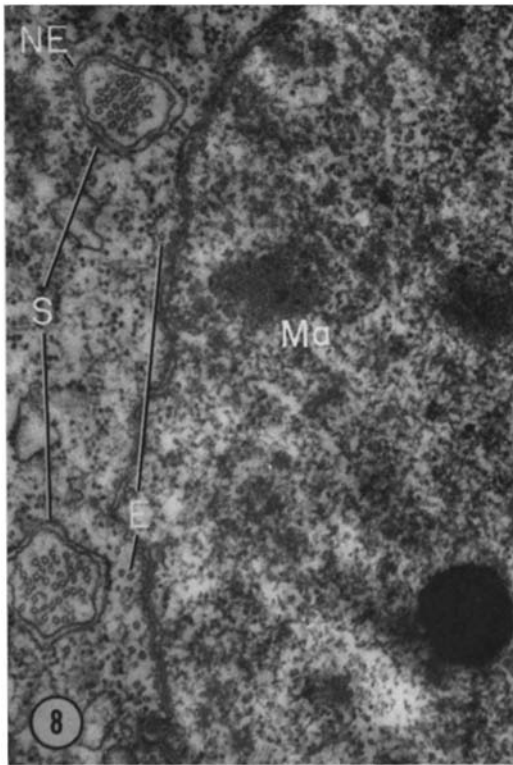


FIGURE 8 A cross-section through the macronucleus (*Ma*) and two micronuclear separation spindles (*S*) at telophase. Cross-sections of spindle microtubules are easily identified within the confines of the intact nuclear envelope (*Ne*). Fine material does not surround the spindle microtubules which are now quite closely packed and often associated as pairs at this stage. Extranuclear SMT (*E*) are located near the macronuclear envelope. Epoxy-embedded; uranyl acetate-lead citrate stain. *Blepharisma* sp. Turtox strain. $\times 45,000$.

completion within an intact nuclear envelope, absence of centrioles, and absence of nucleolar cycle are just further variations on the central theme of equational division of genetic material.

Centrioles are not present either within, exterior to, or adjacent to the micronuclear envelope in *Blepharisma*. Studies by Schuster on meiosis in *Didymium* (40), and by Roth and Shigenaka (37) and Carasso and Favard (4) on ciliates revealed an acentric, membrane-limited figure; however, Berlin and Bowen found centrioles exterior and adjacent to the intact nuclear envelope during the mitosis of *Albugo* (3). Elliott

(7) questioned the nature of dense rods located at the apogee of mitotic nuclei in *Tetrahymena*; that they represented centrioles is doubtful. The absence of centrioles is a consistent feature of mitosis in plant cells and in some animals such as giant amoebae (34). The significance of a mitosis in the absence of centrioles will be considered in a later section of this discussion.

The use of the term "intranuclear mitosis" generally applied to nuclear divisions in which the nuclear envelope is not disrupted has been avoided because a literal interpretation is not meaningful and because the findings presented in this study do not support the descriptive criteria currently used. Grell (13) has designated a division as intranuclear if the divisional centers, usually centrioles in protozoa, are located within the cell nucleus. It is suggested that the terms "closed" and "open", referring to an intact or disrupted nuclear envelope, used in hyphenated form with the terms "centric" or "acentric", indicating a presence or absence of centrioles, would provide a simple and efficient terminology and accommodate the great variability in mitosis, e.g. *Blepharisma* micronucleus, closed-acentric; *Chaos chaos*, open-acentric (34); *Albugo*, closed-centric (3); *Physarum flavicomum*, closed-acentric but becoming open-acentric in anaphase;¹ and HeLa cells, open-centric (32).

The micronuclear division in *Blepharisma* is correlated with the macronuclear division; generally, metaphase figures are found as the macronucleus reaches the condensed stage. However, all micronuclei do not divide at each cell division; in fact, interphase and metaphase nuclei are found in the same organism. Seshachar and Devi (44) have made a similar observation for the micronuclei of *Frontonia* and suggest that proximity to the macronucleus inhibits division. Observations on many dividing giant amoebae have never disclosed nuclei which were not in division synchrony (personal observation). The question which develops from these considerations is directed to the nature of the signal which initiates mitosis and to the observation that in ciliates there is a variable response in nuclear behavior (19). The factors which control division are of considerable interest generally, and further studies of

¹ Aldrich, H. C. 1966. A study of the ultrastructural details of morphogenesis in the myxomycete *Physarum flavicomum*. Unpublished dissertation. University of Texas, Austin.

