

BEHAVIOR OF KINETOCHORES DURING MITOSIS IN THE FUNGUS *SAPROLEGNIA FERAX*

I. BRENT HEATH

From the Biology Department, York University, Downsview, Ontario M3J 1P3, Canada

ABSTRACT

In rapidly growing hyphae of *Saprolegnia ferax*, all nuclei contain arrays of kinetochore microtubules, which suggests that the nuclei are all in various phases of mitosis, with no apparent interphase. In prophase nuclei, kinetochore microtubules form a single, hemispherical array adjacent to the centrioles. This array separates into two similar arrays after centriole replication. The two arrays form by separation of the initial group of microtubules, with no kinetochore replication. During metaphase, between 6.5 and 85% of the kinetochores occur as amphitelic pairs, with a slight tendency for pairing to increase as the spindle elongates. 100% pairing has never been observed. The interkinetochore distance in these pairs is consistently $\sim 0.17 \mu\text{m}$. Throughout metaphase and early anaphase, there is extensive and increasing diversity in kinetochore microtubule length, so that a true metaphase plate has not been found. During metaphase, anaphase, and telophase, kinetochore numbers vary considerably, with a mean of ~ 30 per half spindle. A number of artefactual causes for this variability were examined and discarded. Thus, these results are accepted as real, suggesting either variable ploidy levels in the coenocytic hyphae or kinetochore replication during mitosis.

KEY WORDS mitosis · kinetochores · fungi

The fundamental mode of operation of a universal process such as mitosis may be understood by looking for common denominators among as many variants as possible. The oomycete fungi are a group of eukaryotes that have an uncertain phylogenetic position but that undoubtedly are less complex and, thus, perhaps, less highly evolved than many plants and animals. Previous work on these organisms has revealed a mitotic system that is undoubtedly simpler than that of many other organisms, but that has many features in common with most mitotic systems (5, 11, 12). The value of data from these studies for the overall understanding of mitosis has been discussed elsewhere (2, 4, 7, 10). However, whereas it was clear from these studies that the behavior of the chro-

matin and kinetochores is unusual, this behavior was not fully explored. The purpose of this report is to more accurately describe these aspects of mitosis and, in doing so, to draw attention to interesting but neglected aspects of mitosis in some lower organisms. Preliminary accounts of this work have been published previously (8, 9).

MATERIALS AND METHODS

The observations reported here are of the previously used isolate of *Saprolegnia ferax* (Gruithuisen) Thuret, which was isolated in 1950 (14) and has since been maintained by vegetative subculture. Subcultures are deposited in the Aquatic Phycomycete Culture Collection at Reading University and in the American Type Culture Collection (ATCC 36052). Hyphae were grown in Petri dishes containing ~ 10 ml of liquid organic medium (OM) (11) at 25°C for ~ 24 h.

For electron microscopy, colonies were removed from the incubator, left on the laboratory bench for ~ 1 h, then fixed by

rapidly adding ~10 ml of isothermal 5% glutaraldehyde in 0.067 M phosphate buffer, pH 7.0. After 5 min in this mixture, colonies were transferred to fresh glutaraldehyde for 55 min followed by rinsing in the 0.067 M phosphate buffer, postfixation in buffered 1% OsO₄ for 1 h, dehydration in ethanol, and embedment, via propylene oxide, in Epon 812. Individual hyphal tips were selected from the blocks with the aid of Nomarski interference contrast microscopy. Only hyphae that had the same morphology and cytoplasmic organization (at the level of detectability of a × 10 objective lens) as similarly observed living tips from the margins of growing colonies were selected for sectioning. Blocks were trimmed to contain single hyphal tips that were oriented parallel to the block face. Serial sections were collected on single slot grids, stained with 2% aqueous uranyl acetate at 60°C for 20 min followed by 2.84% aqueous lead citrate for 5 min at 25°C.

Spindles were analyzed from prints of serial longitudinal sections using transparent overlays to track from one section to the next. When differential distortion occurred between adjacent sections, cytoplasmic and nuclear markers as close to the structure of interest as possible were used to identify the area of interest. Although spindles that were highly oblique to the plane of section were discarded, very few series were oriented in such a way that their long axes were completely parallel with the plane of section. As a measure of the degree of obliquity of the spindle long axis relative to the plane of section, an "obliquity factor" was calculated as follows: the pole-to-pole length of the spindle (in μm) was divided by the number of sections lying between the sections that contained the center point of each pair of centrioles multiplied by an "average" section thickness of 0.08 μm. Thus, a spindle with an interpolar distance of 2.0 μm and the center points of the centriole pairs lying in sections one and four would have an obliquity factor of $2.0 / (2 \times .08) = 12.5$. Spindles with the center points of both centriole pairs lying in the same section had a value of infinity and were omitted from the correlation calculations. The pole-to-pole length of the spindle was measured between the center of each "pocket" region of the nuclear envelope in metaphase and later spindles and between the center points of the two centriole pairs during prophase. Simple geometry was used to correct for the obliquity of the plane of section when necessary.

When counting kinetochores and determining the extent of pairing of kinetochores, it was important to analyze all of the series at the same time because of the somewhat subjective nature of the identification of kinetochores. As one becomes more experienced in looking at these spindles, one becomes more able to recognize obliquely sectioned kinetochores. Also, using clearly visualized kinetochores as indicators of where their mates might be, it was often possible to detect kinetochores that otherwise might have been overlooked. Thus, the numbers and pairing data were determined simultaneously, and all of the spindles were analyzed over a continuous sequence of days. To determine the number of paired kinetochores in a spindle, each kinetochore detected was examined to see whether there was a kinetochore facing the opposite direction within 0.2 μm (as measured along the long axis of the spindle) either in the section containing the detected kinetochore or in the immediately adjacent section. If there was a paired kinetochore, the interpair distance was measured from the prints, with the measurement running between the points indicated in Fig. 6. The decision as to the presence or absence of a pair was initially made on a subjective basis only; the measurement was made after the pairing decision. Statistical analysis of the data was based on the correlation coefficient (*r*) program of a Texas Instruments SR-51-II calculator (Texas Instruments Inc., Digital Systems Div., Houston, Tex.). Standard

deviations are given in the text as ± numbers after the means.

Part of the spindle analysis data was obtained during work on antimetabolic agents in *S. ferax* (6). The treatments used were: incubation at 25°C in OM containing camphor (100 μg/ml for 48 h), colchicine (10 mM for 10 min, 24 h, and 24 h followed by 24 h in OM without colchicine), and colcemid (2 mM for 24 h and 5 mM for 0.25 h) and a pretreatment of 3°C for 1 h, all before fixation as described above.

RESULTS

Outline of Mitosis

The general features of mitosis in *S. ferax* and the closely related and very similar *Thraustotheca clavata* have been described previously (5, 11, 12) but need to be outlined here for orientation. Each nucleus is accompanied by a pair of centrioles that are aligned parallel to each other. These centrioles lie adjacent to a variously indented, differentiated region of the nuclear envelope termed the "pocket." At the onset of mitosis, pocket and centriole replicate to produce two pairs of adjacent centrioles, each with a pocket. Centriole pairs and their pockets move apart as the intranuclear spindle microtubules form between the pockets. Initially, the kinetochore microtubules form first one, then two, hemispherical arrays around the pockets as described below. Subsequently, they and the nonkinetochore microtubules reorient themselves to form a typical biacuminate spindle. All stages before the formation of this spindle are defined as prophase. Once formed, the spindle elongates, with the kinetochore microtubules elongating to maintain the position of many of the kinetochores in the general vicinity of the equator of the spindle. As in higher organisms, the kinetochores move to the spindle poles during anaphase and telophase. Metaphase is defined as all stages of spindle elongation after prophase and before all kinetochore pairs separate. Anaphase is defined as the stages of spindle elongation in which the kinetochore pairs have all separated but have not come to within ~0.5 μm of the spindle poles. During anaphase and into telophase, spindle elongation continues to yield an elongated and increasingly medianly constricted nucleus. Telophase is defined as the stages after which the kinetochore microtubules have shortened to <0.5 μm. In favorable series of sections, the complete nucleus can be traced to both poles with a very narrow isthmus containing the nonkinetochore microtubules. However, in the present study, any nucleus that contained the short kinetochore microtubules clustered around the pair of centrioles, and which also

had a clear projection filled with nonkinetochore microtubules such as would either be produced in an incomplete series through the isthmus or would remain immediately after complete constriction and severance of the isthmus was considered to be a telophase nucleus. Because telophase nuclei parallel the long axis of the hyphae, they also lie approximately parallel to the plane of section. Thus, when a "tail" was present it probably was detected. However, it must be pointed out that the centriole-kinetochore cluster of a prophase nucleus would be very similar to that of a telophase nucleus, and there is a possibility that some telophase tails were missed. Thus it is conceivable that a few of the nuclei designated prophase were in fact in telophase.

