MITOSIS IN *BARBULANYMPHA*

II. Dynamics of a Two-Stage Anaphase, Nuclear Morphogenesis, and Cytokinesis

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ABSTRACT

Anaphase in *Barbulanympha* proceeds in two discrete steps. In anaphase-A, chromosomal spindle fibers shorten and chromosomes move to the stationary centrosomes. In anaphase-B, the central spindle elongates and ("telophasic") bouquets of chromosomes, with kinetochores still connected by the shortened chromosomal fibers to the centrosomes, are moved far apart.

The length, width, and birefringence of the central spindle remain unchanged throughout anaphase-A. In anaphase-B, the central spindle elongates up to fivefold. During elongation, the peripheral fibers of the central spindle splay, first anteriorly and then laterally. The remaining central spindle progressively becomes thinner and the retardation decreases; however, the coefficient of birefringence stays approximately constant.

The nuclear envelope persists throughout mitosis in *Barbulanympha* and the nucleus undergoes an intricate morphological change. In prophase, the nucleus engulfs the spindle; in early anaphase-A, the nuclear envelope forms a seam anterior to the spindle, the nucleus thus transforms into a complete sleeve surrounding the central spindle. In late anaphase-A, the middle of the seam opens up in a cleft as the lips part; in anaphase-B, the cleft expands posteriorly, progressively exposing the central spindle. Finally, the cleft partitions the nucleus into two.

The nuclear envelope shows an apparent elasticity and two-dimensional fluidity. Localized, transient deformations of the nuclear envelope indicate poleward and counter-poleward forces acting on the kinetochores embedded in the envelope. These forces appear responsible for nuclear morphogenesis as well as anaphase chromosome movement.

At the end of anaphase-B, the two rostrate *Barbulanympha* may swim apart or be poked apart into two daughter cells by another organism cohabiting the host's hindgut.

KEY WORDS *Barbulanympha* · mitotic spindle · living cell · chromosome movement · nuclear envelope · cytokinesis · birefringence

In Part I we described a preparation of *Barbulanympha* which permits extended observation of mitosis in individual living cells in this unusually favorable material. The optical system we assembled for alternate through-focus observation and recording in differential interference contrast and rectified polarized light microscopy is also described. The formation and fine structure of the magnificent central spindle, the forward hoisting of the nucleus to meet the spindle surface, and the churning of chromosomes in the prophase nucleus are reported.

In Part II we report the behavior of chromosomes, kinetochores, nuclear envelope, mitotic spindle fibers, and astral rays during late prophase, the two anaphases, karyokinesis, and cytokinesis in *Barbulanympha*.

RESULTS

Late Prophase

With progress of prophase, the nucleus is continuously hoisted forward. As detailed later, the nucleus is deformed and eventually wrapped around the central spindle, forming a complete sleeve (schematically shown in Fig. 11). Within this tubular nucleus, the prophase chromosomes point polewards with the chromatid arms still unseparated. Their kinetochores remain embedded in the nuclear envelope. In this configuration, the kinetochores slowly move toward and away from the centrosomes individually, continuing the churning of chromosomes described in Part I. Then, without ever entering a regular metaphase configuration, the cell leaves prophase and enters anaphase.

Anaphase-A

At the onset of anaphase, kinetochores are positioned at varying distances from the centrosomes. In differential interference contrast micrographs, the minute, discrete kinetochores sharply delineate the poleward apex of each chromosome (e.g., Fig. 1*d*; also see Figs. 9, 10, and 12). In Fig. 1*a* and *b*, some kinetochores already appear near the centrosome and others are still seen near the equator of the spindle.

With progression of time, arms of the sister chromatids gradually separate. The chromosomes then move, kinetochore foremost, steadily to the spindle poles (Figs. 1*a* to *d*; also 5*a* to *d*; 13*a* to *f*).

As Figs. 3 and 8 show, each kinetochore moves at similar velocities ranging from 0.3 to 0.5 μm/min at 18 ± 1°C. Inasmuch as there is no metaphase as such, and because the kinetochores have varying distances to travel, the chromosomes move poleward over a considerable span of time.

With the polarizing microscope, the nuclear membrane-embedded kinetochores are seen linked to the centrosome by positively birefringent chromosomal spindle fibers (Figs. 2*a*, *b*, *e*, 4*a*, and *c*). The individual fibers are difficult to document photographically because the birefringence of the central spindle is so overwhelming (10–15 nm in retardation). The chromosomal fibers also tend to lie parallel to the central spindle fibers or to the large bundle of astral rays enveloping the nucleus. Birefringence due to bundles of chromosomal spindle fibers can be seen in Figs. 2*c* to *e* and 4*a* to *d*. With our microscope equipped with rectified polarization optics, the dark-adapted eye can clearly distinguish the individual chromosomal fibers when the λ/25 Brace-Köhler compensator is rapidly turned back and forth. Furthermore, each fiber is seen to terminate on an individual kinetochore which appears as a positively birefringent, miniature spherulite.

During anaphase-A, the weakly birefringent chromosomal fibers shorten. Concurrently, chromosomes move with kinetochores foremost to the centrosomal surface. Finally, the chromosomal fibers become short birefringent stubs which link the kinetochore and centrosomal surface (Fig. 4*d* to *f*; also shown schematically in Fig. 11).

Eventually, all the kinetochores crown the centrosome. In optical sections given by differential interference contrast, the kinetochores can be seen aligned in an arc, as though a string of pearls (Figs. 5*e*, *g*, *h*; and especially 6*b*, *c* left pole). As the kinetochores become aligned the chromosomes shorten, forming a tight ("telophasic") bouquet around the centrosome.

The arc of kinetochores centers around the distal tip of the elongate-centriole. In polarized light, we see a cone made up of thin, radiating, birefringent strands between the tip of the elongate-centriole and the centrosomal surface crowned by the kinetochores (Fig. 2*a*, *b*, *f*; also see Figs. 14, 15, and 16*b*). As discussed in Part I, these strands which show positive birefringence appear to include intracentrosomal portions of the chromosomal spindle fibers (also see text figure 20 in reference 8).

Throughout this somewhat haphazard migration of chromosomes to the centrosome, the bire-

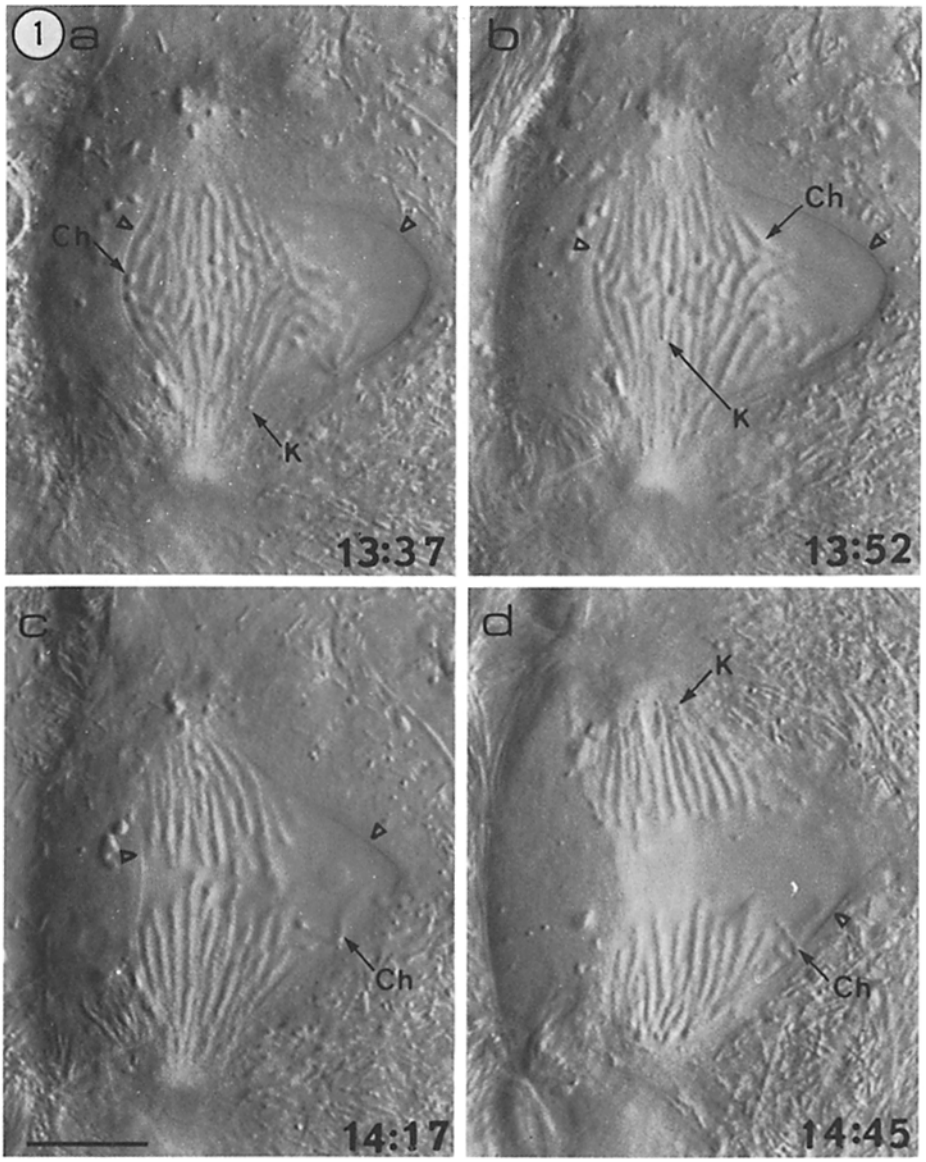

FIGURE 1 Differential interference contrast image of *Barbulanympha* anaphase-A in side view, focused on chromosomes above the central spindle. (*a*), Early anaphase-A. Chromosomes (*Ch*) for the most part are still attached at their distal ends. Kinetochores (*K*, distinct dots at chromosome apices near lower pole) appear at varying distances from the spindle poles. Anterior margin (left side in these photos) of the nuclear envelope is convex outward (triangle). (*b*), Mid-anaphase-A. Arms of several chromosomes are just separating. Their kinetochores are closer to the poles, but others are still lagging. Notice one kinetochore near the axis of the spindle only a fifth of the way from the equator to the lower spindle pole! Anterior margin of the nuclear envelope is less convex than in (*a*). (*c*), Late mid-anaphase-A. Most chromosome arms have separated and their kinetochores are approaching the spindle poles. The anterior margin of the nuclear envelope is now concave (triangle). In the lower right hand part of the nucleus, note the lone pair of chromosomes (*Ch*) that appear as though they had moved away from the lower spindle pole. (*d*), Late anaphase-A. Chromosome arms are contracting. The kinetochores lie near the centrosomal surface but are not yet aligned in a "string of pearls" configuration. The lone pair of chromosomes is moving poleward. h:min in time of day on 74g28. Bar, 20 μm. × 800.

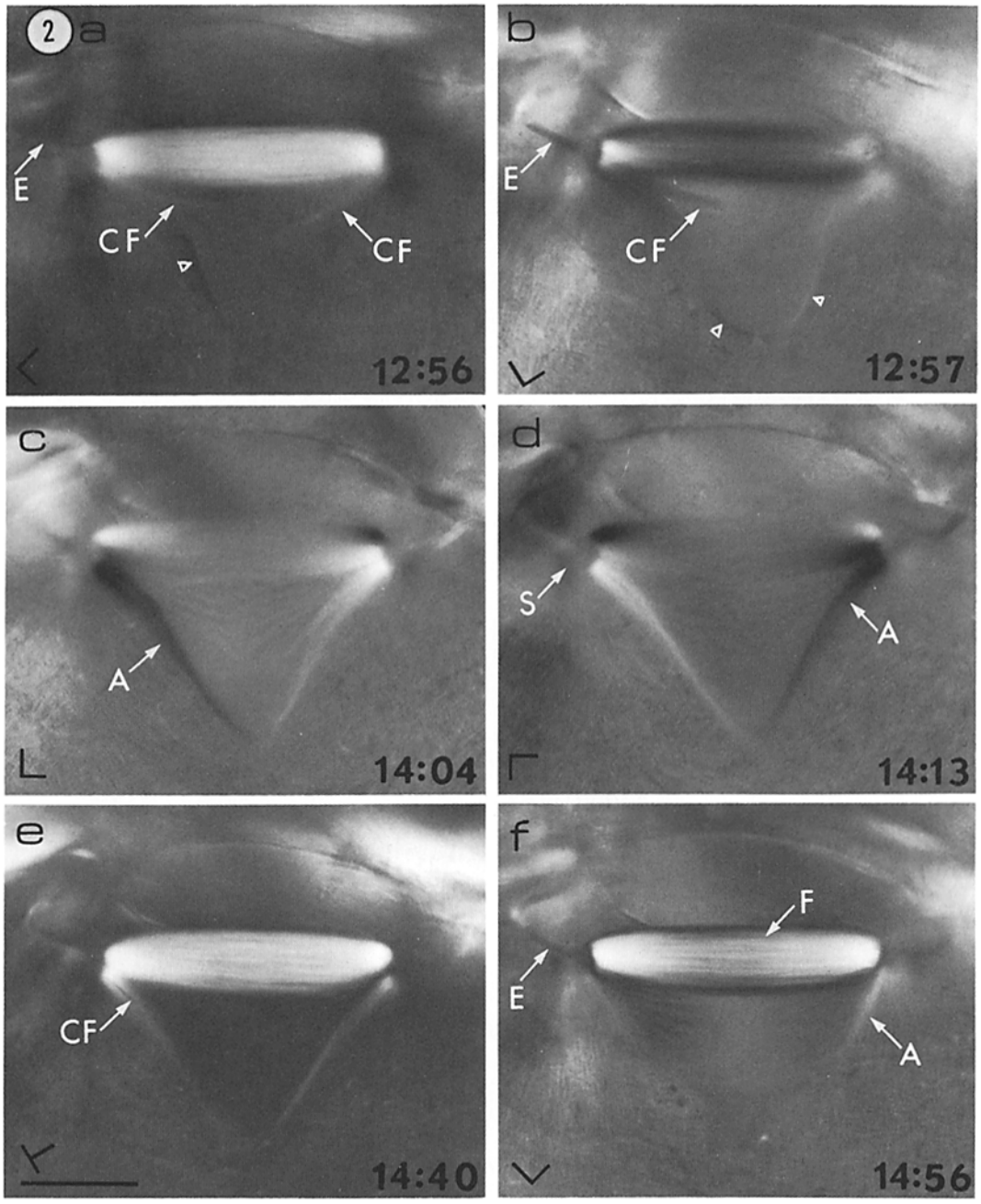

FIGURE 2 Anaphase-A side view of the same cell shown in Fig. 1, observed in polarized light and focused on the central spindle; turned 90° to Fig. 1. Over the 2 h of anaphase-A covered in these photos, the dimensions and birefringence of the central spindle have remained unchanged. Polarizer and compensator axes are oriented variously relative to the spindle axis to enhance different fibers of the mitotic apparatus; central spindle "continuous" fibers (*F* in (*f*), chromosomal spindle fibers (*CF* in (*a*), (*b*), (*e*)) and perinuclear astral rays (*A* in (*c*), (*d*), (*f*)). Compare with Figs. 1 and 3 which indicate location of chromosomes. In (*a*), (*b*), and (*f*), note the cone of weakly positively birefringent strands which link the distal tip of the left hand elongate-centriole (*E*) to the central spindle fibers and astral rays abutting the centrosomes (*S*). The hooked-shape characteristic of the elongate-centriole in anaphase can be seen clearly to the left of the spindle. L, orientation of polarizer and analyzer and quadrant containing slow axis of compensator. Time in h:min on 74g28. Bar, 20 μm. × 835.

