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Control of Spindle Form and Function in Grasshopper Spermatocytes

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Abstract. The influence of the mitotic organizing centers, the kinetochores and the polar organizers, in controlling the dynamic spindle form and function has been investigated in the primary spermatocytes of two grasshoppers, *Arphia xanthoptera* and *Melanoplus differentialis*. A new measure of the total birefringent material in the spindle is introduced – *volume-birefringence*. This measure avoids many of the problems associated with the traditional retardation measurements of spindle organization. – The number of chromosomes (and their kinetochores) in a spindle can be altered with a piezoelectric micromanipulator in three ways: 1) chromosomes can be removed permanently from the cell, 2) chromosomes can be detached from the spindle and allowed to reenter the spindle at a later time, and 3) chromosomes can be transferred from one spindle to another in cells containing two spindles. Such operations show the volume-birefringence of the spindle is proportional to the number of chromosomes in the spindle. A residual volume-birefringence is seen and attributed to the contribution of the polar organizers to spindle structure. The relative polar contribution differs in the two species. Chromosome motion and spindle elongation in anaphase are unaffected by the number of chromosomes in the spindle. The proportion of volume-birefringence associated with a kinetochore is used to estimate the number of microtubules one might expect to see if the birefringence of the spindle is of microtubular origin. These calculations predict about twice the number of microtubules per kinetochore than seen with the electron microscope. Reasons are suggested to explain this discrepancy. – It is argued that chromosome detachment releases spindle component subunits into the total subunit pool, but that these excess subunits do not influence the metaphase form nor the anaphase function of the spindle; therefore, spindle dynamics are under the direct control of the kinetochores and the polar organizing centers.

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Introduction

The mitotic spindle is the transient structure which distributes the chromosomes during cell division. The spindle contains parallel fibers visible in the polarized light microscope. These birefringent fibers consist most notably of microtubules, but other fibrous proteins oriented with the microtubules could contribute to spindle birefringence (cf. Sato et al., 1971, 1975; Stephens, 1971, 1972; Forer, 1969, 1976). Spindle fibers are in a dynamic equilibrium with their subunits in the cell (review and additional data: Inoué and Sato, 1967). As the fibers assemble, the spindle forms and the chromosomes align at the equator; when the chromosomes move poleward in anaphase, the equilibrium shifts from assembly to disassembly. Disassembly either provides the force needed to move the chromosomes (Inoué and Sato, 1967; Inoué and Ritter, 1975) or controls the force(s) causing the movement (Forer, 1975; Nicklas, 1975; Salmon, 1975c). In either case, the transition from fiber assembly to disassembly is an essential feature of mitosis in eucaryotes. Therefore, understanding the cellular control of the dynamic equilibrium of the spindle is crucial to understanding the mechanism of cell division.

The development of successful in vitro conditions for microtubule assembly (Weisenberg, 1972) and subsequent experiments give us candidates for the in vivo, molecular controls: divalent cations (Olmstead, 1976), nucleotides (Jacobs et al., 1975; Weisenberg and Deery, 1976), sulfhydryl groups (Mellon and Rebhun, 1976), polycations (Erickson and Voter, 1976), and microtubule-associated proteins (Whitman et al., 1976) (only the most recent references have been cited; for a review of previous studies on these agents, see Snyder and McIntosh, 1976). Furthermore, the main spindle components, microtubules, can grow on the isolated chromosomes and polar organizers of lysed cells (Weisenberg and Rosenfeld, 1975; McGill and Brinkley, 1975; Telzer et al., 1975; Snyder and McIntosh, 1975). Snyder and McIntosh find that microtubule polymerization in the spindles of lysed tissue culture cells depends on the concentration of added tubulin subunits. Hence, mitotic organizing centers and tubulin pool size both regulate microtubule assembly in these in vitro systems, and both have been suggested as controls of spindle organization in living cells (Inoué and Sato, 1967).

The effects of changing the tubulin pool size in living cells have been studied in the eggs of several organisms. The size of the available tubulin pool in *Strongylocentrotus droebachiensis* eggs depends on the environmental temperature of the organism (Stephens, 1972, 1973). Cells grown at 0° C form a smaller spindle at 12° C than cells grown at 8° C. Since spindle size is temperature dependent and can be rapidly changed by changing temperature (Inoué et al., 1975 and earlier), the 8° C eggs, it is argued, must have a larger supply of available tubulin in order to produce larger spindles at 12° C (Stephens, 1972, 1973). Sluder (1976) reduces tubulin pool size and, consequently, spindle size in *Lytechinus variegatus* eggs by treating with colcemid before fertilization. Colcemid reduces the available pool because it binds only tubulin subunits (Borisy and Taylor, 1967a, b). If Colcemid is converted to its non-binding isomer, lumi-

colcemid, by ultraviolet light (Aronson and Inoué, 1970), the spindle soon reaches its normal size (Sluder, 1976).