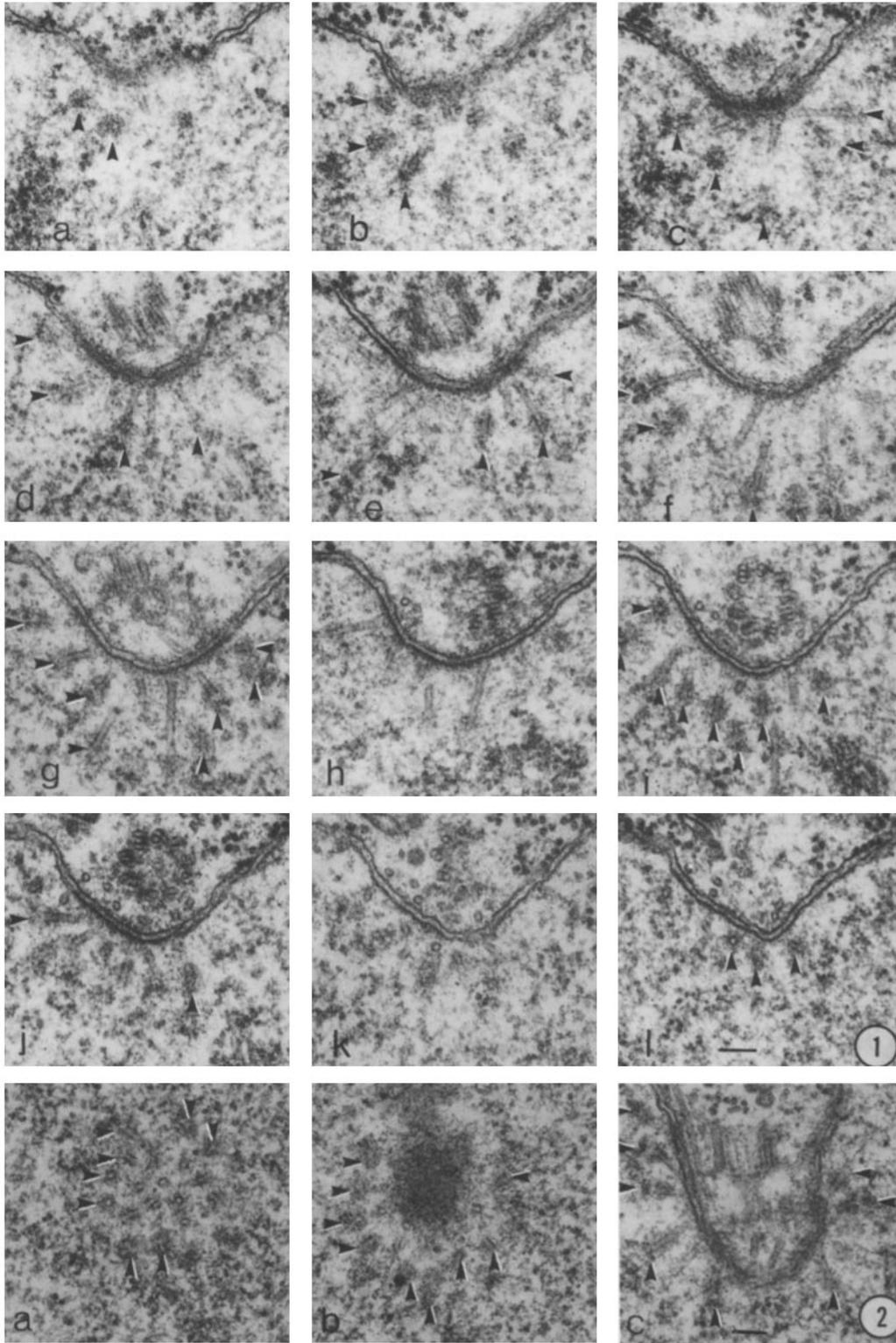
Kinetochores

Because the structures described as kinetochores are so small and poorly defined, some justification should be made for considering them as such. Kinetochores are characteristic terminations of spindle microtubules whose morphology is hard to accurately describe, but they can be seen in Figs. 1-4. This morphology did not appear to alter throughout mitosis. The behavior of these structures during metaphase, anaphase, and telophase, as outlined above, is generally similar to the behavior predicted for kinetochores in any mitotic system. Furthermore, at least in *T. clavata*, their numbers come close to those predicted on the basis of chromosome numbers, if one assumes a one-to-one relationship (5). In the interest of increasing sample sizes, the following discussion treats the results from the various antimetabolic treatments and the controls as a single population. This approach is justified because simple analysis of means and standard deviations for the various treatments shows no difference in kinetochore numbers among the populations. It must be emphasized that this result is contrary to the data presented in reference 11, where it was erroneously suggested that antimetabolic agents induced elevated kinetochore numbers. The reason for this error is that the data in reference 11 was accumulated over a period of time, with the controls being analyzed long before the treatments. As described in Materials and Methods, it was important to analyze all spindles at one time. When I did this I found that there is no increase in kinetochore numbers following treatment with antimetabolic agents. Because the detectability of kinetochores varies with the

obliquity of the plane of section with respect to the long axis of the kinetochore microtubules and also with varying levels of background staining material in the nucleoplasm, only spindles sectioned close to longitudinally and with kinetochores that were well contrasted were analyzed in detail. The results of this analysis are shown in Table I and Figs. 7-10.

KINETOCHORE ARRANGEMENTS: During prophase, kinetochores are located on the end of very short microtubules (mean length, $\sim 0.1 \mu\text{m}$, Figs. 1-5). These kinetochore microtubules are arranged in a hemispherical array with their polar ends adjacent to, but not directly adjoining, the pocket region of the nuclear envelope (Fig. 1). This single array may be found adjacent to either one or two pairs of centrioles (Figs. 1-3), which presumably indicates that centriole replication occurs during prophase while the kinetochores are still in a single group. Although there is a clear indication of a minimum center-to-center spacing of kinetochores within these single groups (Fig. 2), there is no detectable pattern that would indicate either that the kinetochores occur in side-by-side pairs, or that, in fact, the single group is composed of two separate groups. However, after the formation of two pairs of centrioles, never before centriole replication, the kinetochore microtubules do become separated into two distinct groups that form two adjacent hemispherical arrays (Figs. 4 and 5). In some cases, these arrays overlap to a considerable extent, so that they cannot be clearly delineated, whereas in other cases, presumably later stages, they are unambiguously separated. Subsequent to the formation of these side-by-side arrays, there is a major reorientation, so that the kinetochores come to form two interdigitating arrays that diverge from opposite poles and face one another to form a metaphase spindle (Fig. 6). This rearrangement occurs in the absence of any significant further separation of the centriole pairs because, as can be seen in Table I and Figs. 7 and 8, there is overlap in the intercentriole distances between prophase and early metaphase. This reorientation does not affect all kinetochores simultaneously because, during the early phases of metaphase, there are often a few kinetochores that do not face the opposite spindle pole but lie at right angles to, or even face away, from the spindle.