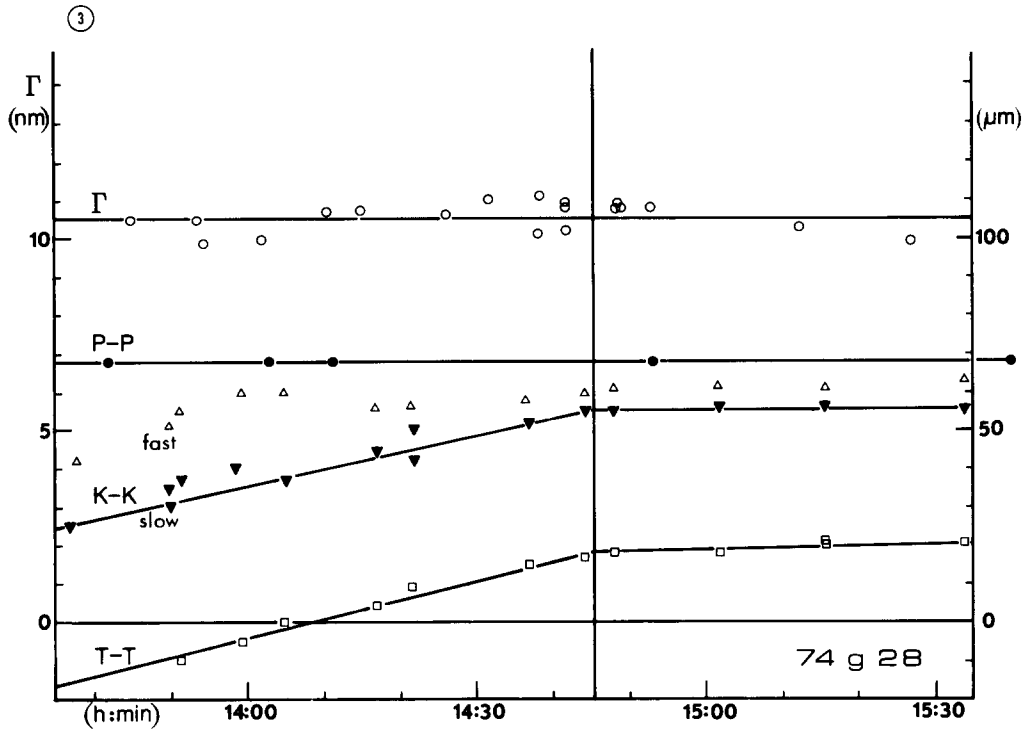

FIGURE 3 Measurements of anaphase-A taken on the same cell 74g28 photographed in Figs. 1 and 2. *P-P*, spindle pole-to-pole length, which clearly remains unchanged throughout anaphase-A. *K-K*, interkinetochore separation for chromosomes moving to poles early (fast), and late (slow). *T-T*, separation of tails of "slow" chromosomes. Γ, retardation of central spindle. h:min in time of day on 74g28.

fringence, shape, and size of the extranuclear central spindle do not change (Fig. 2*a* to *f*; also compare Fig. 8*a* to *c* in Part I and Fig. 5*a* in Part II with Figs. 4*a*, *b*, 5*b*, and *c*, graphed in Fig. 8). The retardation (Figs. 3 and 17), the width (Fig. 17) and pole-to-pole distance of the central spindle (Figs. 3, 8, and 17) remain constant until the chromosomes all reach the spindle poles. While the chromosomal spindle fibers shorten and their birefringence diminishes, their retardation contributes negligibly to the measured retardation of the central spindle.

With the arrival of all of the kinetochores at the centrosomal surface, the first stage of anaphase terminates. This stage thus involves chromosomal segregation and kinetochore poleward migration led by shortening chromosomal spindle fibers. We have named this stage of chromosome segregation, anaphase-A. For *Barbulanympha*, this occurs in the total absence of central spindle elongation.

Some *Barbulanympha* cells, as the one illustrated in Figs. 1-3, did not proceed beyond the end of anaphase-A. Others proceeded to anaphase-B (see below) after varying intervals of time (Figs. 4-6, 8, 9-17). The use of an orange filter in our microscope illuminating system to eliminate even a trace of stray blue light, and other precautions to minimize exposure to intense illumination as described in Part I, Materials and Methods, increased the frequency of cells progressing into anaphase-B. In cells which did not proceed to anaphase-B, the flagellar tufts did not separate farther, but otherwise such cells appeared perfectly healthy. Their flagella beat vigorously and the cells swam about for many hours, continuing to display a normal-looking, birefringent, anaphase-A central spindle.

Anaphase-B

At the end of anaphase-A the chromosomes are well separated, forming ("telophasic") bouquets with their kinetochores aligned at each spindle pole. Up to this stage of anaphase, the length and shape of the birefringent central spindle have not changed since prophase.

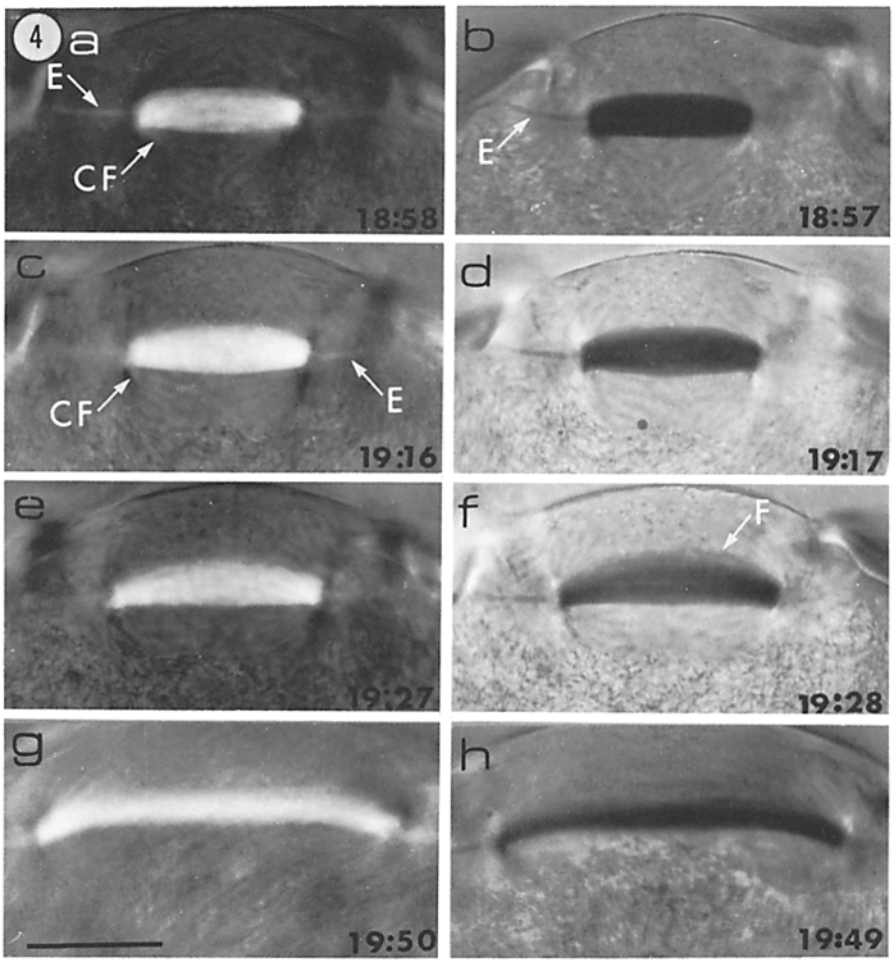

FIGURE 4 Late anaphase-A to mid anaphse-B spindle; side view in polarized light. (*a*), (*b*), Cell in mid-anaphase-A, showing typical anaphase-A spindle. (*c*), (*d*), Anaphase-A is practically completed and the spindle is becoming somewhat banana-shaped. The posterior margin of the spindle is still sharply delineated but the anterior margin (above in these photos) has become fuzzy. (*e*), (*f*), Anaphase-B has already started. The central spindle is longer and the fibers (*F*) have started to splay anteriorly. (*g*), (*h*), Central spindle continues to elongate, becomes thinner and less birefringent. Continued to Fig. 6*a* and *d*. Near the poles, short chromosomal spindle fibers (*CF*) are visible just below the spindle. Spindle axis 45° to polarizer, compensator orientation reversed for each pair of photos. h:min time of day on 75h8. Bar, 30 μm. × 630.

The onset of anaphase-B is signaled in the polarizing microscope by a subtle change in shape and contour of the central spindle. The birefringent fibers at the anterior margin become fuzzier and bowed out, and the spindle's posterior margin remains sharp (Fig. 4*c*, *d*). Characteristically, this transformation to a banana-shaped spindle is seen at the beginning of each anaphase-B.

The spindle then starts growing, and may extend to as much as five times its original length (Fig. 14 in reference 21). This spectacular growth of the central spindle is shown in the polarized light images in Figs. 4*c* through *h* to 6*a* and *d* (also see Fig. 12 in Reference 21). At 18° ± 1°C, the spindle shown here grew at a steady rate of 1.5 μm/min (Fig. 8), and the spindle in the larger species shown earlier reached an extension rate of 5.9 μm/min (Fig. 14 in reference 21).

As the central spindle elongates, the chromosomes are separated farther. The several-fold separation of the chromosome bouquets is clearly seen in the differential interference contrast im-

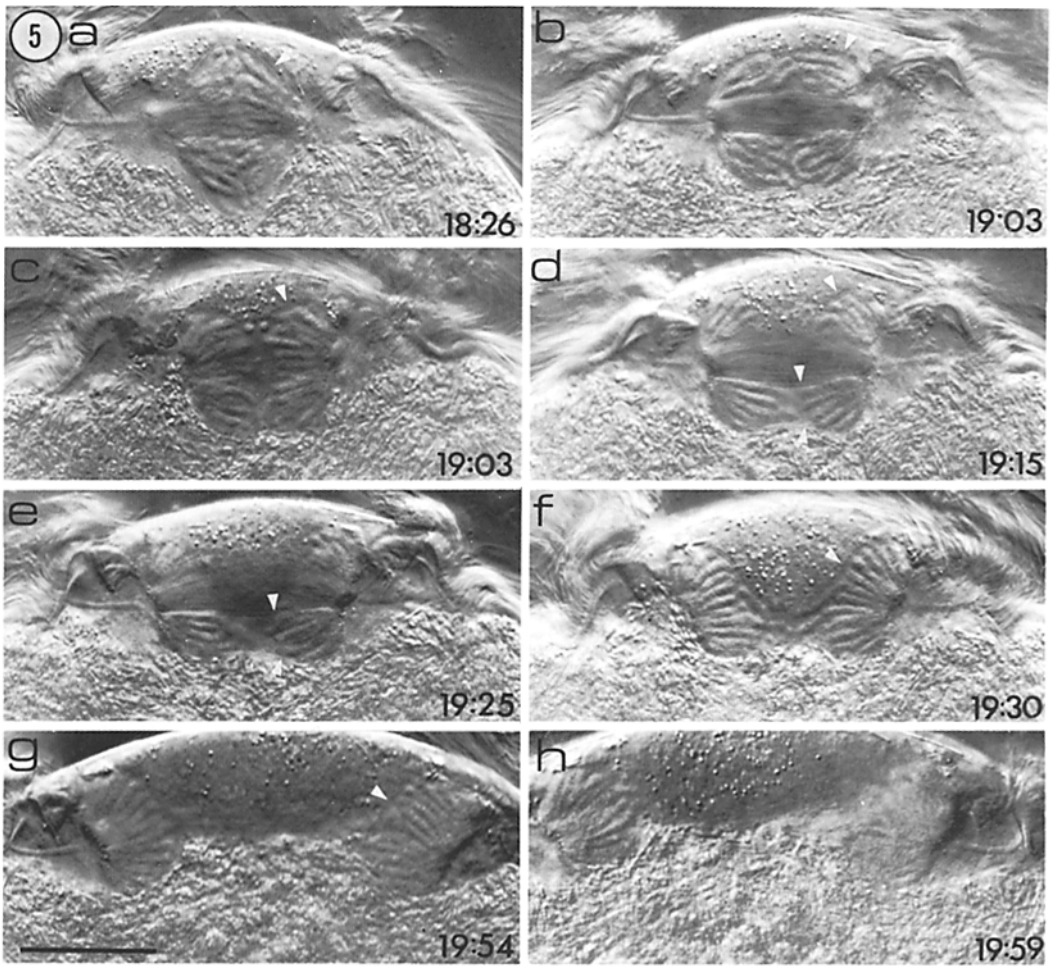

FIGURE 5 Same cell as shown in Fig. 4 but viewed in differential interference contrast. (*a*) to (*d*), Anaphase-A. (*e*) to (*h*), Anaphase-B (continued to Fig. 6*b* and *c*). Note change of nuclear contour (triangles) with progression of mitosis. The nuclear envelope never breaks down in *Barbulanympha* but instead undergoes a complex morphogenesis (see Fig. 11). Time of day in h:min on 75h8. Bar, 30 μm. × 600.

ages (Figs. 5*d* to *h*, 6*b*, *c*; also see Fig. 13 in reference 21). In these figures, the arched row of kinetochores shows clearly (see especially Fig. 6*b*, *c*). Throughout anaphase-B, the kinetochores, which lead the chromosome arms, remain anchored to the centrosomal surface by short stubs of chromosomal spindle fibers (Figs. 4*a* to *h* and 6*a* and *d*).

As the birefringent spindle elongates, its anterior margin becomes fuzzier as though fibers were splaying (Fig. 4*e* to *h*; also Fig. 12 in reference 21). The remaining spindle stem becomes progressively thinner while the retardation also decreases (Figs. 4, 6*a*, *d*, 7*a*; also Figs. 12 and 14 in reference 21).

Decrease in retardation of the central spindle remnant is nearly proportional to the decrease of spindle diameter (Fig. 17; also Fig. 14 in reference 21). Thus, the coefficient of birefringence (= retardation/thickness) remains relatively constant; presumably, fewer microtubules make up the stem of the central spindle while their packing density remains unchanged.