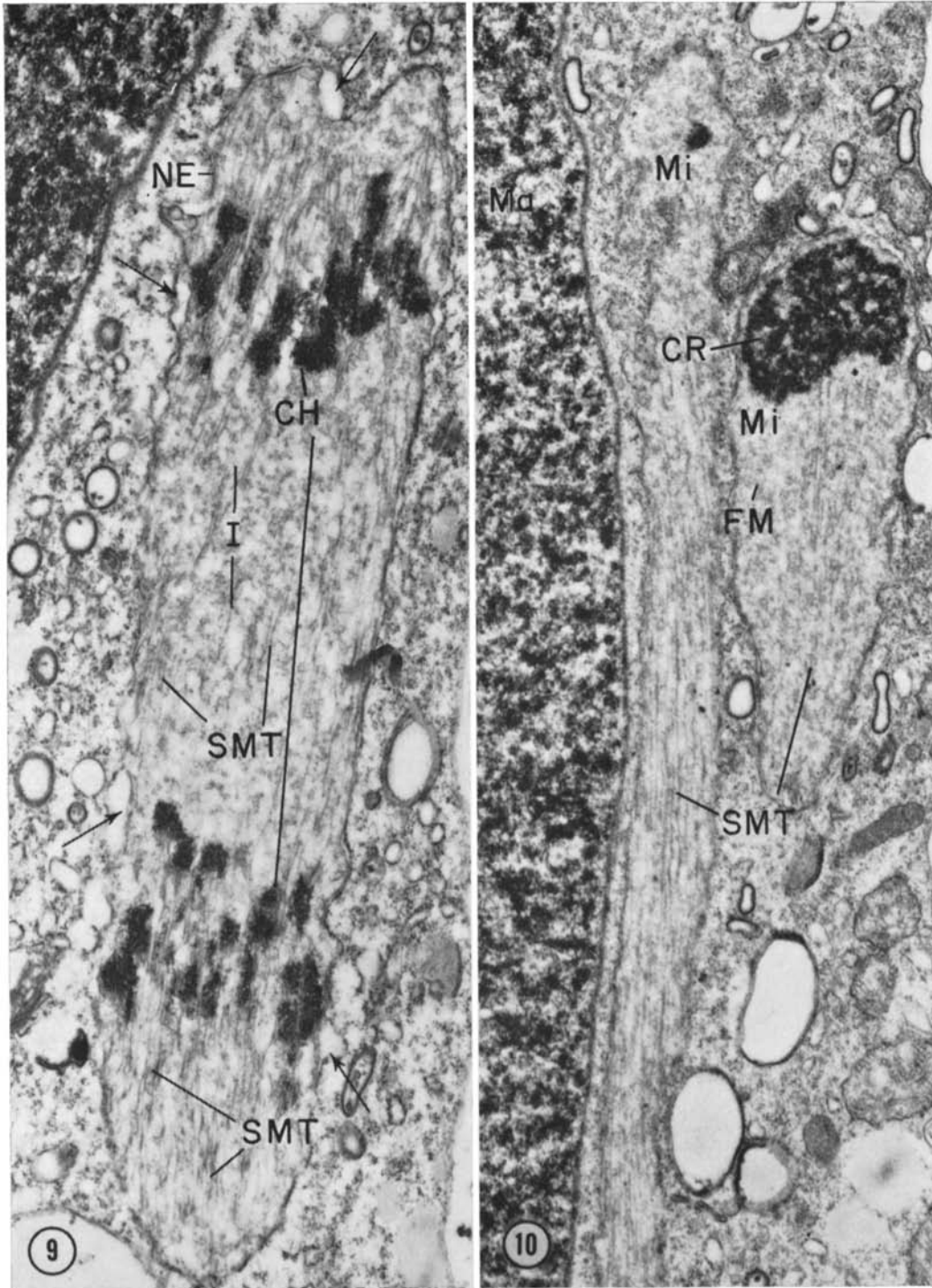


FIGURE 9 Mid-anaphase micronucleus in *B. undulans americanum*. Chromosomes (*CH*) are separated by about 5μ . Numerous spindle microtubules (*SMT*) are present on the poleward side of the chromosomes but are also present in the interzone (*I*). The nuclear envelope (*NE*) remains intact but shows variability in the space separating the two membranes (arrows). A portion of the macronucleus is included at the upper left. Uranyl acetate-lead citrate stain. $\times 24,000$.