Throughout metaphase there is considerable variation in the length of the kinetochore microtubules, so that the kinetochores do not form a



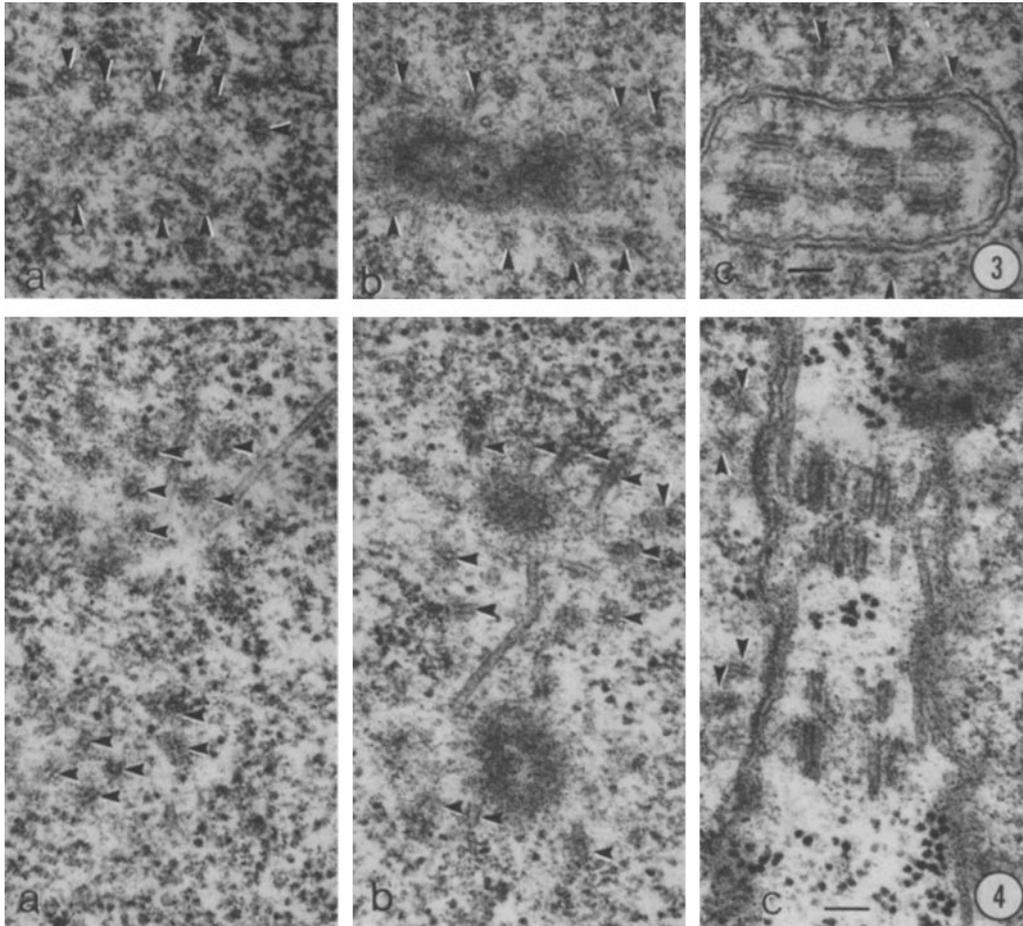


FIGURE 3 Sections 3, 4, and 9 from a series of 16 through a prophase nucleus (F31 in Table I) in which centriole replication has produced two pairs of centrioles (c), whereas the kinetochores (arrowheads) still form a single array. Bar, $0.1 \mu\text{m}$. $\times 57,000$.

FIGURE 4 Sections 3, 5, and 9 from a series of 12 through a prophase nucleus (A7 in Table I) in which centriole migration has begun (c) and the kinetochores (arrowheads) form two adjacent arrays (a and b). Bar, $0.1 \mu\text{m}$. $\times 60,000$.

well-defined equatorial group but instead are located over most of the length of the spindle (Fig. 6). The mean kinetochore microtubule length and the maximum length both increase at the same

rate as the elongation of the spindle (Fig. 7). Furthermore, there is a marked lag during which some of the kinetochore microtubules do not elongate to any significant extent relative to their

FIGURE 1 Sections 4–15 from a series of 21 through the centriole region of a prophase nucleus (A8 in Table I). Adjacent to the single pair of centrioles is a single array of kinetochore microtubules, the kinetochores of which are marked by arrowheads. Bar, $0.1 \mu\text{m}$. $\times 57,000$.

FIGURE 2 Sections 2, 3, and 7 from a series of 12 through a prophase nucleus (F26 in Table I). The single array of kinetochores (arrowheads) is seen in face view in a and was adjacent to a single pair of centrioles, part of which is seen in c. Bar, $0.1 \mu\text{m}$. $\times 57,000$.

prophase lengths (Fig. 7). This lag lasts until the spindle is $\sim 2 \mu\text{m}$ long, at which time all of the microtubules appear to start elongating in step with the elongation of the spindle (Fig. 7). Using standard deviation as an indicator of the variability in the length of the kinetochore microtubules at each stage of metaphase, it is clear that there is an increase in their variability during metaphase (Fig. 7; $r = 0.868$). However, because, in the later stages of metaphase, the shortest kinetochore microtubules have elongated, and the spindle itself is longer, the variability expressed as percent of spindle length is less (Fig. 7; $r = 0.758$), thus giving the impression of a more compact equatorial grouping of kinetochores toward the end of metaphase. In all 24 metaphase figures listed in Table I, and, in all other metaphase nuclei examined in less detail during observations of this species (a total of between 100 and 200), there has never been a true metaphase plate as seen in higher organisms and most chytridiomycete fungi (listed in reference 11).