Other treatments alter spindle size, but the manner of the alterations is less clear. In particular, D_2O (reviewed by Inoué and Sato, 1967; Stephens, 1973) and six-carbon glycols (Rebhun et al., 1974, 1975) augment the spindle in marine eggs, causing an increase in spindle volume many times the normal size. The increased volume is accompanied by an increase in the number and length of the microtubules in the spindle (Inoué and Sato, 1967). D_2O changes the kinetics of microtubule assembly, but also seems to increase the pool of subunits (Stephens, 1973).

Reduction or disappearance of the spindle occurs with chemical inhibitors such as Colcemid (e.g. Sluder, 1976), low temperatures (Inoué, 1964; Inoué et al., 1975), and high hydrostatic pressures (Salmon, 1975a, b, c). With the exception of chemical inhibitors which bind tubulin or microtubules specifically these experimental treatments also affect the whole cell. Hence the activities of the mitotic organizing centers may change as well as the pool size and kinetics of the assembly reaction.

Our knowledge of the function of the mitotic organizing centers—kinetochores and polar organizers—is limited thus far to the in vitro work described above and to experiments in which organizing center number is increased within a living cell. If centriole-containing structures are injected into *Xenopus* spec. eggs (Heidemann and Kirschner, 1975; Maller et al., 1976), a number of large asters is formed. Such injections mimic polyspermic eggs (see Wilson, 1925, Fig. 193). Although the amount of polymerized material is not quantitated in these studies, the large number of asters which can be stimulated (up to 40) (Heidemann and Kirschner, 1975) indicates an active role of the centers in polymerizing new material in the cell. Galeotti's work on human cancer cells (1893; see Wilson, 1925, Fig. 73) hints that spindle size is related to the number of chromosomes attached to each pole.

The present studies examine the role of the kinetochores and polar organizers in spindle organization and function. The hypothesis tested is that spindle organization in vivo depends on the number of organizing centers in the cell. The hypothesis is readily tested by changing the number of organizing centers in a spindle while leaving the subunit pool unaltered. The approach in this study is to remove chromosomes (and their kinetochores) from a spindle or add chromosomes to a spindle and to follow the resulting alterations in the birefringent material in the spindle.

The experiments presented here provide the first quantitative evidence that spindle size and organization can be influenced by the number of kinetochores in the spindle; the amount of birefringent material is proportional to chromosome number even though the size of the tubulin pool is presumably unaltered. These experiments further show that a portion of spindle organization is controlled by the polar organizers. Finally, anaphase movement has been investigated; the amount of birefringent material (and, inversely, the number of subunits) in a spindle has no effect on chromosome velocities or the dynamics of spindle elongation during anaphase.

Materials and Methods

The grasshoppers used in this study were *Melanoplus differentialis* (Thomas), taken from a laboratory colony, and *Arphia xanthoptera* (Burmeister), taken from wild populations near Durham, North Carolina, from July through September of 1974 and 1975. Spermatocytes were cultured according to the methods of Nicklas and Staehly (1967). Briefly, testicular follicles were dissected in a modified Ringer's solution (pH = 6.9), spread on glass coverslips, and covered with a halocarbon oil (Voltalef 10S – similar to the Kel F-10 originally used). The spermatocytes were viewed on an inverted Zeiss microscope equipped with a Zeiss long-working-distance phase condenser and a Zeiss Neofluar, oil-immersion objective (100×, N.A. = 1.30). The chromosomes were manipulated with a piezoelectric micromanipulator (Ellis, 1962). The glass needle used in the manipulations had a tip diameter smaller than the resolution limit of the microscope.

After micromanipulation, the cells were viewed with an inverted Nikon Model M stand modified for polarized light microscopy. The light source was an HBO 100W W/2 mercury arc lamp. Polarizer and analyzer were a Glan-Thompson prism (extinction factor 1×10^5) and a sheet of unlaminated Polaroid film respectively. Nikon rectified, polarized light optics were used: a 40× (N.A. = 0.65) objective and a 16 mm condenser (N.A. = 0.52). A Brace-Koehler rotating compensator (Zeiss, 21.75 nm) was mounted at the back focal plane of the objective. The extinction of the system was $1\text{–}2 \times 10^4$ with the condenser set at a numerical aperture of 0.4 and the field stop closed to its minimal diameter (75 μm in the field of view). A 2.5% aqueous solution of $CuSO_4$ and two Schott filters (KG-1 and BG-12) were used as heat filters. The green mercury line was isolated with a 5461 Å interference filter (Omega Optics).

Spindle retardation was measured in one of two ways: 1) visual estimation of extinction using the full compensation method (Bennett, 1950), or 2) photographic estimation (Swann and Mitchison, 1951; Shirley and Forer, personal communication: described briefly in Forer, 1976).