During the early part of anaphase-B, the caudal margin of the spindle remains reasonably straight (Fig. 4*c*, *d*; also Figs. 12, 12:35, and 12:51 in reference 21). Soon, however, the spindle becomes bowed near the poles as the bouquets of chromosomes tilt outwards (Figs. 4*e* to *h*, 5*e* to *h*; also Figs. 12, and 13, 13:01 *et seq.* in reference

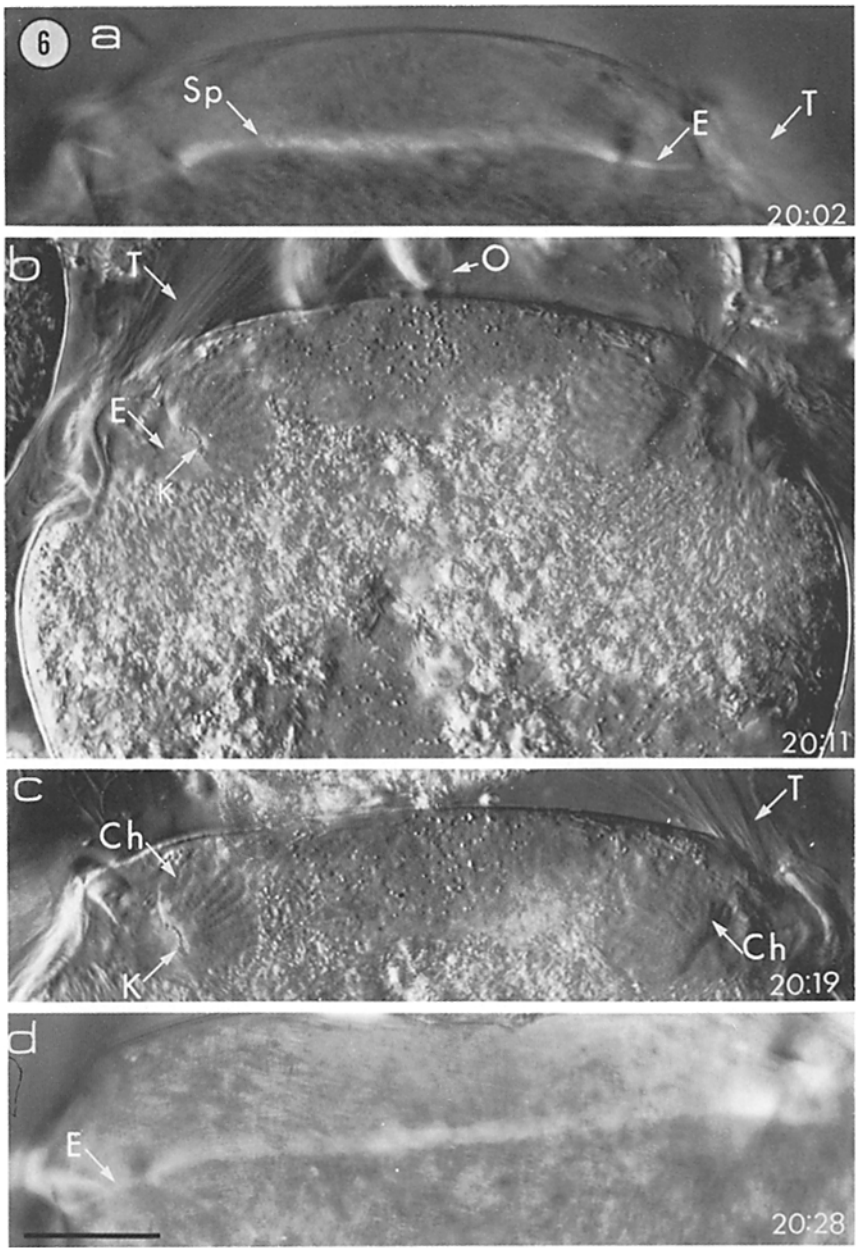

FIGURE 6 Anaphase-B continued from Figs. 4 and 5. (*a*), (*d*), In polarized light showing highly extended central spindle. Elongate-centrioles (*E*) are clear at spindle poles. (*b*), (*c*), Differential interference contrast. Note pearl string configuration of kinetochores (*K*) strikingly displayed by the left hand bouquet of chromosomes (*Ch*). The arc of kinetochores, focused on the elongate-centriole, abuts and outlines the centrosome. The distance between the flagellar tufts (*T*) is progressively increasing from (*a*) to (*d*). Time of day in h:min on 75h8. Bar, 30 μm. × 600.

21). Later, the spindle becomes wavy (Figs. 6*a*, 7*a*) or more or less straight (Fig. 6*d*) depending on transient variations of the interpolar distance. The distance varies in late anaphase-B as *Barbulanympha* swivels its two rostra independently and with increasing frequency.

At the commencement of anaphase-B, two flagellar tufts lie anterio-lateral to the spindle poles.

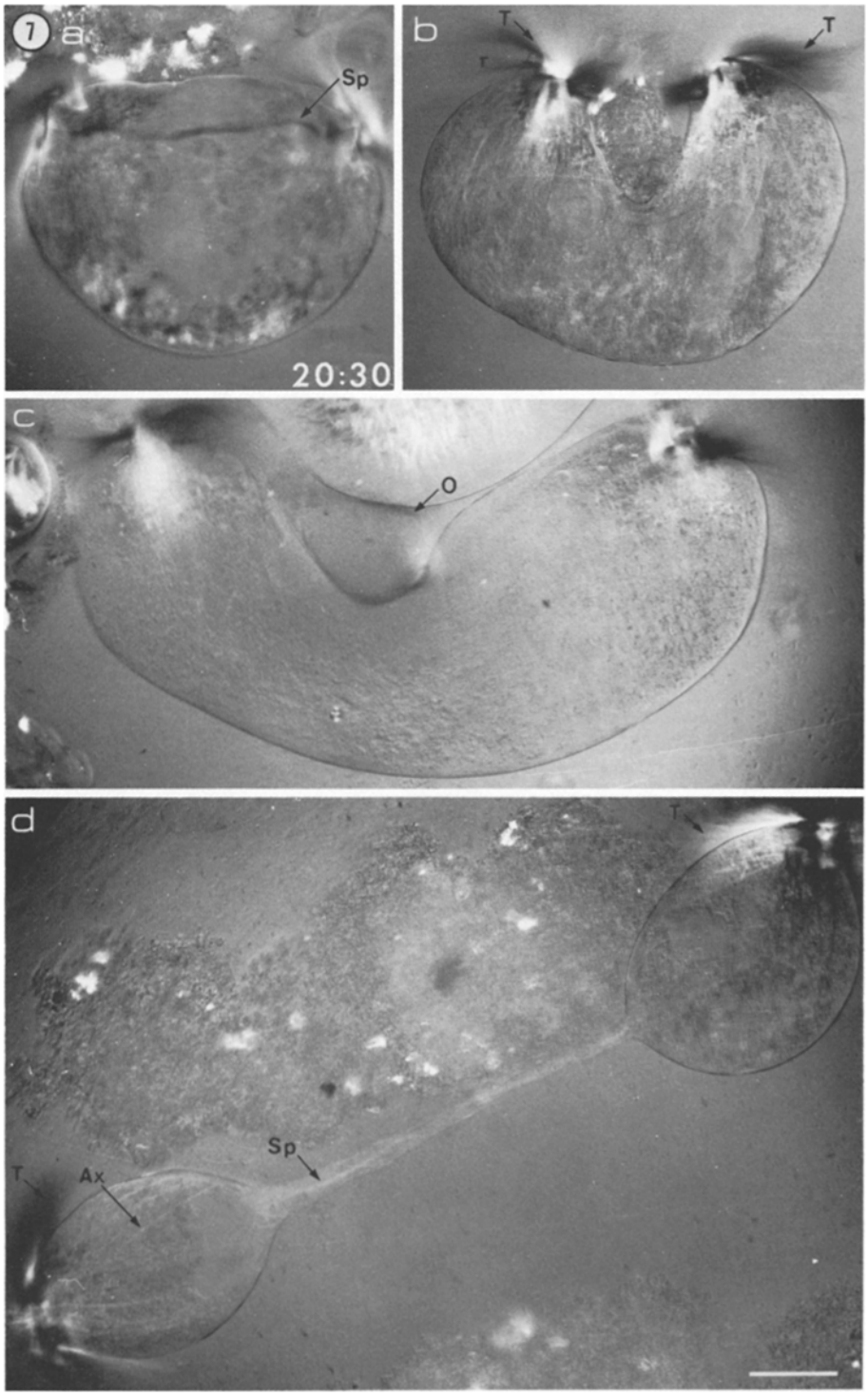

FIGURE 7 Polarized light views of late anaphase-B to cleavage. (*a*), Lower magnification of the same cell continued from Figs. 4 and 6. Spindle (*Sp*) remnant appears in dark compensation. During cytokinesis, *Barbulanympha* are often found pushing against debris as in (*a*), (*b*), or being assisted by another organism (*O*, *Trichonympha* as in (*c*), also Fig. 6*b*), which rams against the side of the *Barbulanympha* and seems to push in a cleavage furrow! (*d*), Terminal cleavage stage. A thin spindle remnant is still visible in bright compensation through the length of the long stalk connecting the two daughter cells. The daughter cells are swimming in opposite directions propelled by their flagellar tufts (*T*). In the daughter cells, parabasals and axostyles (*Ax*) have elongated and appear respectively as twisted ribbons and distinct, positively birefringent threads. Bar, 50 μm. × 250.

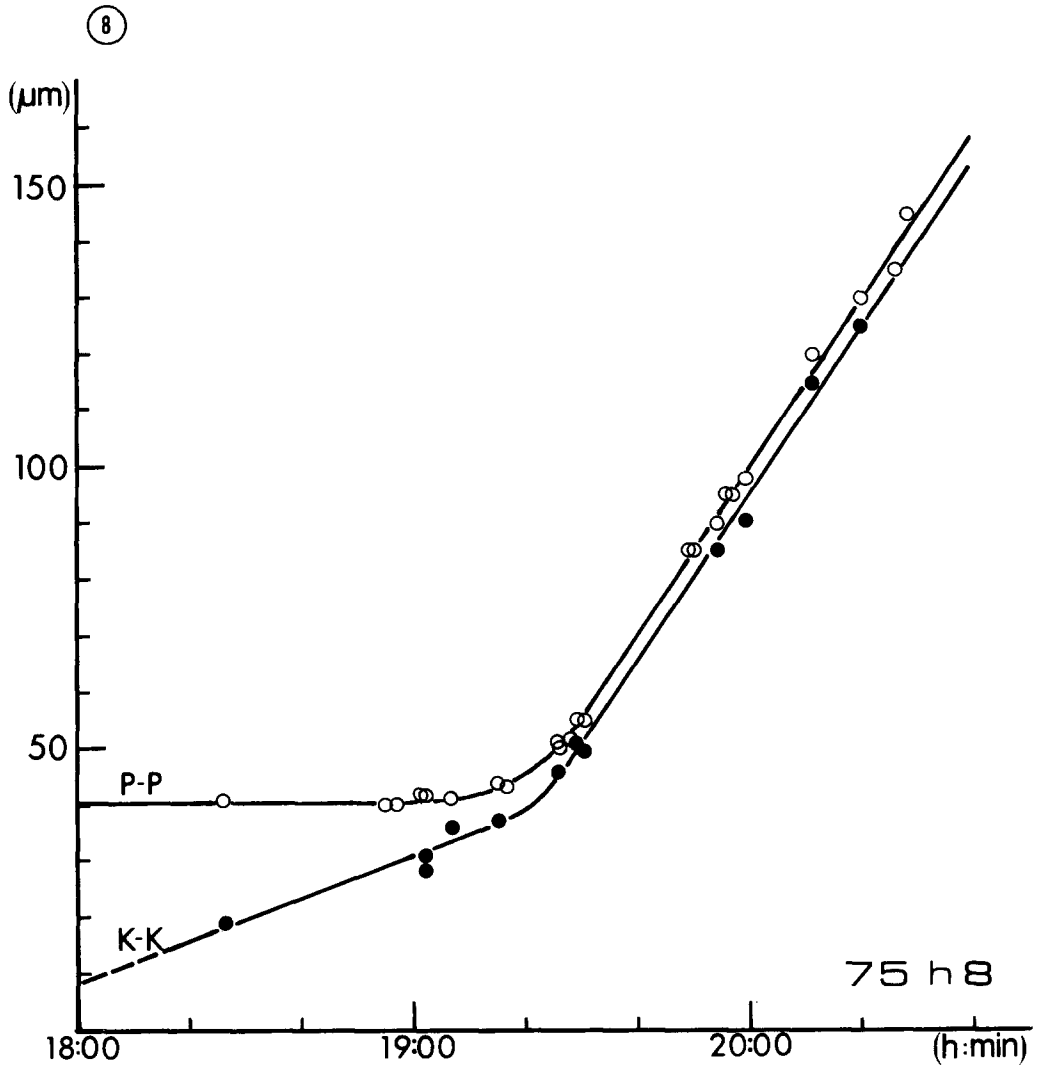

FIGURE 8 Spindle length and kinetochore separation of cell (75h8) shown in Figs. 4–7*a* and Fig. 8 in Part I. *P-P*, spindle pole-to-pole length, which remains stationary in anaphase-A and elongates several-fold in anaphase-B. *K-K*, kinetochore velocity in anaphase-A was 0.4 μm/min, for anaphase-B it was 1.5 μm/min at 18°C.

Each centrosome at the spindle poles is connected by its elongate-centriole to the parabasal axostylar lamella and to the basal structure of the flagellar tufts (Figs. 2*f*, 4*b*; also see Part I and reference 8). As the spindle extends in anaphase-B, the anterior surface of the cell between the flagellar tufts also extends. During spindle elongation the elongate-centrioles sometimes orient in line with the spindle axis as if being pulled into alignment by the diverging flagellar bundles. In other cases, the elongate-centrioles lie at an angle to the spindle axis, suggesting that spindle length and distance between the flagellar bundles each independently increases at nearly the same rate. During anaphase-B, the shape and orientation of the elongate-centrioles change with the temporarily imposed tension as the anterior rostra of the organism actively swivel about.

In the absence of nuclear envelope breakdown, there exists no typical telophase in *Barbulanympha*. The nuclear envelope does not have to be reformed and chromosomes, although contract-

ing, do not decondense as in typical mitosis. Rather, mitosis would appear to terminate undramatically with the end of karyokinesis. In fact, however, the nuclear envelope has undergone a dramatic morphogenetic behavior. This behavior of the nuclear envelope which culminates in the orderly packaging of sister chromosomes will be next described together with changes in the three-dimensional shape of the central spindle.

Nuclear Morphogenesis – Introduction

The exact three-dimensional events of karyokinesis in *Barbulanympha* were clarified through a fortunate position of a cell displaying its spindle in exact anterior view (e.g., Fig. 9*c*). In this position, the cell proceeded from prophase through anaphases-A and -B. Over the entire period, we were able to obtain through-focus time-sequence photographs, alternately in differential interference contrast and rectified polarized light microscopy.[1] While photographically recording the morphological and dimensional changes, we also measured the birefringence retardation of the spindle by adjusting the compensator to visually extinguish the most retarding parts of the central spindle halfway between the two spindle poles. The retardation measurement allows us to determine the concentration of microtubules responsible for the spindle birefringence.

Before describing the anterior view, we shall highlight the shape changes of the nucleus seen in lateral view.