FIGURE 10 Survey micrograph showing portions of two late anaphase micronuclei (*Mi*) and the elongated macronucleus (*Ma*) in *Blepharisma intermedium*. Individual chromosomes are no longer distinguishable but are now combined as the dense chromatin mass (*CR*). Fine material (*FM*) surrounds the spindle microtubules (*SMT*). Uranyl acetate-lead citrate stain. $\times 20,000$.

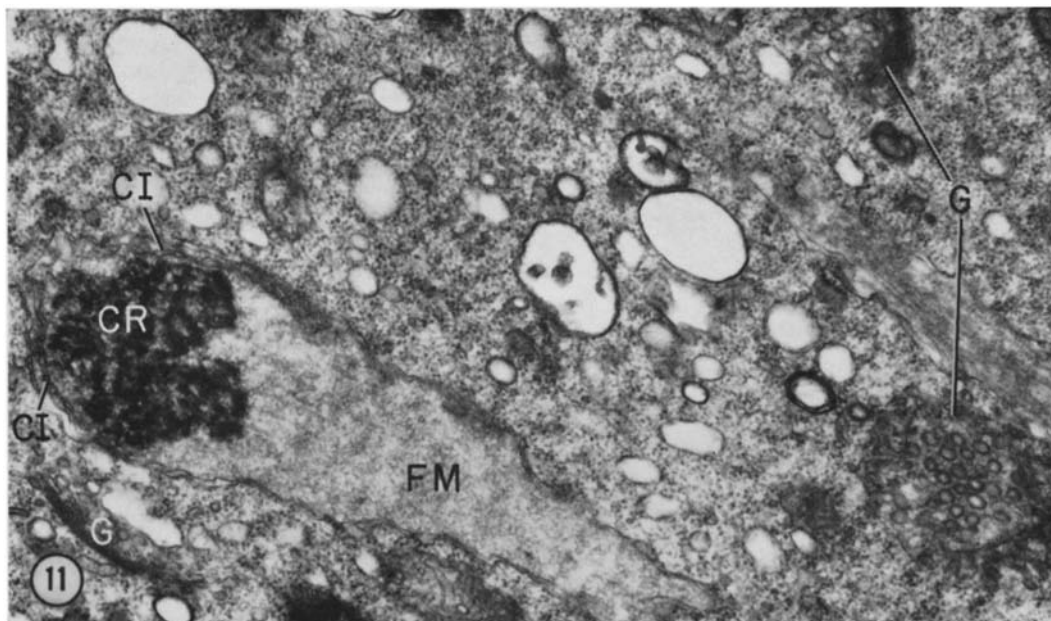


FIGURE 11 Formation of the new nuclear envelope begins with the appearance of flattened cisternae (CI) closely applied to the distal side of the chromatin mass (CR). SMT are nearly obscured by the presence of fine fibrous material (FM). Golgi bodies (G) are present near the nucleus. Uranyl acetate-lead citrate stain. *B. undulans japonicus*. $\times 41,000$.

macronuclear-micronuclear interdependence may yield valuable information.

Formation, Morphology, and Function of Spindle Microtubules

Formation of SMT within the confines of an intact nuclear envelope presents a question of considerable importance: what is the source of the material, presumably protein, which is aggregated to form the SMT? The metaphase-telophase micronuclear, mitotic apparatus represents a large amount of material that is derived without free mixing of nucleo- and cytoplasmic substance. Obvious hypotheses are that SMT precursors are synthesized either within the micronucleus, or within the cytoplasm and are carried into the nucleus across the nuclear envelope. Some combination of these two alternatives might also be considered.