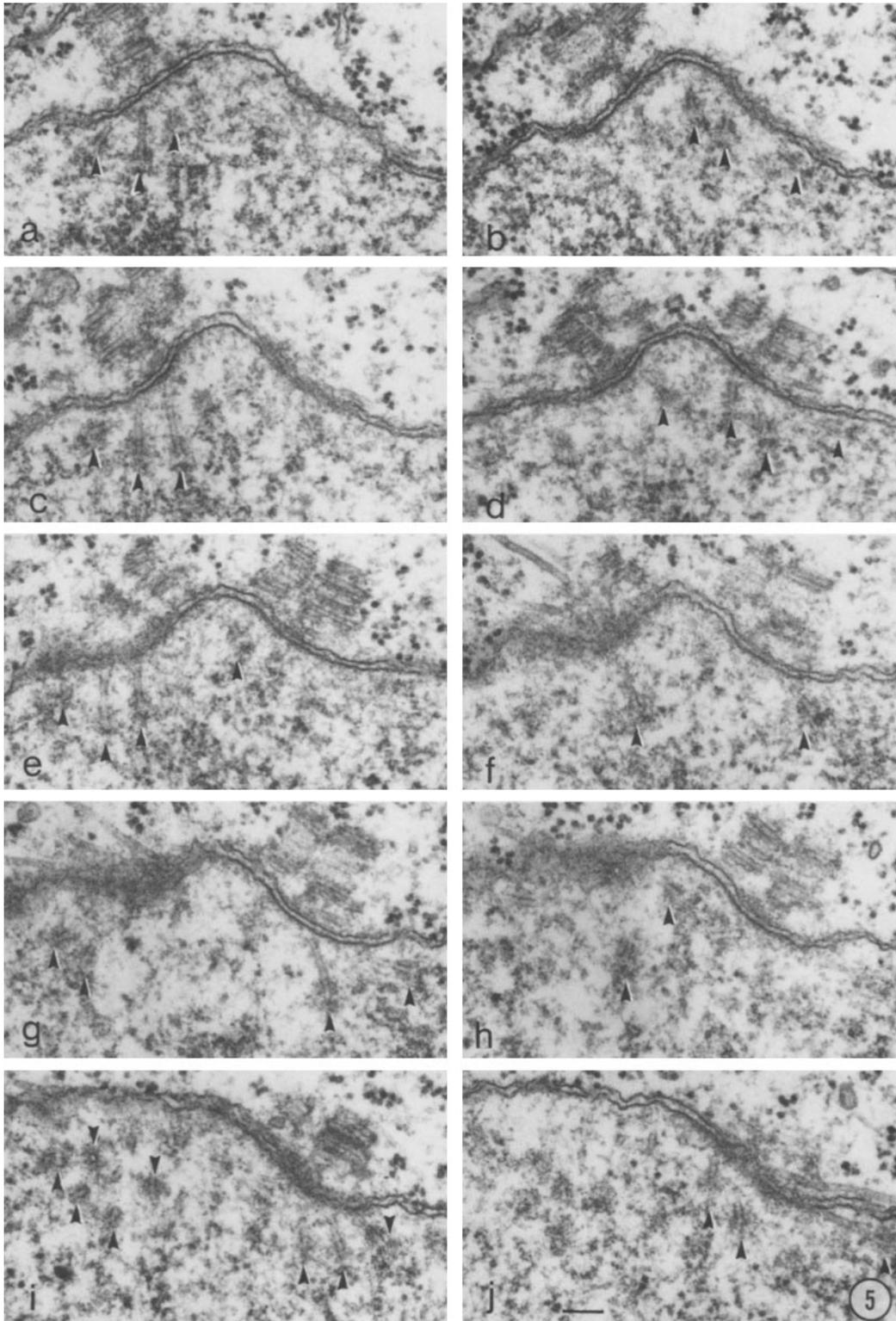
Anaphase is a relatively rare stage in sectioned material, indicating that it is a brief phase. Because there is overlap in the spindle lengths of metaphase and anaphase (Fig. 7), one might suspect the absence of a well-defined transition signal. However, the two metaphase spindles that overlap anaphase the most ($3.1 \mu\text{m}$ and $3.7 \mu\text{m}$, Fig. 7) are derived from cold-treated cells. These spindles may become longer than untreated metaphase spindles (I have insufficient data to confirm or deny this possibility), thus the range of spindle length in the overlap region may normally be lower than is indicated in Fig. 7. As might be expected, the variation in the kinetochore microtubule lengths in early anaphase (defined as such by having mean kinetochore microtubule lengths close to the metaphase lengths, i.e., spindles with interpole distances of $2.7 \mu\text{m}$ and $4.0 \mu\text{m}$ in Fig. 7) is comparable to that found in late metaphase, but, by late anaphase ($3.3 \mu\text{m}$ cell in Fig. 7), the variation has become substantially reduced. By late telophase, the kinetochore microtubule lengths are the same as found at prophase.

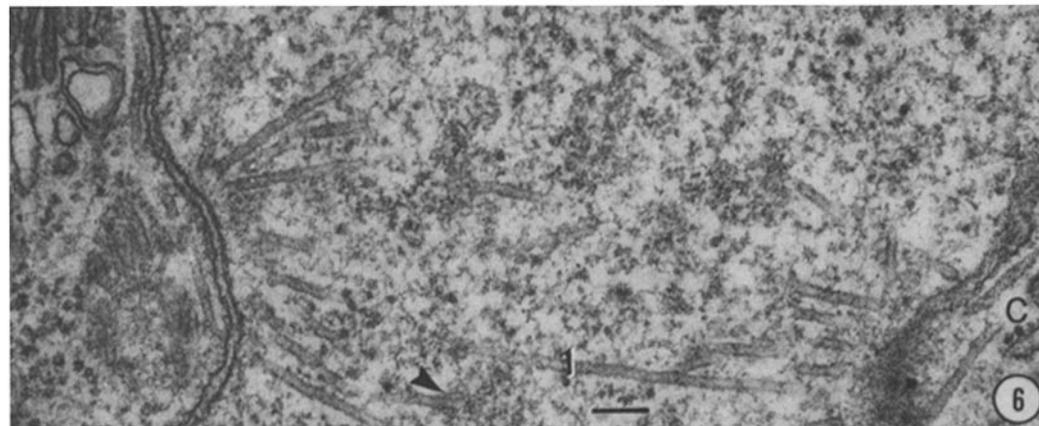
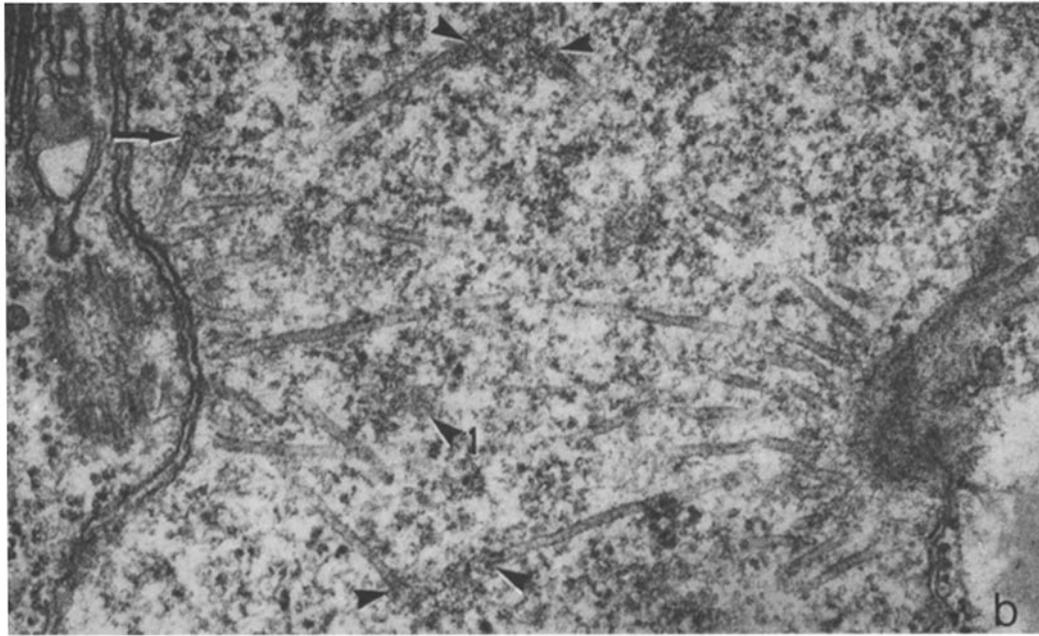
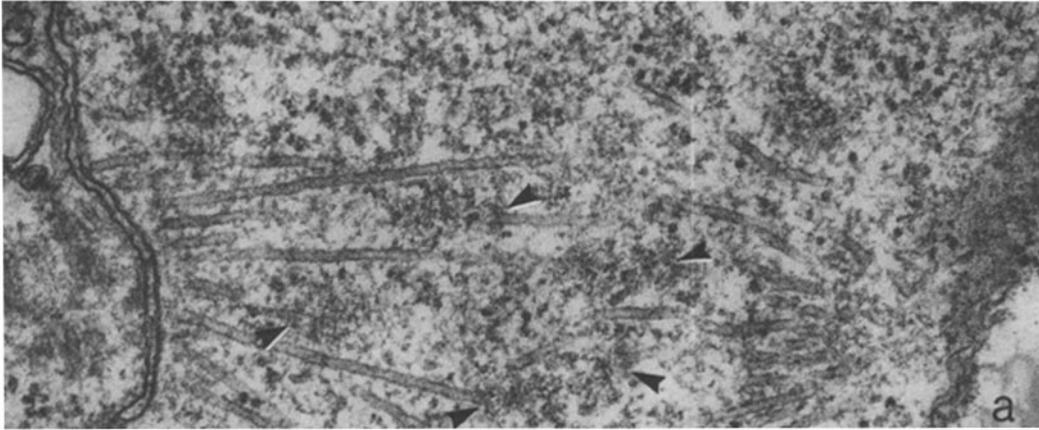
KINETOCHORE NUMBERS: The number of kinetochores found in various stages of division is shown in Table I and Fig. 8. Before biological

conclusions can be drawn from this data, the possible technical sources of error must be considered, inasmuch as there is extensive variability in the data. Technical problems that could give false values are as follows:

Because obliquely sectioned kinetochores are harder to identify than others and because the plane of section with respect to the spindle long axis (and, thus, also with respect to the average kinetochore) varied, one might expect that the number of kinetochores detected would co-vary with the "obliquity factor." Graphical and correlation coefficient analysis showed no such co-variation, thus eliminating plane of section as an explanation of the diversity. Kinetochores are more easily detected at higher magnifications. Thus, variation in magnification of the micrographs analyzed may explain the variability, but again this potential source of error was ruled out by graphical and correlation coefficient analysis. Because the overall "quality of fixation," primarily the level of "background" material preserved in the nucleoplasm, affects the detectability of kinetochores, this problem could explain the observed variability. This hypothesis was tested on the rationale that, if kinetochores could not be adequately detected because of fixation problems, then not only the absolute number, but also the percent of paired kinetochores (see below), would be reduced because one would fail to detect the other number of a pair in a significant number of cases. The correlation coefficient for this comparison (number vs. percent paired) was 0.49, suggesting that there is perhaps some validity to this hypothesis but clearly indicating that all of the variability in either the number or the percent paired is unlikely to be the result of fixation problems. This conclusion is supported by the observation of considerable variability in what were subjectively judged to be equally "well-fixed" nuclei. No other sources of "experimental error" were considered or tested. Because, in general, there is good agreement between the number of kinetochores in each half spindle (the average difference between half-spindle values of 10%, based on a mean difference between half spindles, is 3.35 ± 2.98 , and the mean number of kineto-

FIGURE 5 Sections 5-14 from a series of 19 through a prophase nucleus (A13 in Table I) in which centriole migration has begun and the kinetochores (arrowheads) have separated into two adjacent arrays. Bar, $0.1 \mu\text{m}$. $\times 60,000$.





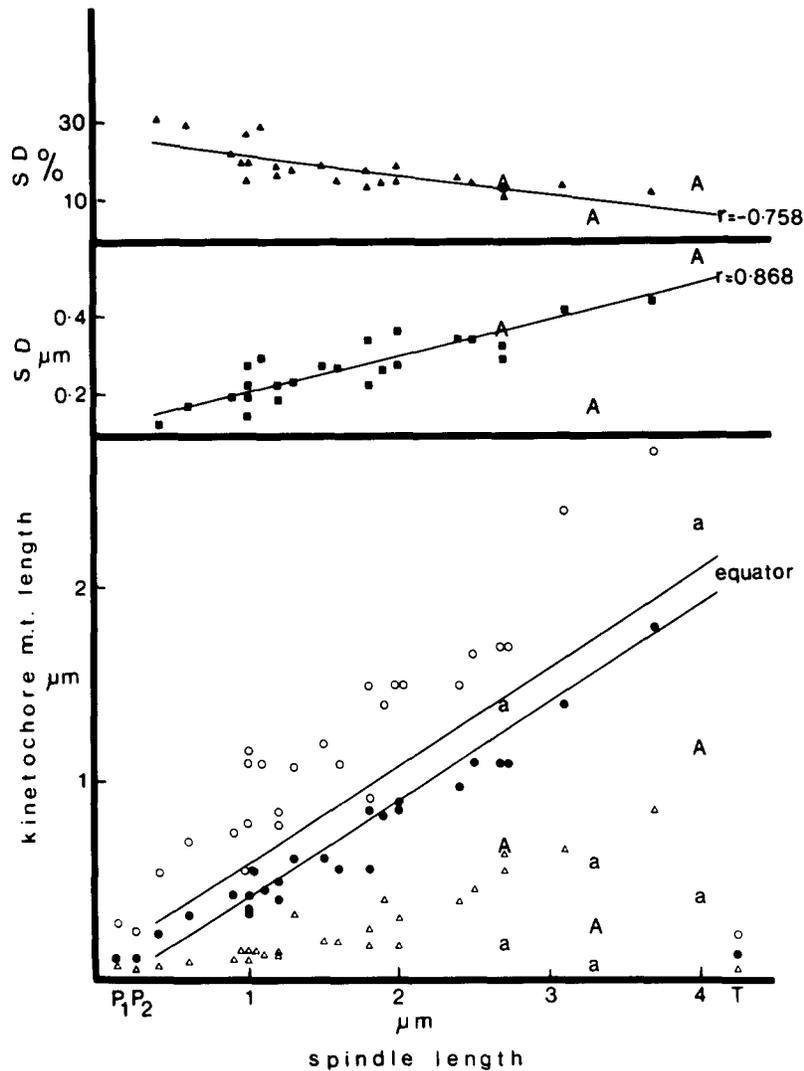


FIGURE 7 The behavior of kinetochore microtubules during mitosis. In the lower panel kinetochore microtubule lengths are plotted as mean (●), maximum (○), and minimum (△) lengths measured from the median sections of each spindle with pole-to-pole distances shown on the abscissa. The data for prophase spindles with single and double groups of kinetochore microtubules is designated by P_1 and P_2 , respectively, for telophases by T and for anaphases by A and a . The spindle equator is defined by the center point of the spindle, plus and minus half of the average interkinetochore pair distance derived from Table I. In the upper panels, the standard deviation of kinetochore microtubule lengths are expressed as a percent of the spindle lengths and as an absolute value in μm . The correlation coefficients (r) of the metaphase values (▲ and ■) were calculated exclusive of the anaphase values (A).

FIGURE 6 Three sections from the series through an early metaphase nucleus (F26 in Table I). Arrowheads indicate kinetochores that were judged to be paired with other kinetochores in this series or in adjacent sections. Kinetochore 1 in b is the termination of microtubule 1 in c . The kinetochore marked with an arrow in b is seen to lack a mate in either a or c and is thus considered to be unpaired. In the sections on either side of the presented series, there was no microtubule running from the right pole to the vicinity of this unpaired kinetochore. Bar, $0.1 \mu\text{m}$. $\times 75,600$.

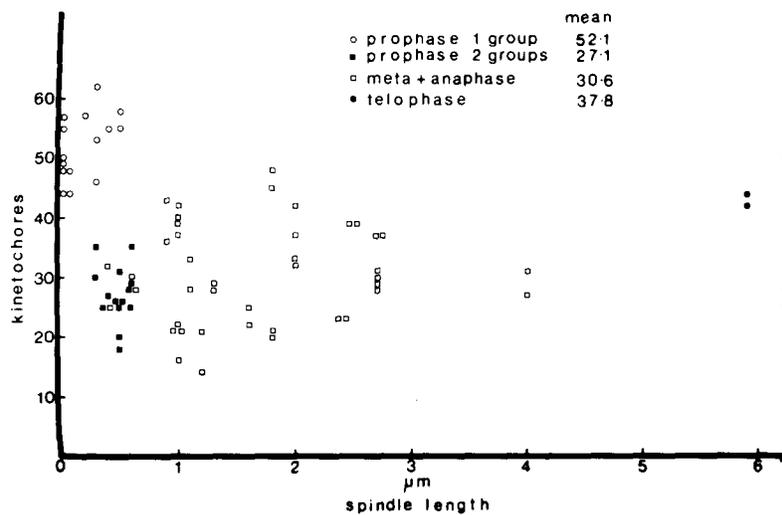


FIGURE 8 The number of kinetochores counted per spindle is plotted as a function of spindle length, with the explanation of the symbols and means given on the graph. The question mark indicates a nucleus that appeared to be in telophase, but this could not be determined with certainty. The number of kinetochores is given per group at prophase and telophase and per half spindle at metaphase and anaphase.

chores per half spindle is 30.17 ± 7.88), it seems likely that the observed variation is real and has a biological not a technical basis. The following possible biological explanations for the variability were examined:

Kinetochores may become more visible or more numerous as mitosis proceeds, and thus, one might expect a correlation between mitotic stage (measured as spindle length) and number of kinetochores detected. As is suggested in Fig. 8, no such correlation exists. In an organism that has been in culture for many years and in samples of nuclei examined over a considerable time span (5.5 yr in this case), the possibility of polyploid nuclei occurring is very real. This polyploidization may be a one-time event occurring in the stock culture, but this was not the case in the present work because there is no correlation between date of fixation and number of kinetochores per spindle. Alternatively, there may be a portion of the nuclear population that is always polyploid, in which case there should be evidence of a number of peaks in the kinetochore number histograms. Because the number of nuclei analyzed is small, the histograms are ambiguous and are not reproduced. However, there are consistent indications of multiple peaks, with interpeak values of 7–10 (the values depending on how the data is grouped). These results would be consistent with a haploid number of 7–10 and with vegetating nuclei rang-

ing from a $2n$ to $6n$ state, with the majority of the nuclei in a $3n$ or $4n$ state, depending on the value of n . Alternative explanations of the variability, especially in the metaphase stage of mitosis, will be discussed below.