Changing Contour of the Nucleus in Lateral View

As the asters grow in prophase, the nearly spherical nucleus migrates anteriorly (In Part I Figs. 1, 2*a, b,* 3*a,* and *b*). Soon the nucleus abuts the posterior margin of the central spindle which has been developing between the centrosomes. The anterior margin of the nuclear envelope flattens and appears as a sharp straight line at the base of the spindle (Part I, Fig. 3*a*).

With time, the nucleus moves farther forward, wraps around the spindle, and becomes horseshoe-shaped in cross section (also see Figs. 51, 53, 54, etc. in reference 8). At this stage, the nuclear envelope abuts the posterior spindle margin along its whole length. The exterior shape of the nucleus changes from nearly spherical to a hanging drop or inverted bell shape (Part I, Figs. 4, 7).

By the onset of anaphase-A, the nucleus completely engulfs the spindle except at the centrosomes. The nucleus therefore becomes a sleeve surrounding the spindle. The sleeve bulges out in the mid-region so that the anterior as well as posterior margin of the nucleus now appears bell-shaped (Figs. 1*a*, *b*, and 5*a*; also Fig. 8*c* in Part I).

At the end of anaphase-A, chromosomes form a ("telophasic") bouquet around each centrosome. The shortening chromosomal fibers align the kinetochores into a crown capping each centrosome. The poleward margins of the persistent nuclear envelope, in which the kinetochores are embedded, consequently balloon out (Fig. 5*a* to *d*).

The astral rays which run from the centrosomes tangent to the nuclear envelope are shoved together by the ballooning nucleus. The hollow cones formed around the spindle poles by these astral rays increase in angle from $< 90°$ to 180°, as with an umbrella turning inside out. Concurrently, the mid-region of the anterior nuclear envelope turns from convex outward to convex inward and becomes saddle-shaped (Figs. 1*a* to *d*, 5*a* to *f*).

At the onset of anaphase-B, the inner posterior surface of the nuclear tube stands out in differential interference contrast as a straight line (Fig. 5*b*; also see Figs. 13, 12:42, and 12:52 in reference 21). Here, the nuclear envelope is apposed tightly to the central spindle; in polarized light the posterior margin of the spindle also displays a sharp contour (Fig. 4*a* to *d*; also Figs. 12, 12:35, and 12:51 in reference 21).

In early anaphase-B, the bouquet of chromosomes tilts anteriorly. Concurrently, the nuclear envelope expands more anteriorly than posteriorly (Fig. 5*e, f*; also Figs. 13, 12:52 in reference 21). The tilting of the bouquet is accompanied by a localized posteriad bending of the spindle axis (Fig. 4*c* to *h*; Figs. 12, 12:51, 13:04 in reference 21).

With progression of anaphase-B, the posterior surface of the nuclear envelope also invaginates

[1] For this sequence, the differential interference contrast objective lenses were especially carefully oriented to align their Wollastron prisms to the condenser Wollaston. A $\lambda/25$ compensator was introduced, and the polarizers and compensator were oriented so as to achieve sufficiently high extinction to simultaneously display the spindle birefringence and the differential interference contrast image.

and becomes saddle-shaped (Fig. 5*d* to *f*). Upon casual observation, the nucleus seems to be simply drawn apart into two. In fact, the nuclear envelope undergoes an intricate topological maneuver, as became evident from the anterior view of *Barbulanympha* mitosis which is described below.

Anterior View and Three-Dimensional Interpretation of Nuclear Morphogenesis

Fig. 9 displays prophase views of the *Barbulanympha* cell that gave the best anterior view of mitosis. An old flagellar tuft and rostrum appear at the left end of the central spindle (Fig. 9*c*), and a developing new flagellar tuft, still lying in the cell's interior, is seen at the upper right of the spindle (see lettered tracing, Fig. 10).

In Fig. 9*c*, a differential interference contrast optical section through the spindle axis, many chromosomes fill the bell-shaped area outlined by the nuclear envelope directly lateral to (above and below) the spindle. Both elongate-centrioles show at the spindle poles. To the left, an elongate-centriole is connected to the parabasal axostylar lamella beneath the row of basal granules at the base of an old flagellar tuft. Many axostyles and parabasals surrounding the nucleus trail to the right. All photographs shown in Figs. 9 through 16 are of this same highly flattened individual whose division in anterior view we recorded for 8 h.

With the microscope focused to give a high optical section (i.e. focused anterior to the spindle), this prophase cell displayed a pair of lines running parallel to the spindle axis (Fig. 9*a*). The lines run about one-third the spindle length in the spindle mid-region. At both ends, the parallel lines open into Y's which soon terminate with apices each marked by a kinetochore (see diagram Fig. 10*a*). Through-focus serial micrographs reveal that the parallel lines are apposed lips of the nuclear envelope which have met anteriad to the central spindle after the nucleus had wrapped around the spindle. We have named this structure the nuclear "seam." The seam is shown in some of Cleveland's drawings (e.g. Figs. 6 and 7 in reference 9), but no mention of such a structure is found in his writings.

The nuclear envelope can be traced from the seam to its lateral aspects (toward the top and bottom of each of these photographs) by following optical sections taken at successive levels (e.g., Fig. 12). There, the exterior contour of the nucleus is more or less bell-shaped, but the smooth contour is interrupted in places by protrusions and recesses. The protrusions contain chromosomes, with kinetochores located at the poleward apex of each pointed protrusion (Figs. 9*a* to *c*, 10*a* to *c*; also see Fig. 35 in reference 10). Also, at the pits of the recesses, kinetochores are found embedded in the nuclear envelope (Figs. 9*a* to *c*, 10*a* to *c*; also see Figs. 50 and 73 in reference 8). In living cells, we see these protrusions and recesses slowly appearing and then disappearing over a course of several minutes. These changes in the nuclear envelope occur in association with the churning of chromosomes mentioned in Part I.

With time, the kinetochores gradually move poleward and the nuclear seam elongates (Figs. 9*d*, 10*d*). In this manner, the nucleus becomes a more complete sleeve surrounding the central spindle. Our three-dimensional interpretation of the mitotic apparatus at this stage (less the chromosomes) is shown in Fig. 11, top.

In the individual *Barbulanympha* recorded here, the chromosomes continued to churn and the cell would not proceed to anaphase-A for several hours. So at 4 h and 45 min after initial observation, we cold-shocked the cell hoping to nudge it into anaphase. The first chilling cycle started at 22:13.5 h and lasted 5 min in a 12.5°C refrigerator. No change of spindle birefringence was observed, and the chromosomes continued to churn. The slide was then placed in a 7°C refrigerator at 22:44.4 h for 4 min. Immediately after the slide was removed from the refrigerator, and the condensed moisture was blown off from the slide surface with a jet of dry Freon 12, no difference in central spindle birefringence was observed (cf. Figs. 9*e* and 14*c*). However, this time the chromosomes appeared somewhat thinner, the kinetochores started to recede from the poles, and the nuclear envelope became recessed in several places where kinetochores were inserted (22:56 h to 23:02 h). Some chromosome arms started to separate at 23:00 h and several kinetochores became more or less aligned at 23:05 h. The cell then entered anaphase (see graph, Fig. 17).

Progression of anaphase-A is shown in Figs. 12 and 13*a* to *f*. Anaphase-B is shown in Figs. 13*g*, *h*, 15*c*, *e*, *g*, and 16*a*. The advancing locations of chromosomes and kinetochores are summarized in the graph, Fig. 17.

In general, the chromosomes and kinetochores moved as described for the lateral views. How-

ever, presumably due to extreme cell flattening, spindle elongation was limited and the chromosomes did not move rapidly in the latter phases of anaphase-B, especially after 00:19 h.

At the onset of anaphase-A, the nuclear seam spans about three-quarters of the anterior spindle margin between the two centrosomes (Fig. 12*a, b*). Because the kinetochores have not yet quite reached the centrosomes, a short gap remains between the centrosomal surface and the apex of the nuclear envelope (follow the rows of kinetochores in the successive optical sections in Fig. 12). As anaphase-A proceeds and kinetochores reach the centrosomes, the seam spans the full length of the spindle (Fig. 13*a*). But just after that, the seam opens up in the middle, as though with parting of lips (Fig. 13*c*). The gap thus formed rapidly opens laterally and towards the spindle poles (Fig. 13*e, g*). This gap which appeared in the seam, we call the nuclear "cleft."

In the microscope image, only those regions of the nuclear envelope that lie along the direction of sight show up in optical section. By mentally reconstructing the optical sections, we thus arrive at the three-dimensional shape of the *Barbulanympha* nucleus at an early nuclear cleft stage (Fig. 11, middle).

Proceeding into anaphase-B, the cleft, which outlines the anterior margin of the nuclear envelope, progresses latero-posteriad exposing the lateral surface of the central spindle (Figs. 13*h*, 15*c, e*, and *g*). As the cleft passes farther posteriad, the tapered connections, earlier seen in median optical section between the left and right lobes of the nucleus, disappear (cf. Fig. 15*c* with *e*). At this focus, the nucleus takes on the shape of two pairs of insect wings (Fig. 15*g*). The three-dimensional structure of the mitotic apparatus and nuclear