The possibility that the protein is nuclear in origin must be considered remote since the ciliate micronucleus apparently lacks both nucleoli and ribosomes and is thought to be metabolically inert. Sound evidence for a protein-synthesizing

capacity relates to the presence of RNA. In an early study, the presence of considerable RNA in the micronucleus of *Paramecium* was reported (25), whereas more recent investigators have not reported the detection of RNA (11, 28). Under conditions in which massive incorporation of labeled RNA precursors into the macronucleus occurs, little or no incorporation into micronuclei was found (19). These evidences, plus the observation that the micronucleus of *Blepharisma* shows no evidence for the presence of either nucleoli or ribosomes, and similar observations reported for micronuclei of *Diplodinium* (37) and *Tetrahymena* (8), strongly suggest that the micronucleus lacks a protein biosynthetic capacity. As for the presence of significant protein in interphase micronuclei, the data are too sketchy to be of much value (28). Therefore, because information currently available points toward a lack of biosynthetic function of micronuclei, a cytoplasmic synthesis of SMT precursor materials and its subsequent entrance across the intact nuclear envelope seem most logical.

The import of large amounts of material into the micronucleus is possibly made more difficult

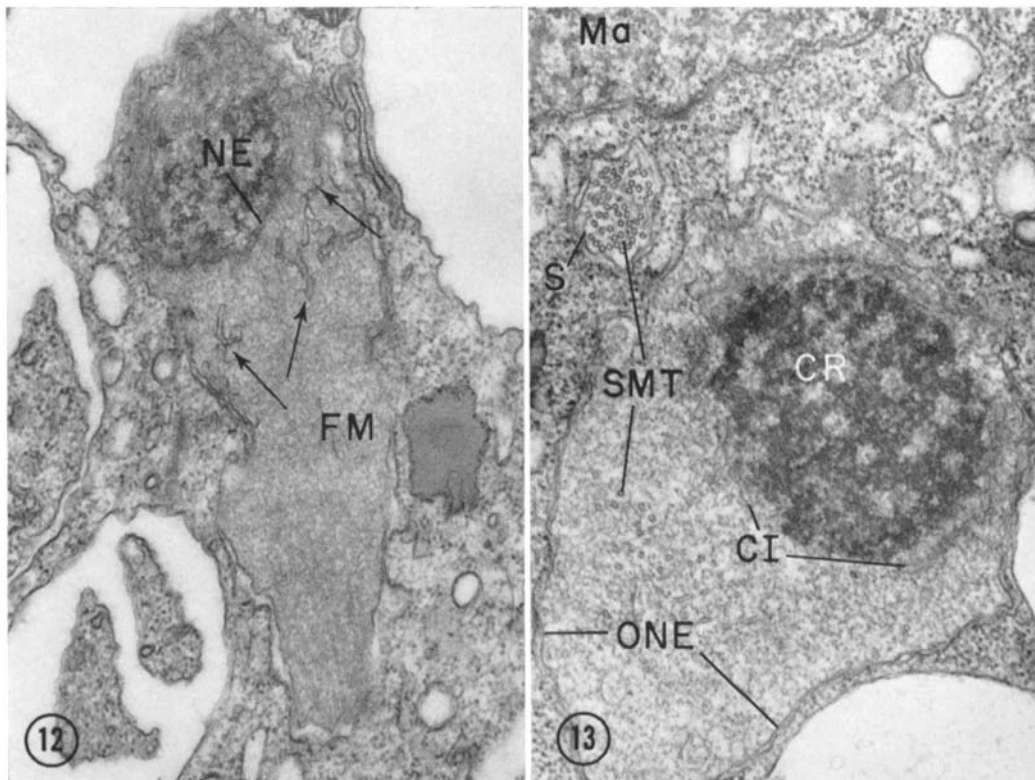


FIGURE 12 A slightly later stage of nuclear envelope formation than shown in Fig. 11. The new envelope is essentially complete (*NE*) but difficult to visualize because the membranes are sectioned tangentially. Numerous small vesicles (arrows) are present. Note the great amount of fine material (*FM*). Organism and stain as in Fig. 11. $\times 43,000$.