The characteristic number of kinetochores present in the single hemispherical arrays of early prophase (mean, 52.1 ± 5.5) is consistently almost double the number present in each of the two adjacent arrays of late prophase (mean, 27.1 ± 4.8). There is no evidence for other than a normal distribution about this mean in each population. When the kinetochore microtubules undergo the prophase to metaphase transition, there is no change in their numbers (mean, 27.1 ± 4.8 vs. 30.6 ± 8.3), nor is there a change during anaphase (Fig. 8). Because of the sparsity of data, it is uncertain whether there is a change in the number of kinetochores during telophase. The mean number of kinetochores increases from 30.6 ± 8.3 at metaphase-anaphase to 37.8 ± 9.6 at telophase, but, given the observed variability in each population, it is impossible to determine the validity of this difference. A summary diagram of the arrangement and number of kinetochores found during the early phases of division is shown in Fig. 9.

KINETOCHORE PAIRING: At late prophase, the two groups of kinetochores lie side by side, and, thus, there is little possibility for the kinetochores to form amphitelic pairs. At all stages of

TABLE I
Kinetochore Characteristics

Hypha no.*	Treatment‡	Stage	Spindle length§ μm	No. of kinetochores		Percent paired	Magnification $\times 10^{-3}$	Obliquity factor	Interpair distance		
				L	R				mean	min.	max.
A 1	OM	M	2.0	33	32	68	76.1	12.5	1.8	1.5	2.1
A 1	OM	M	2.7	37	37	54	76.1	8.4	1.8	1.2	2.4
A 2	OM	M	1.6	22	25	55	76.1	4.0	1.7	1.3	2.1
A 2	OM	M	1.8	21	20	39	76.1	5.6	2.0	1.8	2.4
A 3	OM	M	1.2	14	21	46	76.1	3.8	1.9	1.3	2.2
A 4	OM	A	3.3	—¶	—¶	0	48.6	20.6	0	0	0
A 5	OM	M	1.3	29	28	75	89.6	16.3	2.0	1.5	2.5
A 5	OM	M	1.0	16	21	43	89.6	4.2	1.8	1.3	2.1
A 6	OM	P	0.6	35	28	0	114.2	na	0	0	0
A 6	OM	P	0	50		0	114.2	na	0	0	0
A 6	OM	P	0.5	31	26	0	114.2	na	0	0	0
A 6	OM	P	0.4	55		0	114.2	na	0	0	0
A 6	OM	P	0.3	53		0	114.2	na	0	0	0
A 7	OM	P	0	44		0	114.2	na	0	0	0
A 7	OM	P	0.5	25	26	0	114.2	na	0	0	0
A 7	OM	P	0	44		0	114.2	na	0	0	0
A 8	OM	P	0	48		0	114.2	na	0	0	0
A 9	OM	P	0.5	58		0	114.2	na	0	0	0
A 9	OM	T	5.9	44	42	0	51.0	6.7	0	0	0
A 10	OM	T	?	25	—	0	114.2	—	0	0	0
A 11	OM	P	0.5	56		0	76.1	na	0	0	0
A 11	OM	P	0.5	20	18	0	114.2	na	0	0	0
A 12	OM	T or P	? or 0	28	—	0	114.2	—	0	0	0
A 13	OM	P	0.4	25	27	0	114.2	na	0	0	0
A 14	OM	T	45	37	37	0	114.2	56.3	0	0	0
B 14	Camphor	M	2.5	39	39	82	48.6	7.8	1.6	1.0	1.8
B 14	Camphor	M	1.9	34¶	37¶	85	72.8	50	1.8	1.2	2.5
C 15	Cold	M	3.7	—	—	—	—	—	—	—	—
C 15	Cold	M	3.1	27¶	31¶	59	48.6	38.8	2.0	1.0	2.7
C 15	Cold	M	2.4	23	23	57	48.6	10.0	2.0	1.7	2.7
C 16	Cold	M	2.7	31	29	83	72.8	11.3	1.9	1.4	2.8
D 17	Colcemid 5	M	1.0	42	37	76	72.8	12.5	1.6	1.2	2.2
D 18**	Colcemid 5	M	1.1	33	28	6.5	76.1	50	—	—	—
D 18	Colcemid 5	M	0.6	30	28	31	76.1	2.5	1.4	1.1	1.7
D 18	Colcemid 5	M	0.4	32	25	49	114.2	50	1.4	1.0	1.8
D 19	Colcemid 2	A	4.0	31	27	0	35.1	50	0	0	0
E 20	Colchicine R	M	1.0	22	21	19	114.2	2.5	1.6	1.2	1.9
E 20	Colchicine R	T	?	50	—	0	114.2	?	0	0	0
E 21	Colchicine R	M	0.9	36	43	37.5	114.2	5.6	1.5	0.9	1.8
E 21	Colchicine R	P	0.3	46		0	114.2	na	0	0	0
E 22	Colchicine R	P	0	48		0	114.2	na	0	0	0
F 23	Colchicine 10	M	2.0	37	42	66	76.1	2.3	1.5	0.9	2.2
F 23	Colchicine 10	M	1.8	48	45	58	76.1	5.6	1.9	1.3	2.4
F 23	Colchicine 10	A	2.7	30	28	0	51.0	4.8	0	0	0
F 24	Colchicine 10	M	1.5	32¶	31¶	38	76.1	9.4	1.7	1.1	2.2
F 24	Colchicine 10	T	?	38	—	0	114.2	?	0	0	0
F 25	Colchicine 10	M	1.2	31¶	30¶	46	76.1	5.0	1.9	1.5	2.6
F 26	Colchicine 10	M	1.0	39	40	81	114.2	3.1	1.5	1.1	2.2

TABLE I—Continued

Hypha no.*	Treatment‡	Stage	Spindle length§	No. of kinetochores		Percent paired	Magnification × 10 ⁻³	Obliquity factor	Interpair distance		
				L	R				mean	min.	max.
F 26	Colchicine 10	P	0	55		0	114.2	na	0	0	0
F 27	Colchicine 10	P	0.6	25	29	0	76.1	na	0	0	0
F 28	Colchicine 10	P	0	49		0	114.2	na	0	0	0
F 29	Colchicine 10	P	0.3	30	35	0	114.2	na	0	0	0
F 30	Colchicine 10	P	0	57		0	114.2	na	0	0	0
F 30	Colchicine 10	P	0.3	62		0	114.2	na	0	0	0
F 31	Colchicine 10	P	0.2	57		0	114.2	na	0	0	0

* Letters designate the treatment for cross reference to Fig. 10; numbers designate the hypha (thus, two nuclei from hypha number 1 were analyzed).

‡ Refers to growth medium with *R* denoting recovery from colchicine and numbers denoting mM concentration (see Materials and Methods).

§ *O* indicates the presence of only one pair of centrioles; other lengths determined as described in Materials and Methods.

|| *L* and *R* figures denote the values for each half spindle. A single entry denotes a single group.

¶ Series incomplete. Such incomplete series were very nearly complete, so that they do not introduce a significant error into the pairing data. They do, however, influence the count data from which they were excluded.

** The interpair distances were not recorded for this spindle because the number of paired kinetochores was too low to make a mean value worthwhile.