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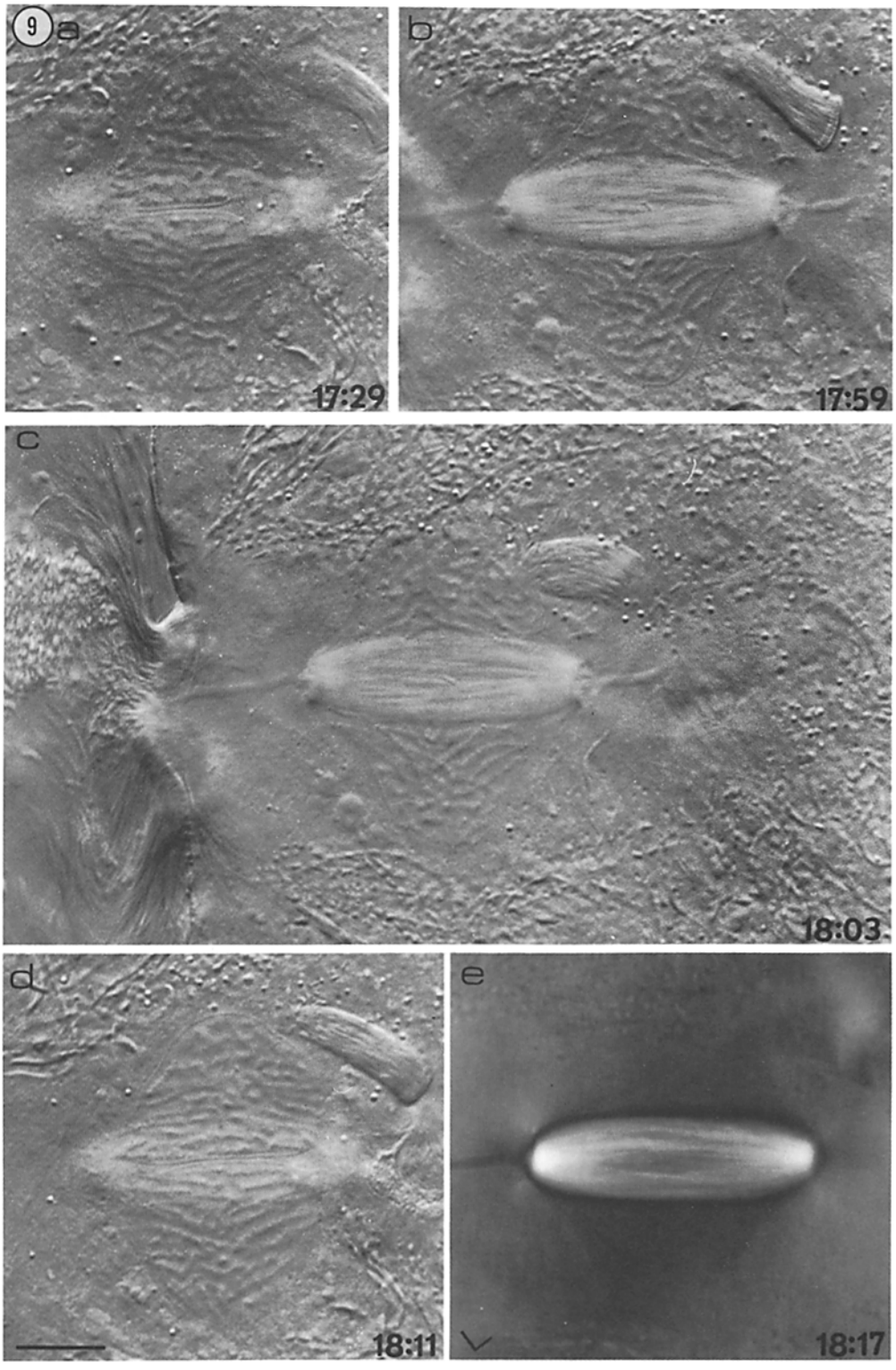
FIGURES 9 and 10 Anterior view of prophase and nuclear seam formation in *Barbulanympha*. (*a*), Mid-prophase. The nucleus has already moved anteriad (up towards viewer in these photos) and wrapped around the central spindle. (cf. schematic drawing in Fig. 11). In this high focus view, a pair of lines appears parallel to the spindle axis in the mid-region of the nuclear envelope (between arrows in Fig. 10*a*). This is an optical section of the "nuclear seam", which is found anteriad to the spindle where the nucleus has completely wrapped around the spindle and come into contact with itself. Laterally, the nuclear envelope appears shield-shaped. Within the nucleus many chromosomes are visible. In the tracing Fig. 10, those parts of the nuclear envelope visible in Fig. 9 are indicated by broken lines, and kinetochores are indicated by solid dots. (*b*), (*c*), Median focus optical section showing birefringence of central spindle outlined by differential interference contrast image of the nucleus. The lateral aspects of the nuclear envelope appear bell-shaped. Chromosomes are moving apart, but their arms have mostly not yet separated. Their kinetochores remain embedded in the persistent nuclear envelope. Some of the kinetochores appear at protrusions (*Kp*) and in recesses (*Kr*) of the nuclear envelope. (Note: some of the kinetochores can be seen more easily by turning the page by 90°.) In (*c*), one of the two old flagellar tufts appears prominently on the left side of the picture (*F-F*). At its base, the left hand elongate-centriole (*E1*) joins the parabasal axostylar lamella (*L*) and from there runs to the left pole of the central spindle. The centrosome here is surrounded by a large, granular-inclusion-free spherical zone, roughly twice the diameter of the centrosome (cf. Fig. 12*h*). A new flagellar tuft (*T*), still not yet externalized but already beating within the cytoplasm, appears above the right hand elongate-centriole (*E2*). The old flagellar tuft on this side can only be seen at a much higher focus. (*d*), Higher focus view 42 min after (*a*). As the kinetochores are pulled closer to the centrosomes, the seam is extended (between arrows in Fig. 10*d*; compare with 10*a*). (*e*), Median focus in polarization optics. Elongate-centriole (*E*) and margin of the central spindle are compensated (by subtractive action of the compensator) or are extinguished (by being oriented parallel to the polarizer or analyzer axis) and appear dark. The central spindle was completely formed before the stage showed in (*a*), and from that time on has maintained this shape, size, and birefringence. Asters radiating northerly and southerly from the centrosomes (*S*) appear in bright (additive) compensation. *L*, polarizer-analyzer axes and quadrant containing slow axis of compensator. Fig. 9*a* to *d* and other similar figures in Part II were obtained by carefully aligning a Leitz Smith T system differential interference contrast 40× objective and condenser on a high extinction polarizing microscope. We were thus able to display the birefringence of the central spindle simultaneously with the differential interference contrast image. The exceptionally shallow depth of focus of the differential interference contrast image yields striking optical sections of the nuclear envelope, chromosomes, etc, and the polarized light image brings out the spindle fibers. Time in h:min of day on 74g30. Bar, 20 μm. × 720.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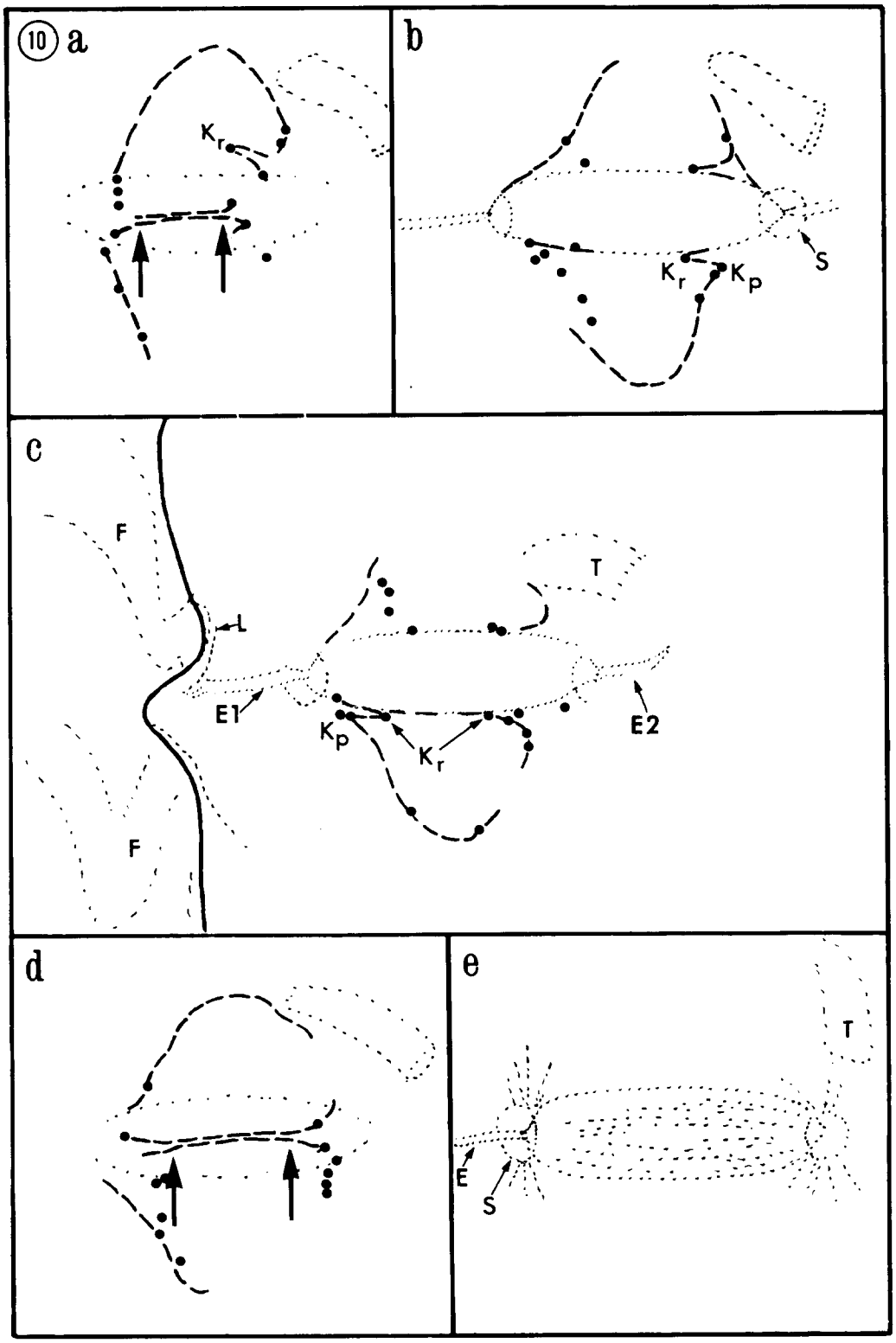
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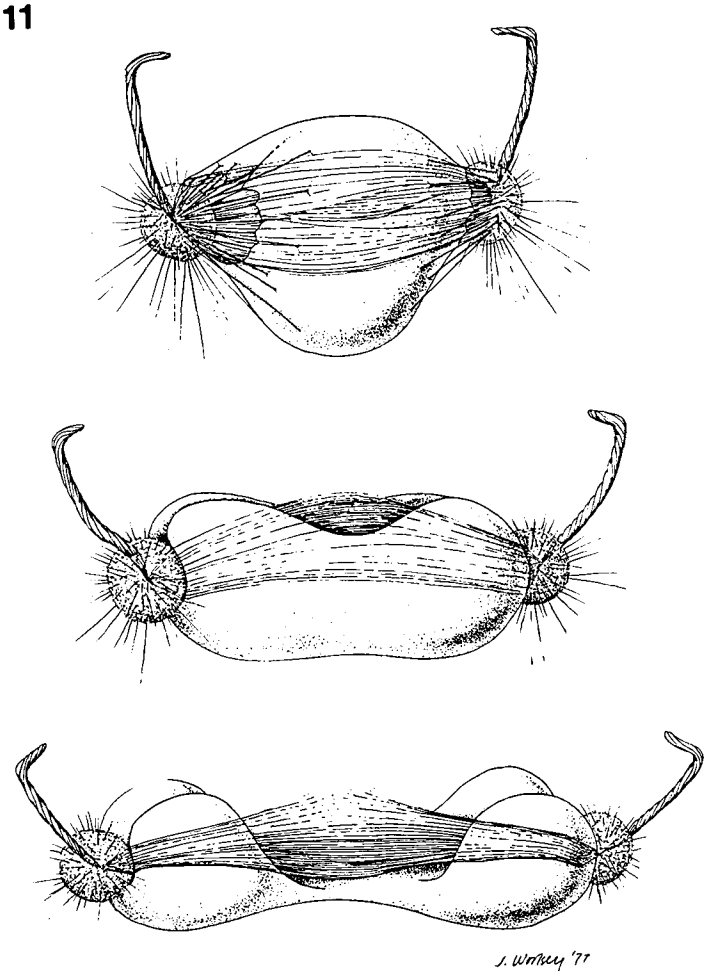


FIGURE 11 Schematic diagram of seam and cleft formation in *Barbulanympha*. *Top*, prophase to early anaphase-A. Those astral rays which have become attached to the kinetochores, embedded in the persistent nuclear envelope, become the chromosomal spindle fibers (chromosomes not shown). These fibers have repetitively shortened, hoisted up and deformed the nucleus, and wrapped it around the central spindle. The nuclear seam is formed where the nucleus contacts itself anterior to (above in these pictures) the central spindle. (For optical sections of the seam, see Figs. 9*a*, *d*, 12*a*, *b*, *c*, and 13*a*.) *Middle*, early to mid-anaphase-B. Chromosomal spindle fibers have shortened during anaphase-A, and the kinetochores as well as the poleward surface of the persistent nuclear envelope now crown the centrosomes. The nuclear seam has extended all the way to the centrosomes and has started to part in the middle. This opening, in the shape of parting lips, has been called the "nuclear cleft." (For optical section, see Fig. 13*c*, *e*, and *g*.) *Bottom*, mid to late anaphase-B. As the central spindle elongates, the kinetochores and nuclear envelope continue to adhere closely to the centrosomes. The nuclear cleft progresses towards the spindle poles and then caudad (towards bottom of picture) around the spindle. A horizontal slice through the middle of the spindle at this stage makes the nuclear envelope appear bilobed at both spindle poles (e.g. Figs. 13*h*, 15*e*) but continuous at a lower level (Fig. 15*c*). The spindle fibers splay where they are no longer constrained by the nuclear envelope, first anteriorly and then laterally (see Figs. 14 and 15). Artwork by John Woolsey.

envelope at this stage is interpreted in Fig. 11, bottom.

Eventually, the cleft progresses posteriorly around the spindle until the nucleus is separated into two parts. Karyokinesis is thus accomplished by an intricate topological maneuver of the persistent nuclear envelope.

At the completion of karyokinesis, each daugh-

ter nucleus in median optical section appears to be made up of two parts connected with a strand of kinetochores (Fig. 16*a*). However, from the foregoing description of karyokinesis, it can be inferred that each daughter nucleus is in fact horseshoe-shaped in vertical section. As one focuses the microscope, the narrow space seen in optical section between the "fly wings" opens anteriorly and the nucleus merges into "one piece" posteriorly. This groove in the daughter nucleus contains a ribbon of birefringent fibers attached at their base to the centrosomes (Fig. 16*b*). These are remnants of the central spindle fibers as will be described next.

Shape Change of the Central Spindle in Anaphase—Anterior View in Polarized Light

In anterior view as in lateral view, the width, length, and shape of the central spindle remain unchanged from the time the spindle is completed in prophase until the end of anaphase-A (Fig. 17; see Fig. 3 for graph of lateral view). For the individual *Barbulanympha* recorded here, this spanned a period of over 6.5 h.

The bilaterally symmetric, anaphase-A central spindle is composed of a bundle of fibers that emanate radially from the centrosomes and run parallel to the spindle axis towards the equator (Fig. 14*a* to *d*). The packing density and birefringence of spindle fibers are greatest at the centrosomal surface, and decrease away from the centrosome as the spindle width increases. Toward the lateral margins of the spindle, where the light rays traverse decreasing thicknesses of the spindle, the retardation is proportionately reduced. In Fig. 14*a* and *c*, the compensator slow axis is oriented perpendicular to the spindle long axis so that these less retarding spindle regions are compensated and appear dark (for an illustration of how spindle fiber contrast changes with compensator orientation, and for the numerical relation of spindle birefringence to microtubule packing density, see reference 36). The mid-region of the spindle also shows less birefringence and gives the appearance of possessing a longitudinal gap. The spindle seems to be made up of left and right bundles of spindle fibers with less fibers running through the middle, possibly because of an interfering nuclear sleeve (Fig. 13*b, d, f, h*? In Part I, Fig. 3*a* to *d*. See Fig. 37 and text Fig. 16 in reference 8 for illustration of the nuclear sleeve, a progressively narrowing funnel running anteriorly from the nucleus).

The microtubular origin of the spindle fiber birefringence was described in Part I. The microtubular bundles which make up the positively birefringent fibers of the central spindle are concentrated at the centrosomal surface where they are disposed radially. When the spindle axis is oriented parallel to one of the polarizer axes, the diagonally oriented microtubules contribute to the high contrast near the spindle poles (Fig. 14*b, d*). When the spindle axis is oriented at 45° to the polarizer axes, those same microtubules which now lie parallel to the polarizer axes, are therefore extinguished, and appear dark (Fig. 14*a, c, f*).

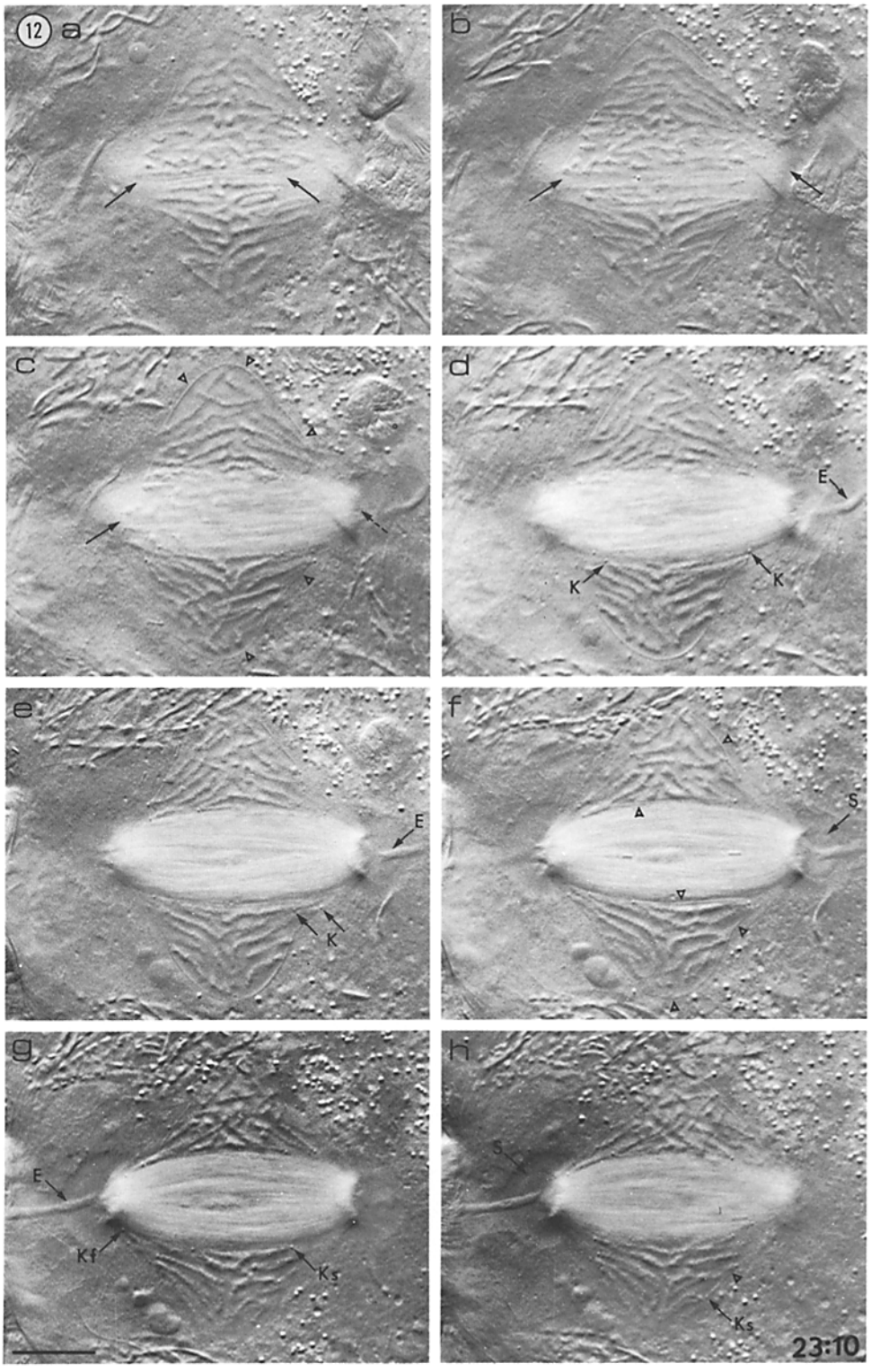
Those asters which appear bright in Fig. 14*a* are made up of rays lying in a quadrant at right angles to the spindle axis. Here, their slow axis lies in the same quadrant as the compensator slow axis in additive compensation, hence the asters appear as white fans with greatest brightness adjoining the surface of the centrosome where their microtubules are most concentrated. When the microscope stage is turned 45°, the bundle of astral rays and chromosomal fibers running tangent to the nuclear envelope show in striking bright or dark contrast (Fig. 14*b, d*).