FIGURE 13 Cross-section through a separation spindle (*S*) and the daughter nucleus of a second mitotic figure in *Blepharisma* sp. Turtox strain; cross-sections of spindle microtubules (*SMT*) are evident in each. The original nuclear envelope (*ONE*) remains intact while the chromatin mass (*CR*) shows close apposition of flattened cisternae (*CI*). A portion of the macronucleus (*Ma*) is also included. Epoxy-embedded; uranyl acetate-lead citrate stain. $\times 34,000$.

by the apparent absence of pores in the nuclear envelope. Recently, pores have been reported for the micronuclear envelopes of *Blepharisma* (18) and *Urostyla* (15), and reported as absent from the micronuclear envelopes of *Paramecium caudatum* (17) and *Blepharisma intermedium* (42). Many dozens of micronuclei were examined in this investigation and at no time were obvious pores seen in the micronuclear envelope. The disparity in observations from different studies could be due to physiological changes of the envelope related to a suggested short stage function of the micronucleus (8); however, the present author is somewhat inclined to the view that pores

are not a feature of the micronuclear envelope in *Blepharisma*. The absence of detectable pores would not be of absolute consequence in the matter of transport except that a generally impermeable nature of the micronuclear envelope is suggested by its failure to pass vital dyes; in contrast, the macronucleus is easily stained by vital dyes. The observed swelling of premitotic micronuclei could represent, if not the actual uptake of SMT precursor (assuming little or no protein biosynthetic capacity for the micronucleus), an indication of nuclear envelope permeability changes that are related to the sequestering of division protein; morphological evidence of such

changes was not obtained in this study. In micronuclei of *Nassulopsis* the increase in dimension occurs only during the G₂ phase, after DNA synthesis is completed (29); therefore, in this organism at least, the swelling cannot be attributed to DNA synthesis directly and is more probably a result of some predivision activity of the micronucleus. Morphological features that would support either micronuclear biosynthesis or facile membrane transport of spindle precursor are not demonstrated in this study. Thus an explanation for the immediate source of SMT material cannot be advanced at this time.

Let us turn our attention to the initiation of SMT formation. Inoué has continually emphasized that fibers of the mitotic apparatus are organized and oriented by "centers" such as kinetochores and centrioles (16). Neither centrioles nor kinetochores have been observed in micronuclear mitosis in *Blepharisma*; the only likely "center of aggregation" in a closed-acentric mitosis is a region of the chromosome, here not distinguished by defined structure. However, the role of centers of formation might be questioned on three major counts. First, the nearly ubiquitous occurrence of microtubules in cells and particularly in areas of cells where direct association with a center cannot be demonstrated (22, 47, 48) indicates a more independent scheme. Taylor (47) has suggested that, if microtubules do originate from organizing or initiating molecular configurations, these centers may reside on membrane surfaces. Roth et al. (38) emphasize the significance of the association of microtubules with membrane surfaces. The present study as well as that of Tilney and Porter (48) on axopodia demonstrate the termination of great numbers of microtubules near the nuclear envelope, albeit to opposite sides; there is, however, no indication of any elaboration at this site. Manton (23) reports the apparent termination of SMT on pieces of nuclear envelope in *P. parvum*. The significance of the association of microtubules with membrane surfaces is not yet clear but seems worthy of further consideration. Second, the electron microscopic observation of well-defined kinetochores with any indication of tubular morphology is not consistent, nor is a firm connection of SMT to centrioles ever clearly shown. It may be, however, that the structure of each of these junctions (centers) is highly labile and that fixation sufficient to preserve the formed or forming SMT is not sufficient to preserve the fine detail of a struc-

ture (region) representing a site of aggregation. Third, within the acentric mitotic apparatus SMT are present which are continuous from pole to pole and lack connections to either chromosomes or centrioles and, in the case of micronuclei, lack direct contact with the cytoplasm. It is probable that these continuous SMT are responsible for the greater separation of daughter nuclei in many cells (for survey see Roth et al. 38). Since these SMT are essentially free from direct association with known centers, it is appropriate to implement a hypothesis which considers their formation as resulting from changes in the microenvironment that are known to control microtubule assembly in vitro such as pH, ionic strength (1), or the presence of a specific linker substance (RNA; 39, 49, 50); such a concept might entail growth of the microtubule by insertion of material along its length or addition at the ends (33) as well as the presence of initial seeds.