—, not recorded.

na, not available.

metaphase, however, a variable percent of the kinetochores are so paired (Table I and Figs. 6 and 10). As with kinetochore numbers, so with the pairing data: there could be both technical and biological causes of the observed variability. Simple observation showed that unpaired and paired kinetochores occurred throughout all spindles. Thus the variability is not the result of the inability to detect pairs in one region of the spindle as opposed to another nor of the inability to detect pairs in some spindles and not others. Graphical and correlation coefficient analysis showed that there was no co-variation between the percent of kinetochores that were paired and the following parameters: obliquity factor, magnification, mean interpair distance, and minimum interpair distance. However, similar analysis showed that there was some slight correlation between the percent of paired kinetochores and both the maximum accepted interpair distance ($r = 0.51$) and the spindle length ($r = 0.43$). Interpretation of this data is difficult because the maximum accepted interpair distance co-varies with spindle length ($r = 0.70$) and magnification ($r = 0.61$). The simplest interpretation of this data is that the variation in the percent of kinetochores that are paired during metaphase is caused by two independent variables. One is a technical problem, whereby, in the longer

spindles, which were examined at a lower magnification, kinetochores that would have been deemed too far apart to be paired in high magnification pictures were accepted as paired. The other cause may be a real tendency of more kinetochores to become paired as the spindle elongates. However, the important points are that, as seen in Fig. 10, pairing never reached 100%, nor was the correlation between pairing and any measurable parameter very close, indicating a considerable amount of variability resulting from unknown sources. The latter point is made clear in Fig. 10, where it can be seen that in the spindles closest to anaphase (e.g., 2.7 μm pole to pole), the percent of paired kinetochores varied from 54 to 83, and, similarly, in early metaphase (e.g., 1 μm pole to pole) the range is from 19 to 81%. Although there is variation in the percent of paired kinetochores between different prefixation treatments (Table I), the variability within each treatment and the small sample sizes make it impossible to determine whether there is any significant difference between treatments. There probably is not.

KINETOCHORE INTERPAIR DISTANCE: As seen in Table I, the measured values of interpair distances in different spindles vary. Some of this variation is the result of technical problems. For example, no correction was made for the under-

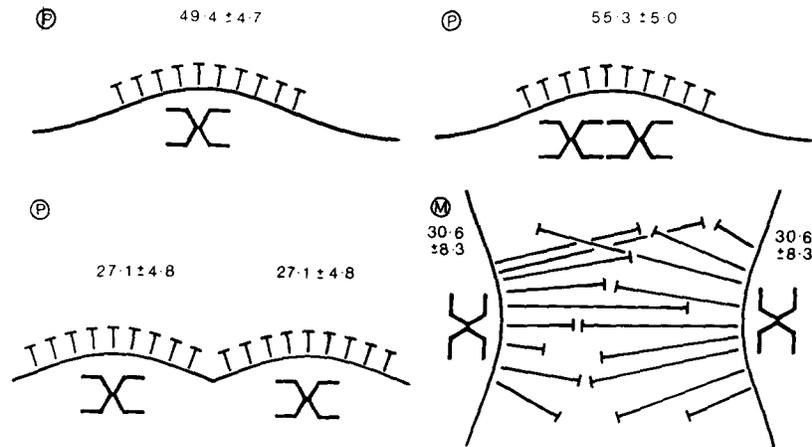


FIGURE 9 Summary diagram showing the changes in kinetochore numbers (with standard deviations) and arrangement during prophase (P) and metaphase (M). The behavior of the centrioles external to the nuclear envelope is also shown.

estimate of length resulting from geometrical problems when the members of a pair were not in the same section. Furthermore, there is often difficulty in accurately locating the correct structure of each kinetochore from which to take the measurement. As noted above, there is also a tendency to accept as paired higher interpair distances in lower magnification pictures. Estimating the contribution of each of these errors to the observed variability is difficult, but the latter source is negligible because the full range of maximum interpair distance values was recorded from spindles analyzed at essentially the same magnification (Table I). Further evidence that the variability reflects real variation between spindles comes from the observation that minimum, mean, and maximum interpair distances show a high degree of co-variation ($r = 0.726$ and 0.805 for minimum vs. mean and mean vs. maximum, respectively). Only two generalizations may be made about the interpair distances. Firstly, there is a tendency toward greater maximum interpair distances in longer spindles ($r = 0.620$ for those spindles analyzed at 72.8×10^3 and 76.1×10^3). This probably reflects a move toward increasing separation of kinetochores before their total separation at anaphase. Secondly, there is little correlation between the percent of kinetochores that are paired and their interpair distances (percent paired vs. maximum interpair distance and mean interpair distance; $r = 0.480$ and 0.055 , respectively). Considering the sources of variability mentioned above, it is noteworthy that the interpair distance values are remarkably constant

from one spindle to the next. Although the absolute range runs from 0.09 to $0.28 \mu\text{m}$, the means for different spindles only range from 0.14 to $0.20 \mu\text{m}$ (Table I) with an overall mean of $0.17 \pm 0.02 \mu\text{m}$. Since these measurements were made after a subjective decision on the presence or absence of pairing had been made for each kinetochore, the uniformity of distances supports the argument that the kinetochore pairs do indeed reflect a real in vivo structure with some constant organization. It should also be pointed out that if, as noted below, there is independent behavior of kinetochores during mitosis, some of the variability in interpair data could be due to various degrees of chromatin separation and various degrees of tension on the chromatids before their separation.

DISCUSSION

One of the primary observations of the present work is that the kinetochores¹ are replicated and present in a single group at the onset of prophase, and that they become sorted into two separate groups before metaphase. The behavior is in some respects similar to that reported in *Trichonympha* by Kubai (13). However, contrary to that situation,

¹ Because, by definition, kinetochores are part of the chromosome, and because the structures described here may not always be attached to the chromosomes, as argued in this section, the term "kinetochore microtubule" might be more appropriate. This potential terminological inexactitude is acknowledged, and the term "kinetochore" is used here for the sake of brevity.

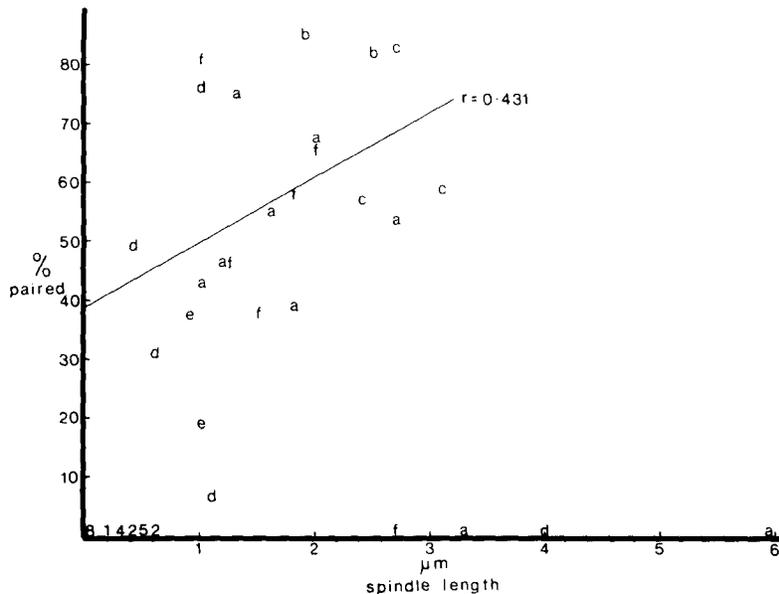


FIGURE 10 Changes in the percent of kinetochores paired at various stages of mitosis expressed vs. spindle length. The values for each spindle are indicated by letters that indicate the treatment group in Table I. The letters on the abscissa around 3 μm are anaphase spindles and the letter at 6 μm is a telophase. The numbers on the abscissa show the number of prophase spindles analyzed at the relevant intercentriole pair distance, with eight being the number of prophases analyzed with only a single pair of centrioles present. The correlation coefficient (r) was calculated only for the metaphase spindles with the d spindle, with 6.5% paired being deemed too aberrant to be considered a part of the normal population. These were excluded from the correlation coefficient analysis.

in *Saprolegnia* they do come to lie in the typical amphitelicly paired, back-to-back configuration characteristic of other metaphase spindles. Two possible explanations fit this behavior:

The kinetochores may be permanently attached to the chromosomes (chromatids) throughout the nuclear cycle. In this case, the essential feature of mitosis, the equipartitioning of the chromatids into two groups (at least at their points of attachment to the spindle), has already occurred at prophase. If this is true, the role of the subsequent behavior of the kinetochores is obscure. The objectives of mitosis could in principle be achieved by simple separation of the two groups of kinetochores, with no further changes in the organization or length of the kinetochore microtubules. In support of this interpretation (i.e., continuous connection), is the observation that the kinetochores undergo no morphological change such as might occur if they changed with respect to their attachment to chromatids. Furthermore, even at prophase, there is as much material associated with the kinetochores as is detectable at later stages of division. However,

these arguments are weak given the poorly defined nature of the kinetochores and their associated material.