In early to mid-anaphase-A, these astral bundles and chromosomal fibers form a 90° hollow cone surrounding the nucleus at the spindle poles (Fig. 14*b*). Toward the end of anaphase-A, the nuclear envelope becomes ballooned poleward by the chromosomes as they congregate (Fig. 13*b* to *f*) and the astral rays are accordingly displaced (Fig. 14*b* to *h*).

The sleeve-shaped nucleus ensheathes the lateral margins of the central spindle up to early anaphase-B. The spindle in anterior view retains a sharp, boat-shaped contour (Fig. 14*a* to *d*). At this stage, the anterior spindle margin has already started to bulge and become fuzzy as seen in lateral view (Fig. 4*c, d*).

As the nuclear cleft moves posteriorly and exposes the lateral aspect of the spindle (see models in Fig. 11), the birefringent fibers start to splay laterally (Fig. 14*e* to *h*). As seen in Figs. 14*c* to *h* and 15*a, b, d, f,* and *h,* the fibers act as though they were no longer bundled together in the middle of the spindle. The fibers appear to first straighten out (Figs. 14*e* to *h*, 15*a, b*) and then curve outwards as the contour length of each fiber increases (Fig. 15*d, f*).

As the fibers splay, the retardation of the spin-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dle mid-region decays precipitously (Figs. 14*g*, *h*, 15*b*, *d*, *f*, and *h*). The measured retardation and width of the birefringent core at the mid-region of the spindle decrease proportionately as plotted in Fig. 17.

Eventually the retardation in anterior view becomes very low at the mid-region of the spindle. Only near the poles, where the spindle remnant is sandwiched in the groove of the daughter nucleus (Figs. 15*g*, 16*a*), is appreciable birefringence observed (Figs. 15*h*, 16*b*). As the daughter nuclei are rocked by the undulating flagella, the contrast of the spindle remnants fluctuates. The retardation is greatest when the spindle remnant appears thinnest, undoubtedly because the light ray then has to traverse the maximum distance through the ribbon-shaped spindle remnant.

Within the centrosome, between the centrosomal surface where the birefringent ribbon is anchored and the tip of the elongate-centriole, a set of weakly positively birefringent radial strands persist (Figs. 15*h*, 16*b*). As described for the lateral view, these strands appear to include the intracentrosomal portions of the chromosomal spindle fibers which continue to link the kinetochores to the elongate-centriole tips.

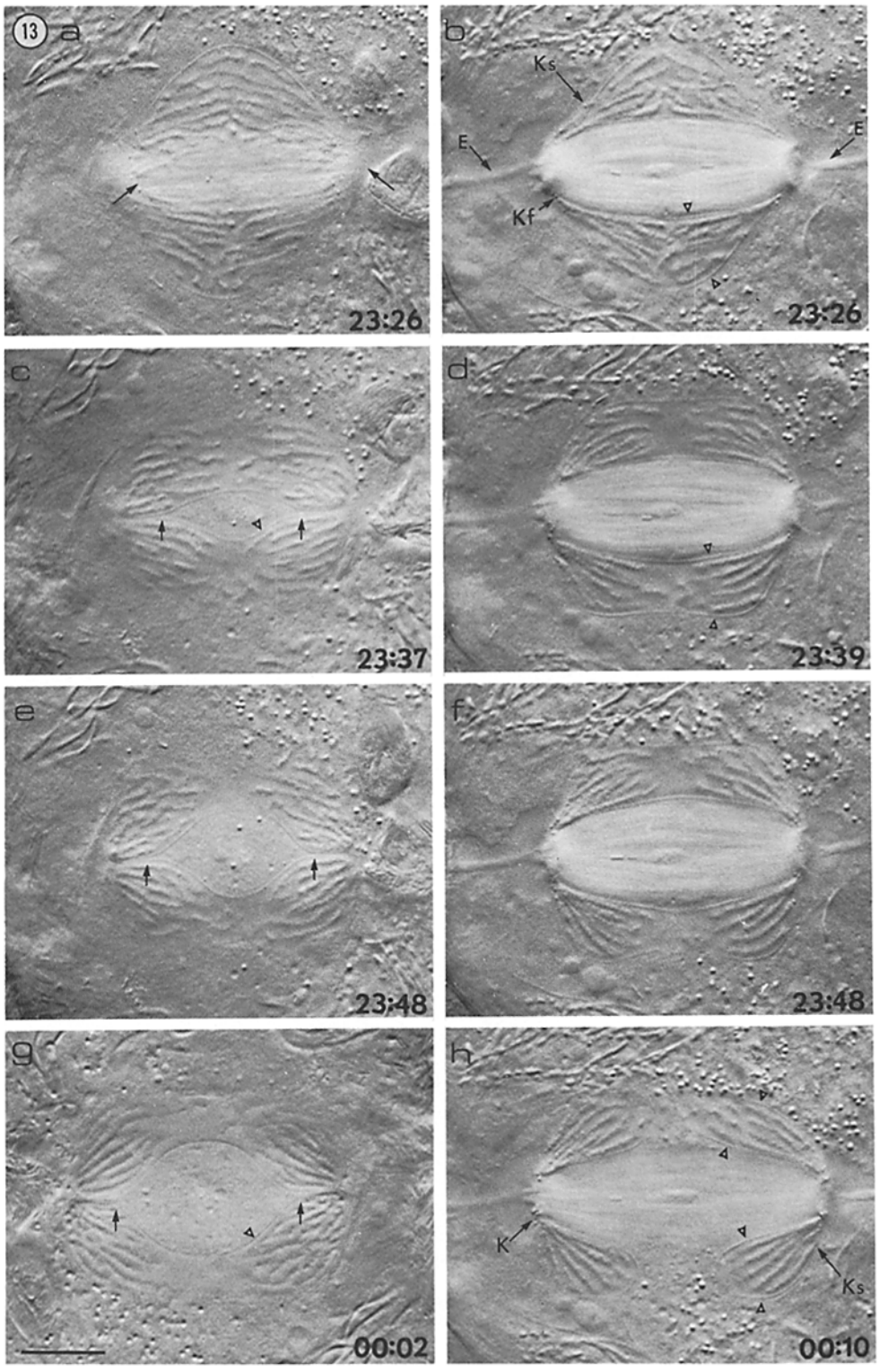
Cytokinesis

In late anaphase-B, the *Barbulanympha* eventually transforms into an organism with two heads (the rostra and flagellar tufts) pointing in opposite directions (Figs. 6, 7). Flagellar activity has increased to a point where, in spite of the compression between slide and cover glass, the two-rostrate organism swims about. It then often comes to rest in a position with the former anterior end pushing against a mass of debris (Figs. 6*c*, 7*a*, *b*, and *d*). Conversely, smaller organisms such as *Trichonympha* may come and poke against the same region (Figs. 6*b*, 7*c*). With assistance from these organisms or after considerable extension of a cytoplasmic bridge, the cell eventually pulls apart into two (Fig. 7*d*). It should be noted that *Barbulanympha* in their natural habitat in the *Cryptocercus* hindgut live in a thick soup of organisms. Only in our culture slides are they so sparsely distributed.

DISCUSSION

In Part II of this report, we have described with respect to spindle function: (*a*) chromosome movement occurring in two sequential steps, anaphase-A (poleward migration of kinetochores) and anaphase-B (kinetochore separation by spindle pole-to-pole elongation); (*b*) the contiguity throughout anaphases-A and -B of the positively birefringent chromosomal spindle fibers with the centriole and individual kinetochores (which exhibit a radially positive spherulitic birefringence); (*c*) protrusion and recessing of the nuclear envelope, with kinetochores positioned in the apex or pit of the deformation; (*d*) the repetitive poleward migration of the chromosomes before and during anaphase-A, led by their kinetochores embedded in the persistent nuclear envelope; (*e*) at the end of anaphase-A, the eventual arrival and alignment of all the kinetochores very close to or up against the centrosomal surface, forcing the nuclear envelope and kinetochores into a crown (string of pearls in optical section) and the chromosomes into a ("telophasic") bouquet configuration; (*f*) the constant length, width, and birefringence of the central spindle throughout anaphase-A; (*g*) in anaphase-B, an elongation of up to fivefold of the central spindle leading far apart the daughter chromosomes already partitioned in anaphase-A;

FIGURE 12 Through-focal series anterior view at onset of anaphase-A. Same cell as in Fig. 9, shown here at 23:10 h after the cell was exposed to cold at 22:44 h (see text). Combined birefringence and differential interference contrast image. In successive optical sections, the elongate-centrioles [*E*, (*c*)-(*f*), right pole; (*f*)-(*h*), left pole], centrosomes [*S*, (*f*), right pole; (*h*), left pole], the nuclear seam [arrows, (*a*)-(*c*)] and other features of the nuclear envelope (triangles) can be traced. Chromosomes and their kinetochores (*K*) show at varying distances from the spindle poles. (Turn left side of page towards you to better visualize kinetochores). Note that the chromosomes have rejoined their arms and have moved farther away from the spindle poles than before the cold treatment (cf. Fig. 9*d*). Similar behavior of chromosomes was observed in anaphase pollen mother cells of Easter lilies when their chromosomal spindle fibers lost their birefringence upon exposure to cold (Fig. 52 in reference 18). The regions of the chromosomal spindle fibers within the centrosomes show exceptionally clearly in (*f*) (right pole) and (*g*) and (*h*) (left pole). 74h8. Bar, 20 μm. × 620.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(*h*) persistence of the kinetochore grouping and the bouquet configuration of chromosomes to late anaphase-B; and (*i*) the splaying of the central spindle fibers initially anteriorly then progressively laterally which results in the proportionate decrease in width and retardation of the central spindle remnant.

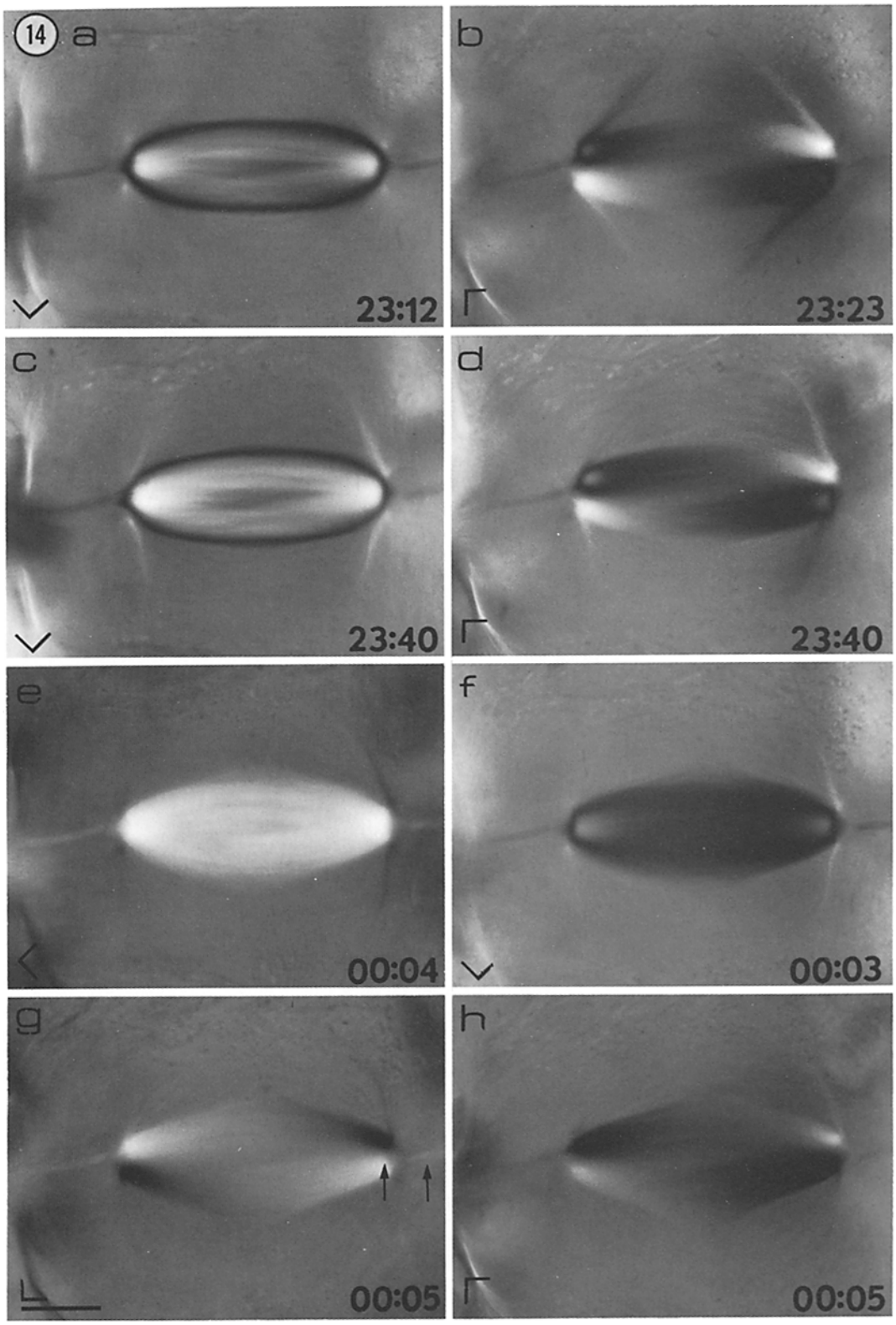
In Part II of this report, we have described with respect to nuclear morphogenesis and cytokinesis: (*a*) the formation and poleward extension (led by apical kinetochores) of the nuclear seam, the juxtaposing surface of the nuclear envelope anterior to the central spindle; (*b*) appearance in late anaphase-A of the nuclear cleft, a parting gap in the middle of the nuclear seam; (*c*) the laterad and posteriad expansion of the nuclear cleft in anaphase-B, culminating in the total partitioning of daughter chromosomes and completion of karyokinesis; and (*d*) the swimming apart of the two daughter cells, whose cytokinesis is often assisted by a clump of debris or by smaller organisms pushing against the prospective furrow region.

The Generality of Anaphase-A and -B

In *Barbulanympha,* we have described a clear-cut separation of anaphases-A and -B. We have demonstrated unambiguously that chromosomes in anaphase-A proceed to the centrosomal surface in the complete absence of central spindle elongation. Shortening of the chromosomal spindle fibers alone is associated with chromosome movement. In anaphase-B, there is no further shortening of the distance between the kinetochores and the tips of the elongate-centrioles. Anaphase-B chromosome separation proceeds hand-in-hand with central spindle elongation. Some cells are blocked at the end of anaphase-A and do not progress into anaphase-B but otherwise appear to be perfectly healthy. Other cells pass directly from anaphase-A into anaphase-B. Therefore, in *Barbulanympha,* anaphase chromosomes are moved apart by two spatially and temporally divisible events, chromosomal spindle fiber shortening and central spindle elongation.