Spindle microtubules of the dividing micronucleus are morphologically identical with microtubules described for the great number of cells previously referred to. Attention is called, however, to the fact that reported differences in the diameter of SMT are apparently due to the use of different preparative techniques, as earlier suggested by Ledbetter and Porter (22). Organisms handled in an identical manner up to the embedding step showed SMT approximately 22 μ m in diameter when in epoxy and 16 μ m in diameter when in methacrylate-DVB; considerable caution should be used in attaching significance to differences in diameter. As is the case in other cells studied with the electron microscope, no changes in SMT diameter or morphology can be determined for different stages of division.

That the fine material found adhering to the surface of SMT is an integral part of the microtubular morphology has been suggested (34). There is a great amount of fine, fibrous material associated with the SMT of the micronucleus in *Blepharisma*, but it is not clear whether this material is wholly a lateral component of the microtubule or represents, at least in part, an unstructured phase serving possibly as precursor material. The material closely resembles that found surrounding the microtubules of the ciliary shaft.

Single SMT of early telophase separation spindles are found to be continuous for several microns; the logical conclusion is that individual continuous SMT undergo a great increase in length during the course of mitosis. In this regard, SMT

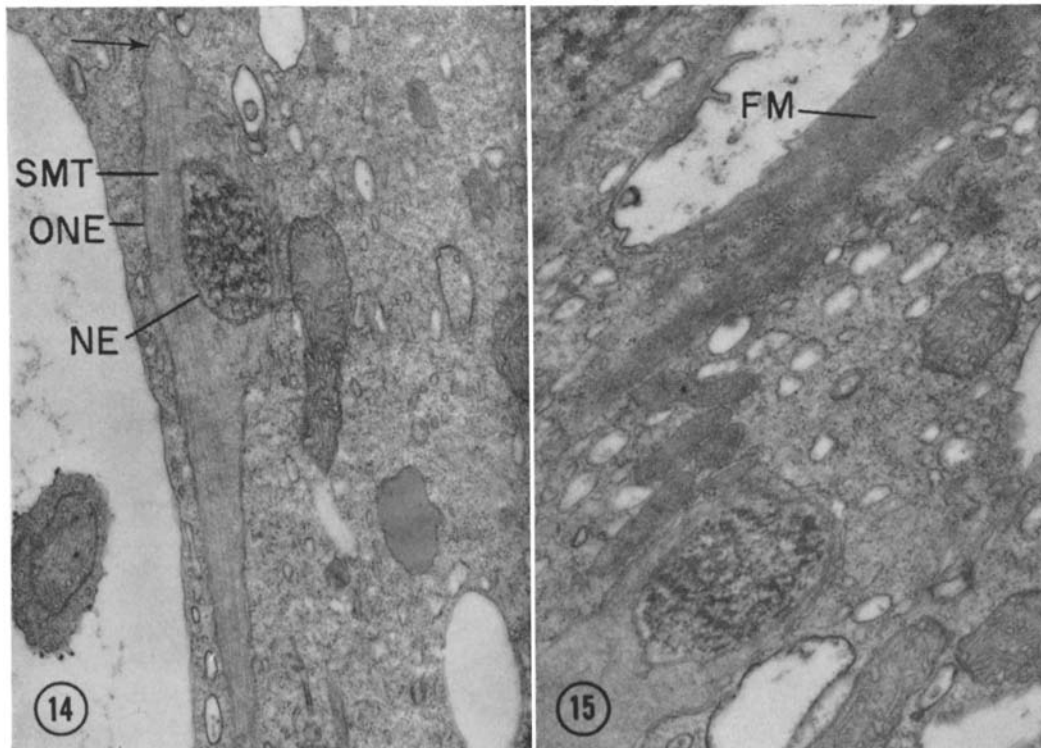


FIGURE 14 A survey micrograph of a telophase micronucleus in *B. undulans japonicus*. The new nuclear envelope (NE) is apparently continuous and the original envelope (ONE) remains intact. Spindle microtubules (SMT) appear to have displaced the micronucleus to one side and distended the original nuclear envelope (arrow). Uranyl acetate-lead citrate stain. $\times 20,000$.

FIGURE 15 Very late in division, dense masses of fine fibrous material (FM) are found oriented parallel with the long direction of previously existing separation spindles. Spindle microtubules are not usually seen at this stage. Organism and preparation as in Fig. 14. $\times 29,000$.

in dividing *Prymnesium* are not connected to chromosomes and lengthen conspicuously during anaphase (23).