The other hypothesis that is consistent with the data is that the kinetochores do not attach to the chromatids until metaphase, and that those kinetochores defined as paired are indeed held together by being bound to the appropriate sites on unseparated chromatids. This would require considerable organization in the nucleoplasm to bring the kinetochore attachment sites to the kinetochores. However, conventional meiotic prophase is similarly complex, and, thus, it is conceptually believable. Both hypotheses require some mechanism for sorting out the kinetochores at prophase, a process that seems to occur with the retention of the attachment of the kinetochore microtubules to the pocket region of the nuclear envelope (although separation and reinsertion of one or two at a time would be undetectable). However, in the latter hypothesis, the need for accuracy to ensure that kinetochores with complementary chromatids are segregated to opposite groups is eliminated. At

present, there is no available evidence to differentiate between these two hypotheses. However, the problem is probably common to *Saccharomyces*, where the spindle forms as two side-by-side arrays that reorient themselves (16). In yeast the question can potentially be resolved because during meiotic prophase the parallel half spindles coexist with synaptonemal complexes (19). If the kinetochore microtubules can be demonstrated in these half spindles, then they would not be associated with the chromatids, which are attached to the complexes unless the kinetochore regions of the chromatids form extensions away from the complexes. Such a situation should be detectable by high-resolution autoradiography.

After the formation of the metaphase spindle, the behavior of the kinetochores is again unconventional. Although the percent of kinetochores in the paired configuration tends to increase as the spindle elongates, the correlation is poor. The data available seems to preclude the interpretation that eventually, at the end of metaphase, all of the kinetochores are paired. However, the consistency in the interpair distances does support the reality of the pairing process as a functionally important phenomenon rather than a mere chance encounter resulting from space constraints within the spindle. Two explanations of the pairing data are tenable. Some kinetochores may never pair at any stage in mitosis. Intuitively, this seems unlikely because the factors determining the formation of pairs likely apply to all chromatids and kinetochores. Alternatively, there may be substantial independence in the behavior of individual kinetochores, so that any two kinetochores that are destined to pair do so at some stage during metaphase, whereas the population as a whole behaves asynchronously. Thus, one would envisage variation in the time at which each pair of kinetochores comes together and similar variation in the duration of pairing and asynchrony in loss of pairing, i.e., the beginning of anaphase separation. Apart from the obvious numerical data showing the absence of 100% pairing at any stage in mitosis, there are two further pieces of evidence to support the interpretation of independent kinetochore behavior. (a) If all of the kinetochores were paired, the mean kinetochore microtubule length should equal the half-spindle length less half of the average interpair distance. As seen in Fig. 8, this condition is not met; consistently during the later stages of metaphase, the mean kinetochore microtubule length is short of the half-spindle length, as would

be expected if there were a population of unpaired kinetochores on short kinetochore microtubules. (b) The lag in the elongation of the shortest kinetochore microtubules during early metaphase is most simply explained by independent control of part of the kinetochore microtubule population. The concept of independent control of individual kinetochore microtubule polymerization is further supported by the considerable variation in kinetochore microtubule length, which becomes increasingly variable throughout metaphase and into early anaphase (Fig. 7). If there were an overall tendency for kinetochores to pair and to locate in a central position on the spindle by the end of metaphase after an asynchronous beginning of the process, one would expect a tendency to less variation in kinetochore microtubule length, but this is not the case. Furthermore, the failure to detect a metaphase plate configuration in the large number of nuclei examined in this species in this, and other work, means that such a state is so rare, and, thus, of such short duration, as to be effectively absent. Whether the absence of a clear metaphase plate is the result of a static configuration or of the halting (during fixation) of a dynamic, oscillating system such as occurs in prometaphase in higher organisms, has been discussed previously (10) and cannot be resolved at present.

In principle, the kinetochore numbers should indicate the time of kinetochore, and, thus, perhaps, of DNA, replication. Such a conclusion is hard to draw for a number of reasons. Clearly, replication does not occur during prophase (i.e., during the transition from one group to two), which is the time of centriole replication. After that stage, the numbers present at metaphase, anaphase, and telophase are too variable for firm conclusions to be drawn. It is notable that at telophase the range encountered is consistent with the presence of both prereplication and postreplication numbers, but a similar range occurs during metaphase. It is tentatively suggested that replication occurs during mitosis from metaphase to telophase. Thus, if kinetochore replication were an indicator of DNA replication, one would, in effect, have a cycle lacking G_1 , as occurs in *Physarum*, *Schizosaccharomyces*, and other species (17). However, because the variation in kinetochore numbers could be a function of polyploidy (discussed below), this suggestion cannot be substantiated.

As mentioned above, one explanation for the variability in kinetochore numbers in different spindles is the existence of variable levels of ploidy

within the nuclear population of even a single hypha. If this were so, one would expect the kinetochore number histograms to show multiple peaks. This they do, but the small sample sizes render the data inconclusive. However, light microscopy has provided some evidence for the existence of a general plasticity in ploidy levels among the oomycetes (18), and a microspectrophotometric study of *Saprolegnia terrestris* revealed a 4.5-fold range of DNA values in hyphal nuclei (1). At present, the question of variable ploidy levels within a nuclear population of *Saprolegnia* hyphae must remain a distinct but unproven possibility.

Description of the nuclear cycle of *Saprolegnia* is difficult because of the ambiguous behavior of the chromatin and, to some extent, because of the problem of semantics and definition raised by the present observations. Although nuclei lacking an array of kinetochores have been seen in *Saprolegnia* (15), recent experience is that all nuclei in rapidly growing hyphal tips contain one of the arrays of kinetochores described above. Work in progress supports this conclusion. If hyphal tips are serially sectioned in such a way that the kinetochore complement of all nuclei can be ascertained, all nuclei do indeed contain an array of kinetochores. At the very least, this shows that the kinetochores, and, thus, part of the spindle, may persist throughout the nuclear cycle (as does the spindle in haplosporidians [15]). Because interphase is usually defined as the period between chromatin condensation, spindle formation, and the converse of these processes, one may conclude that interphase in *Saprolegnia* can be entirely suppressed in rapidly growing vegetative hyphae. Alternatively, one must redefine interphase. Because the doubling time for nuclei in *Achlya*, a close relative of *Saprolegnia*, has been reported as being 51 min (3), this suppression of interphase may be an evolutionary response to selection for a rapid growth rate. The apparent suppression of interphase, or the continuous presence of kinetochores, does at least render DNA replication during mitosis more probable, as discussed above.

Although it might be argued that this investigation has raised as many questions as it has answered, it has put some aspects of the mitotic system of *Saprolegnia* on a more quantitative basis and has demonstrated a number of unexpected features. It has also more clearly defined the problems that require resolution in this, and other fungal mitotic systems.

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Note added in proof: Recent work on another isolate of *S. ferax* indicates that kinetochore replication does occur during metaphase.

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