In cells other than *Barbulanympha,* the involvement of these two events, either singly or in overlapping sequence, has long been recognized (e.g., in *Amoeba proteus,* 26; aphids, 34; newt fibroblast, 5; fungus, 2. For reviews, see 29, 35, 37). In polar body division of oocytes, chromosomes commonly separate by anaphase-A alone with no detectable anaphase-B. Moreover, Ris has shown, on grasshopper spermatocytes, that chloral hydrate can block spindle elongation without affecting chromosomal fiber contraction (35). Oppenheim, Hauschka, and McIntosh report a similar observation in tissue-cultured cells treated with colchicine (32). In such material, the two processes of anaphase-B and -A normally take place in overlapping sequence (32, 35). With the addition of the *Barbulanympha* example, we should now seriously consider the likelihood that anaphase-A and -B are driven by two distinct physiological or molecular mechanisms.

FIGURE 13 Nuclear cleft formation and anaphase-A anterior view. Same cell continued from Figs. 9 and 12. Smith T system differential interference contrast 40× objective adjusted to simultaneously display birefringence of central spindle fibers. (*a*), High focus. The nuclear seam now runs much of the distance between the two spindle poles (hold the page sidewise and sight between the tips of the arrowheads). Anaphase-A is progressing and chromosome ends are just separating. Several kinetochores have reached approximately one-half spindle diameter away from the poles. (*b*), Median focus of same stage. Triangles, optical section of nuclear envelope. Both elongate-centrioles are visible (*E*). "Fast" kinetochores (*Kf*) are not quite at centrosomal surface, "slow" kinetochores (*Ks*) lag behind. (*c*), High focus. In the middle of the seam, an opening cleft is seen as though with parting of lips [short arrows in (*c*), (*e*), and (*g*)]. Chromosomal arms have separated. (*d*), Median focus mid-anaphase-A. Many kinetochores have approached the centrosomal surface. Optical section of the nuclear envelope is clear adjacent to spindle surface (upper triangle) and on the outer face of the nucleus, where its shape is now concave (lower triangle). (*e*), High focus. The cleft is considerably wider than in (*c*), but the seam persists near the spindle poles. (*f*), Median focus of late anaphase-A. Chromosome arms are well separated. (*g*), High median focus. The cleft has advanced further. At a slightly lower focus, the inner aspects of the nuclear envelope appear as two arcs which do not reach the poles. (*h*), The cleft has now advanced past the median margin of the spindle. In the lower part of the picture, continuous lines resembling insect wings depict median optical sections of the nuclear envelope (triangles). Many kinetochores (*K*) have clustered at the surface of the centrosome but some "slow" kinetochores (*Ks*) are still lagging. h:min in time of day on 74g30. Bar, 20 μm. × 620.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Anaphase-A

At the light microscope level, we confirm Cleveland's observation that *Barbulanympha* chromosomal spindle fibers are generated by growth of astral rays. The astral rays attach to each kinetochore embedded in the persistent nuclear envelope, thus establishing a connection between the chromosome, centrosome, and centriole (e.g., 8).

At the fine-structural level, we still do not know how the microtubules, which make up the linear elements of each chromosomal fiber, arise. Growing astral microtubules may directly attach to the kinetochore, or they may interact and align with other microtubules growing from the kinetochore.

Be that as it may, the chromosomal spindle fibers are dynamic. While fibers of the mature central spindle and the spindle pole-to-pole distance remain unaltered, chromosomes are individually observed to both approach and recede from the spindle poles before and during early anaphase-A.

During the several minutes of poleward movement, the poleward pull on a kinetochore is manifested by the point protrusion of the nuclear envelope immediately surrounding that kinetochore. The chromosome arms within the nucleus follow as the particular chromosomal fiber shortens and the kinetochore moves poleward.

When the chromosome moves away from the pole, the kinetochore follows, lying at the pit of a dimple in the nuclear envelope.

As expressed by the cyclic point protrusion and dimpling of the nuclear envelope, the poleward force on each kinetochore varies independently and recurrently after the central spindle has reached its final, stable full length.

Therefore, the chromosomal fibers (astral rays) appear able to (*a*) repeatedly grow and lengthen; (*b*) repeatedly pull the chromosomes poleward; and (*c*) possibly, repeatedly establish new contact with a single kinetochore, thereby establishing new kinetochore-to-pole connections.

The forces exerted on the kinetochores by chromosomal spindle fibers may be produced directly by polymerizing or depolymerizing micro-

FIGURE 14 Anterior view of birefringent central spindle and astral rays in late anaphase-A [(*a*) to (*d*)] and early anaphase-B [(*e*) to (*h*)]. Rectified polarized light image continued from Fig. 9*e*. In (*a*), (*c*), and (*f*) the spindle is oriented at 45° to the polarizer axis. The slow axis of the Brace-Köhler compensator lies at various angles within the quadrant perpendicular to the spindle axis. Therefore, the elongate-centrioles and the (optimally compensated) less retarding regions of the central spindle, as well as those fibers parallel to the P-A axes, appear dark. Astral rays which point northerly or southerly appear bright, especially adjacent to the centrosomes where their microtubule density is high. In (*e*) the spindle axis also lies at 45° to the polarizer but the compensator slow axis is oriented parallel to spindle long axis. The central spindle fibers and the elongate-centrioles now appear bright. Astral rays at right angle to the spindle appear dark. Near each pole, the spindle is brightest where the microtubules converge to the centrosomal surface (cf. Fig. 5 in Part I). A less bright, positively birefringent cone connects this region to the tip of the centriole [also see (*g*), (*h*), and (*f*) in which the compensator orientation is reversed]. The intracentrosomal microtubules described in Part I are believed to contribute to the birefringence of this cone. Extent of centrosome is indicated by two arrows in (*g*). In (*b*), (*d*), (*g*), and (*h*), the spindle is oriented parallel to the polarizer axis so that spindle fibers and astral rays at any single spindle pole may appear bright or dark depending on their orientation and retardation. In (*b*) and (*d*), bright "eyes" appear in the dark NE and SW quadrants near the poles of the central spindle. Here, as along the spindle axis in (*a*), (*c*), and (*f*), the spindle retardation is too great to be (subtractively) compensated; microtubules are packed with a high density (cf. Fig. 5 in Part I) and they are oriented in a direction that introduces maximum alterations of the polarized light beam. In (*a*), the nucleus remains bell-shaped as seen in Fig. 12*c* to *f*. So long as the nucleus is thus shaped, the astral rays, except for those lying tangent to the nuclear envelope, fan out as seen here and in Fig. 9*e*. The rays which lie tangent to the nuclear envelope show clearly in (*b*), but not in (*a*), as they run parallel to the polarizer-analyzer axes in (*a*) and therefore contribute no contrast. As anaphase-A progresses and the shape of the nucleus changes (cf. Figs. 11 and 13), astral rays are pushed poleward as seen in (*b*) through (*h*). Concurrently, their birefringence decreases. (*e*) through (*h*) show the progressive splaying of the lateral spindle fibers as the nuclear cleft progresses caudad and exposes the lateral aspects of the central spindle in anaphase-B (cf. Figs. 13*h*; 11 bottom). The central spindle is growing in length and its birefringence is diminishing, initially anteriorly then progressively laterally and caudally. Nikon rectified 40× objective. h:min in time of day on 74g30. Bar, 20 μm. × 630.

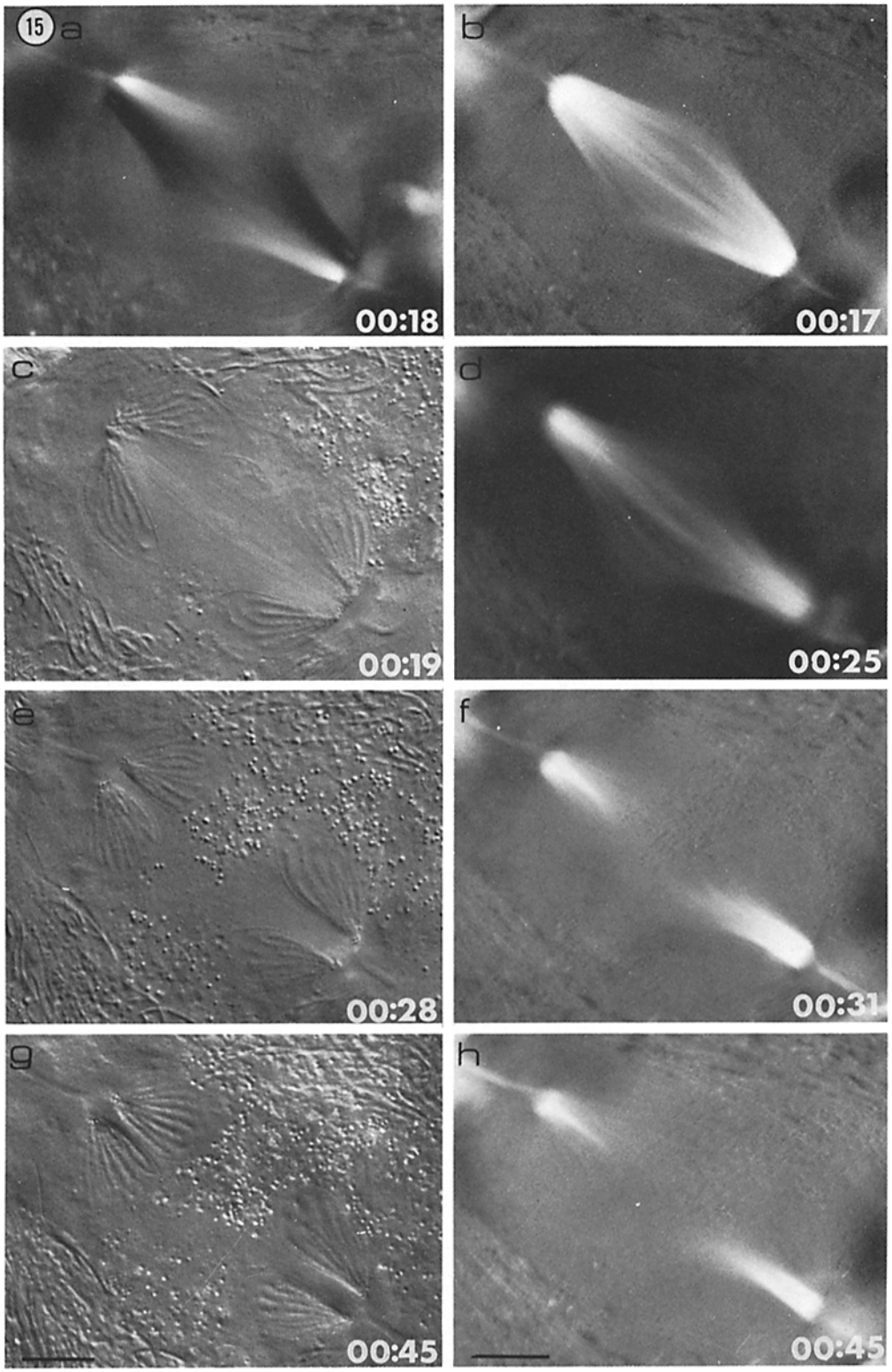

FIGURE 15 Anaphase-B continued from Figs. 13 and 14. The polarized light images, (*a*), (*b*), (*d*), (*f*), and (*h*) show progressive lateral splaying of the central spindle fibers and loss of their birefringence. The central spindle is elongating. Differential interference contrast images (*c*), (*e*), (*g*) show progressive separation of the nuclear envelope as the nuclear cleft traverses caudad (cf. lower diagrams in Fig. 11). In (*c*), at this low median focus, the nuclear envelope still appears connected; in (*e*) and (*g*), they appear separated. At the upper spindle pole in (*e*), note the clusters of kinetochores and their apposition to the centrosome around the tip of the elongate-centrioles. h:min in time of day on 74g30. Bars, 20 μm. × 600.

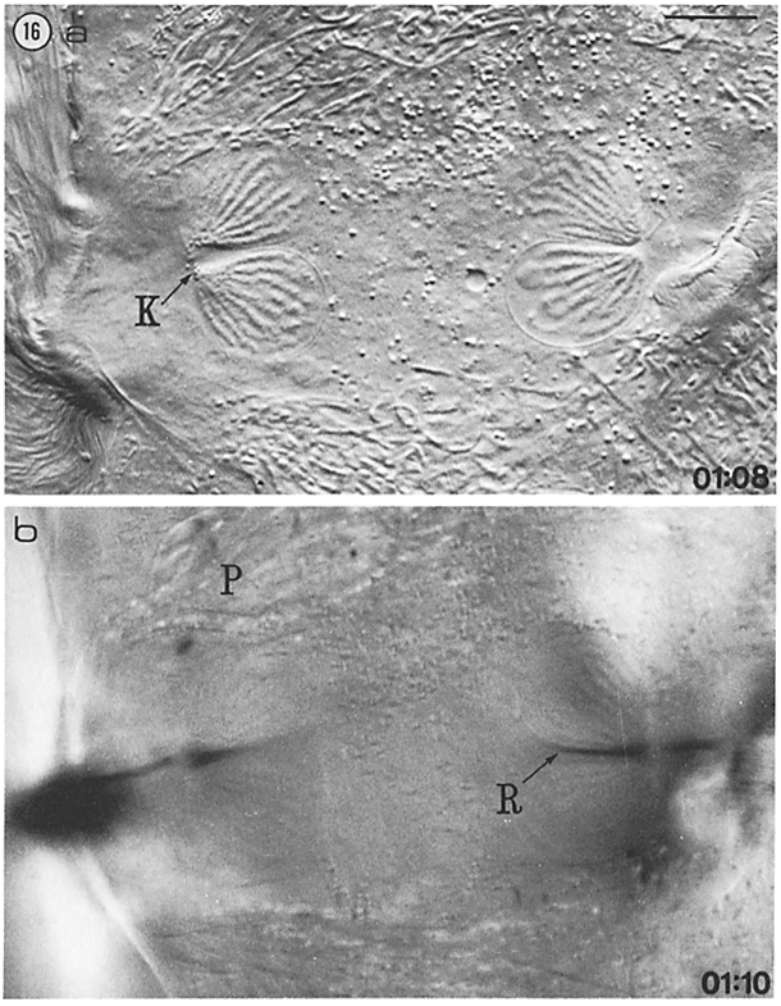

FIGURE 16 Late anaphase-B anterior view continued from Fig. 15. Karyokinesis is now complete (*a*). In differential interference contrast (*a*), the row of kinetochores (*K*) shows prominently at the left spindle pole. (For lateral view of kinetochore rows, see Fig. 6*b*, *c* left pole.) The shortening chromosome arms, nucleoli, and the wing-shaped optical section of the nuclear envelope are also clear. Parabasals and axostyles have regrown. Only a thin remnant (*R*) of the central spindle persists (*b*). In the polarized light image (*b*) the (negatively birefringent?) helical parabasals (*P*) appear as though rows of bright birefringent dots, and the dark, positively birefringent thin axostyles curve gently. At this stage, the flagella start beating vigorously again and contribute to cytokinesis. The new flagellar bundle (to the right) is externalized and only its basal structures are visible in this picture. h:min in time of day on 74g30. Bar, 20 μm. × 620.

tubules. Or, if there are two sets of microtubules, astral and kinetochoric, they may slide relative to one another. Alternatively, polymerizing microtubules may extend or align another component or components that subsequently draw the chromosomes poleward as the microtubules slowly depolymerize. We have already stressed the occurrence and need for microtubule depolymerization in anaphase-A generally (19–22). Because the dynamic changes take place after the central spindle is established, chromosomal fibers almost certainly could not contract as proposed by Cleveland (p. 27 in reference 12) by release of elastic energy stored in them through prior extension by the growing central spindle.