A hypothesis, consistent with the observations, for the mechanism of micronuclear mitosis in *Blepharisma* might include the following elements: (a) chromosomes are held in register by chromosomal SMT, and chromosomal and continuous SMT are linked together (lateral projections) in the poleward region; (b) separation of chromatids is effected primarily by the elongation of continuous SMT, either at their ends, in which case the cross-connections must be formed progressively in the direction of the lengthening microtubule, or along their lengths, in which case the cross-connections might remain fixed to a greater extent. In either case, the chromosomal SMT and the attached chromosomes are moved by a lengthening

of the continuous SMT. When separation is achieved, the chromosomal SMT disperse first and account for the rolling to the side of the chromatin mass; nuclear envelope formation and dispersal of continuous SMT follow shortly.

Nuclear Envelope Formation

In the closed micronuclear mitosis of *Blepharisma*, the chromatin material is isolated at telophase from the kinetic apparatus by the formation of a new nuclear envelope in close apposition to the chromatin. Nuclear envelope reformation is characteristic of the reconstruction of daughter nuclei, but in this case an envelope has remained intact throughout division and a new one forms within its limits. Also, it is apparent that a considerable elaboration of membrane must occur throughout division in order to account for the great increase

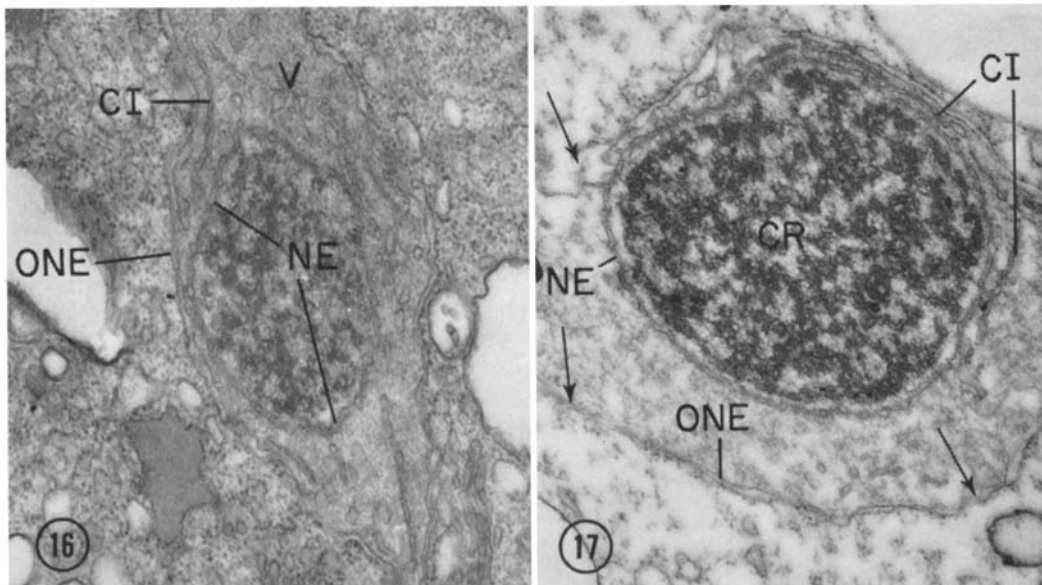


FIGURE 16 At the time the new nuclear envelope (NE) is completed, numerous small vesicles (V) and flattened cisternae (CI) are present within the confines of the original nuclear envelope (ONE) which now shows a greater than usual separation of its component membranes. Organism and preparation as in Fig. 14. $\times 39,000$.

FIGURE 17 A very late telophase micronucleus in *B. intermedium*. The original nuclear envelope (ONE) is less distinct and begins to show discontinuities in its component membranes (arrows). Flattened cisternae (CI) are found in proximity to the new nuclear envelope. The dense chromatin mass (CR) shows separation from the newly formed nuclear envelope (NE). Uranyl acetate-lead citrate stain. $\times 40,000$.

in envelope surface area as the interphase sphere converts to the greatly lengthened structure formed as division is completed. These phenomena are further examples of the dynamic nature of cell membranes and may provide useful systems for the study of membrane elaboration and specialization.

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