Polarization of Kinetochores

The polarization of kinetochores to opposite

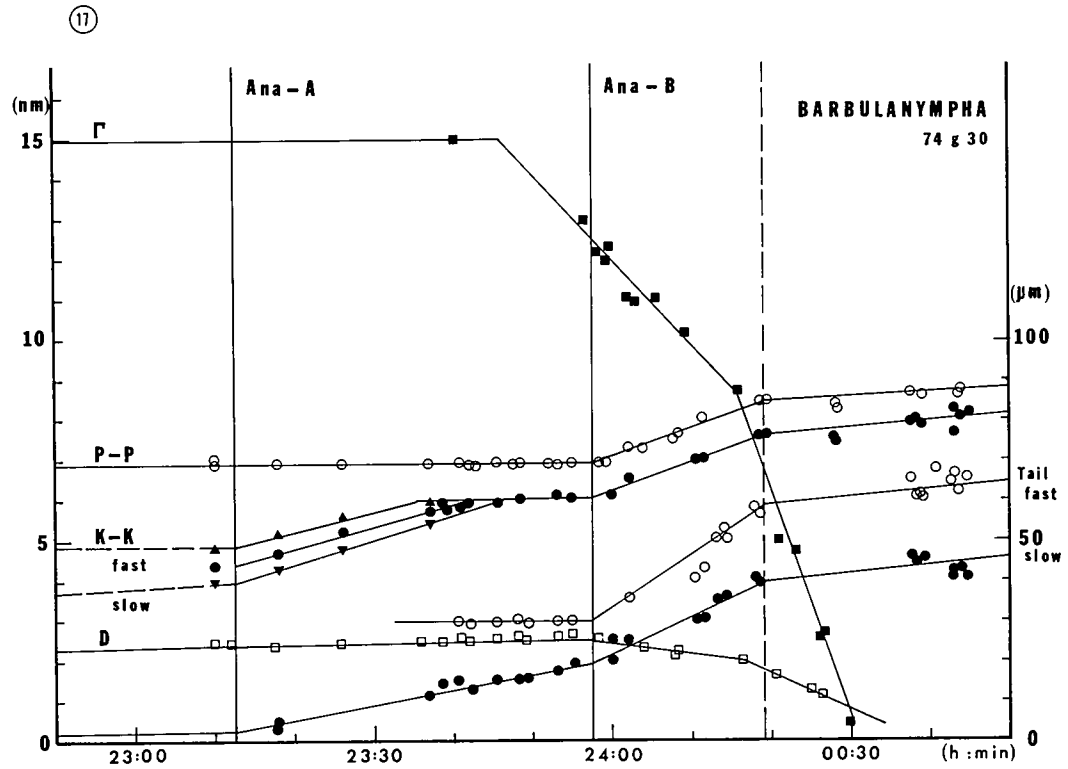

FIGURE 17 Birefringence retardation, dimensions of central spindle, and chromosome positions measured on cells shown in Figs. 9-16. Γ, retardation of spindle mid-region. *P-P*, spindle pole-to-pole length measured between distal tips of centrioles. *D*, width of unsplayed portion of central spindle. *K-K*, distance between kinetochores migrating to opposite poles, for "fast" and "slow" chromosomes. *Tail*, separation of tails of chromosome arms, for "fast" and "slow" chromosomes. Note: presumably because of compression of the cell which made possible recording of this fortuitous anterior view, the extreme elongation of the central spindle commonly found in anaphase-B was not seen. Instead, spindle elongation and chromosome movement slowed down after 00:19 h.

spindle poles is essential for successful completion of mitosis. In *Barbulanympha,* the mechanism for this important event cannot be understood by simple application of Östergren's otherwise elegant metaphase equilibrium hypothesis (31, 33). There simply is no normal metaphase stage in *Barbulanympha* mitosis, nor does Kubai's proposal (24) for kinetochore segregation in the *Trichonympha* nuclear envelope without aide of the astral rays appear applicable. Cleveland (Figs. 11 and 12 in reference 12) shows that where the astral rays fail to reach a nucleus, there is no tendency for the paired chromosomes or indeed their kinetochores to move apart. In the same cell, kinetochores in a second nucleus within reach of the asters do separate.

Cleveland further shows that a monopolar spindle is sufficient for kinetochores to separate from their sisters. After initial separation of kinetochores and chromatids, the chromosomes appear to all polarize more or less away from the single centrosome or from the tip of the elongate-centriole, somewhat reminiscent of movements described for the paternal set of chromosomes in the monopolar division of *Sciara* spermatocytes (30, 37).

Cleveland goes on to state (reference 12, p. 27) that "the anaphase daughter chromosomes – do not move to opposite poles when they have a choice of more than two poles (Fig. 10 of reference 12). When only two poles are present they have no choice; they must move to one or the other—". In a multipolar mitotic figure, the kinetochores may even polarize and separate towards two poles not joined together by a central spindle, and in a direction more or less at right angles to

the existing central spindles.

We view Cleveland's work on *Barbulanympha* to strongly suggest that (*a*) kinetochore separation does not take place in the absence of (astral) microtubules; (*b*) initial separation of kinetochores requires only the presence of astral rays and is not dependent on the presence of two centrioles or centrosomes; and (*c*) a bipolar spindle is essential for the orderly, functional segregation of kinetochores and chromosomes. A normal spindle provides the two poles required, and the central spindle fibers presumably function as support which keeps the two poles apart.

Anaphase-B

At the beginning of anaphase-B, the extending central spindle fibers push apart the bouquet of chromosomes formed at the end of anaphase-A. The kinetochores by then firmly crown the centrosomes. Short chromosomal spindle fibers persist throughout anaphase-B and hold the packed crown of kinetochores, the bouquet of chromosome, and nuclear envelope up against or close to the centrosomal surface.

Later in anaphase-B, the central spindle becomes highly attenuated. One may wonder whether such a slender structure could continue to push the daughter nuclei apart. Or might the spindle itself be passively drawn apart by the cortical cytoplasm extending between the two rostra bearing the flagellar tufts (Figs. 6, 7)? At times, the elongate-centrioles and central spindle are pulled into a straight line when the two-rostrate organism swivels its rostra. At other times, the elongate-centrioles and central spindle are each gently curved and no tension on them is apparent. During the later stages of anaphase-B, the central spindle continues to elongate and the rostra and flagellar tufts separate as though the two events were proceeding at a parallel pace but not because they were mechanically coupled together. According to Cleveland (11), the central spindle of related hypermastigote *Macrospironympha* also attenuates and elongates extensively, but in the absence of cortical changes or any other indication of extensile forces acting on the poles of the spindle that are embedded deep in the cytoplasm (also see reference 26). It seems most likely that throughout anaphase-B the central spindle actively elongates and "pushes apart" the chromosome bouquets.

The birefringence retardation of the *Barbulanympha* central spindle decays in anaphase-B, more or less proportionately with the width of the central spindle. The coefficient of birefringence, or the packing densities of microtubules (number per unit spindle cross section, see reference 36), thus remains approximately constant as the spindle remnant becomes longer and thinner. These data for anaphase-B are not incompatible with the proposal that central spindle fibers extend by the sliding of microtubules. However, the morphology and degree of extension (as much as five times) of the central spindle suggest that the extension cannot be brought about by the relative sliding of two sets of nongrowing microtubules each anchored at their poles (28). Instead, the central spindle microtubules must be telescoping or are in fact growing longer. In other words, anaphase-B in *Barbulanympha* appears to be brought about by (*a*) the growth of central spindle microtubules per se, (*b*) microtubular growth coupled with their sliding, or (*c*) telescopic sliding of microtubules.

In thin-section electron micrographs of the anaphase-A spindle of *Barbulanympha* taken by Dr. Kubai, we have demonstrated by a new optical enhancement method the presence of extensive intermicrotubular periodic structures (21). Because the anaphase-A spindles are extremely stable in dimension and birefringence, these intertubular structures may well be structural linkers of the type found between axopodial microtubules in heliozoa (23, 40) and within the rows of hypermastigote axostyles (15, review in 27). We find that *Barbulanympha* central spindles in anaphase-A do not show cold lability (down to 4°C) as is often found in spindle fibers in cells of other organisms (e.g., 18, 19, 22). Generally, the nonkinetochoric microtubules such as the "continuous" central spindle fibers are more sensitive to cold or colchicine and depolymerize faster than kinetochore microtubules (3, 7, 13).

Instead of being stable linkers, some of the intertubular structures in *Barbulanympha* might be arms, such as dynein, involved in force production. They may then generate relative sliding of central spindle microtubules, telescoping in a manner similar to the trypsin-treated flagellar axonemes demonstrated by Summers and the Gibbons (14, 38, 39). Establishment of the nature and functional role of this intermicrotubular material in the gigantic central spindle of *Barbulanympha* may well resolve the question generally of whether central spindle elongation, i.e. anaphase-B, can or does take place by sliding in addition to growth of microtubules.

Morphogenesis of Nuclear Envelope

The morphogenetic movement of the nuclear envelope we recorded may at first appear unique. In fact, however, this may reflect a general pattern of karyokinesis in evolutionarily embryonic eukaryotes. In prokaryotes, the daughter DNA strands are thought to be carried along by parting mesosomes connected to the cell membrane. The mechanism of mesosome partitioning itself is not clear. As reviewed by Kubai (25), mitosis in many dinoflagellates, lower algae, and hypermastigotes is believed to be mediated by kinetochores, or functionally equivalent regions of chromosomes embedded in or attached to the nuclear envelope. As discussed above, polarization of *Barbulanympha* kinetochores is governed by microtubule bundles. If the association of microtubules with kinetochores in the nuclear envelope reflects an early evolutionary event, we may in *Barbulanympha* witness an ancient experiment of nature. Despite the "permanence" of the nuclear envelope, the pattern of mitosis involving the shortening chromosomal fibers and elongating central spindle is essentially that seen in "orthomitosis" (17) of "higher" organisms. The latter may have abandoned the complex maneuver necessitated by the persistence of the nuclear envelope in a multichromosomal organism.

The nuclear seam that we describe is pictured in some of Cleveland's drawings, although without comment. On the other hand, the appearance and opening of the cleft appears never to have been noticed before our report. The later stages of karyokinesis depicted by Cleveland (9) and by Hollande and Valentin (16) are nevertheless totally consistent with the mode of karyokinesis via cleft expansion that we describe.

The deformation of the nuclear envelope during anaphase-A and -B, including the point protrusions, recesses, and the overall shapes (also see reference 10), suggests that the nuclear envelope possesses an apparent elasticity. In fact, the envelope may well be a constrained interfacial membrane. Interfacial membranes such as soap bubbles do give the impression of possessing elasticity. A constrained "hanging drop" or "rising bubble" takes on a bell shape similar to that of the *Barbulanympha* nuclear envelope (reference 1, pages 365–367; also, see reference 6 for an illuminating discussion on the properties of soap bubbles). In addition to the apparent elasticity, the movements of embedded kinetochores suggest a fluidity to the nuclear envelope. Many aspects of the shape change observed in the *Barbulanympha* nuclear envelope as well as its fluidity in two dimensions can be seen or inferred by comparison with patterns exhibited by soap bubbles.

As discussed earlier, there is little doubt that shortening chromosomal fibers acting on individual kinetochores are responsible for the poleward point protrusion of the nuclear envelope and for the associated chromosome movement. On the other hand, the dimples away from the pole may reflect (*a*) a drop of poleward tension and concomitant elastic recoil of the undissociated chromatid pairs, (*b*) an active extension and push by chromosomal spindle fibers, or (*c*) a disequilibrium resulting from a greater tug on the oppositely directed kinetochore.

The sharp protrusions found at each poleward apex of the nuclear seam (Figs. 9*a*, *d*, 12*a*, *b*, and 13*a*) suggest that the seam may also be formed by poleward forces acting on the kinetochores. The recurrent movement of the prophase and early anaphase kinetochores suggests that new astral rays may sequentially attach to the kinetochores. The poleward forces exerted on the kinetochore by the shortening astral rays (chromosomal fibers) would be counteracted by a radial resistive force on the nuclear envelope arising from the presence of the central spindle and of the chromosomes within the nucleus. The balance of poleward and radial forces would eventually wrap the nucleus around the spindle. Where the nucleus abuts itself, a seam would appear.

Likewise, it is not unlikely that the opening of the cleft also results from the interplay between (*a*) poleward forces acting on kinetochores, (*b*) the tendency of the chromosome bouquet to resist collapse of the nuclear envelope, (*c*) radial support provided by the central spindle, and (*d*) the tendency of the elastic nuclear envelope to maintain, with minimum nuclear volume change, a minimum surface area in the presence of these constraints.

Central Spindle Splaying

We observed the splaying of the central spindle which progressed concurrently with, and conformed with the shape of, the opening nuclear cleft and its posteriad extension. The elongating (or sliding) microtubular bundles may be bulging laterally as a constraining girdle is released or, in turn, spindle fiber splaying may contribute to the

formation of the cleft. In either case, the integrity of the early anaphase-A spindle, before envelopment by the nucleus and formation of the seam, suggests that there is a cellular mechanism that operates independently to control the lateral affinity of spindle microtubules (also see discussions by Bajer and Molè-Bajer, 4).

Cytokinesis

The astonishing observation that *Trichonympha* assists the cytokinesis of *Barbulanympha* appears not to be accidental. *Trichonympha* have repeatedly been observed to poke their rostra specifically against the mid-region of the late anaphase-B *Barbulanympha* as though attracted to the region. One of us (H.R.) has on several occasions seen a *Trichonympha* literally ram through the two-rostrate *Barbulanympha,* completing the *Barbulanympha's* cytokinesis. For organisms which must have attained obligatory symbiotic relationships so long ago, perhaps we should not be surprised at the occurrence of even such an amazing mutualism.

Concluding Remarks

In conclusion, extended observations of individual *Barbulanympha* cells in mitosis is now possible. Sequential through-focus observations with differential interference contrast and rectified polarized light microscopy have provided insight into aspects of mitosis that had not been revealed in other species in such complete detail. In addition to points of interest for mitotic mechanisms generally, the unique features displayed in this presumably ancient organism may shed light into the evolution of mitosis. Some striking features displayed in *Barbulanympha* suggest intriguing opportunities for further experimental studies of mitosis utilizing this unusual but revealing cell type.

Received for publication 29 July 1977, and in revised form 3 January 1978.

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