Visual estimates of retardation were made by using the Brace-Koehler compensator to introduce a retardation equal to that of the spindle, but of opposite sign. At the point which the spindle appeared darkest, four or five measurements were made in less than a minute, recorded electronically (see below), and averaged. After each series of measurements, the slide was moved to an area without cells, and another series of measurements averaged to determine the background extinction point. The difference between values at the two points, measured in degrees, was the angle θ. The retardation of the spindle was computed from the formula

$$\Gamma_{sp} = \Gamma_{comp} \sin 2\theta \tag{1}$$

where Γ_{sp} is the spindle retardation, Γ_{comp} is the compensator retardation (21.75 nm), and θ the angle determined as above. The area of maximal retardation in the spindle was measured in this manner, with the axis of the spindle oriented ±45° to the polarizer-analyzer axes. Maximal retardation usually occurred one-third to one-half the distance from the equator to the pole of the spindle.

Measurements were recorded electronically as follows: a ten-turn, 10,000 ohm variable resistor (linearity 0.1%) was attached to the rotating dial of the compensator so that the resistor value changed as the compensator dial was turned (G.W. Ellis, personal communication). A 5.4 volt, alkaline battery (Mallory, TR134R) was attached across the constant terminals of the resistor. One of these terminals and the variable terminal were then attached to a Photovolt chart recorder. Any turn of the compensator was recorded as a change in potential across the variable resistor. The gain control of the chart recorder was adjusted so that one degree of compensation equaled one division on the chart paper. For the sake of convenience a switched circuit was added which fed a constant voltage to the chart recorder at times when measurements were not being made.

Photographic measurements were made in the manner of Swann and Mitchison (1950). Kodak Plus-X film was exposed for one second at various degrees of compensation with a blank field. The magnification on the film was 155×. The film was developed in Diafine and the resulting negatives were scanned with a Joyce-Loebl microdensitometer. A calibration curve was plotted of the optical density of the negatives versus the degrees of compensation. For this system, compensation from three to six degrees (2.3 to 4.5 nm) gave a sraight line. Exposures of experimental cells were made for one second at three degrees compensation. The negatives were scanned perpendicular to the spindle axis and immediately in front of the chromosomes – as close to the equator

as possible. The size of the spot used to scan the negatives was about 3 μm in terms of the actual cell size. Variations in spot size changed only the noise level of the measurements, not the values of the measured retardations. The density of the cytoplasm was subtracted from the maximal density of the spindle and the difference divided by the slope of the calibration curve. The result was the compensator angle, θ. Retardation was then calculated from formula (1). Photographic retardation measurements were lower than visual measurements (see Ross, 1967, pp. 180ff), but the two methods were consistent within themselves.

H & W VTE Panchromatic film was used for 16 nm cinemicrography in both phase and polarized light microscopy. In phase cinemicrography, the film was exposed for 0.5 s and developed with Kodak D-76 for 4 min at 23° C. For polarized light cinemicrography, the exposure time was 6 s (three degrees compensation) and the film was developed with Kodak D-19 for 5 min at 23° C.

Length and width measurements were made from enlarged 16 mm or 35 mm prints. Measurements of chromosome rates in anaphase were made from either phase or polarized light films as described by Nicklas (1963). Spindle length and the distance of chromosome separation were measured and the difference between the measurements divided by two, which gave the chromosome-to-pole distance. Chromosome velocities were computed as the rate of change of the chromosome-to-pole distance per minute. This rate was determined by a least squares regression analysis of data.

The use of retardation as a measure of spindle organization was inappropriate for this study. A new measure of organization has been used: Volume-Birefringence. Volume-birefringence measures the total amount of polymerized, birefringent material in the spindle and is a function of spindle length, width, and retardation. A complete explanation of the justification, derivation, and use of volume-birefringence will be given in the Discussion.

After each operation length, width, and retardation were measured every 10 to 15 min for a period ranging from one to four hours. Two *Melanoplus* cells in the first series of experiments were followed for a shorter period of time because they entered anaphase during the first hour of the operation. The measurements of all the time points were averaged to provide the final value for each cell.

Results

The primary spermatocytes of both *Melanoplus differentialis* and *Arphia xanthoptera* have a complement of eleven autosomal bivalents and one sex univalent. The normal complement has been altered in three ways: 1) by removing a number of chromosomes permanently from the spindle and the cell, 2) by removing chromosomes from the spindle for a period of time and then allowing them to rejoin the spindle, or 3) by transferring chromosomes from one spindle to another in cells which contain two spindles. The first two operations gave a spindle with a smaller than normal number of chromosomes. The third operation gave one spindle with a greater number of chromosomes.

Permanent Removal of Chromosomes from the Spindle

The Operation and Two Examples of the Consequences

The detachment of chromosomes from the spindle was easily accomplished by micromanipulation (Nicklas and Staehly, 1967; Nicklas, 1967). Carolyn Staehly (unpublished) first succeeded in removing the detached chromosomes completely from the cells, but she did not follow spindle alterations after the operation.

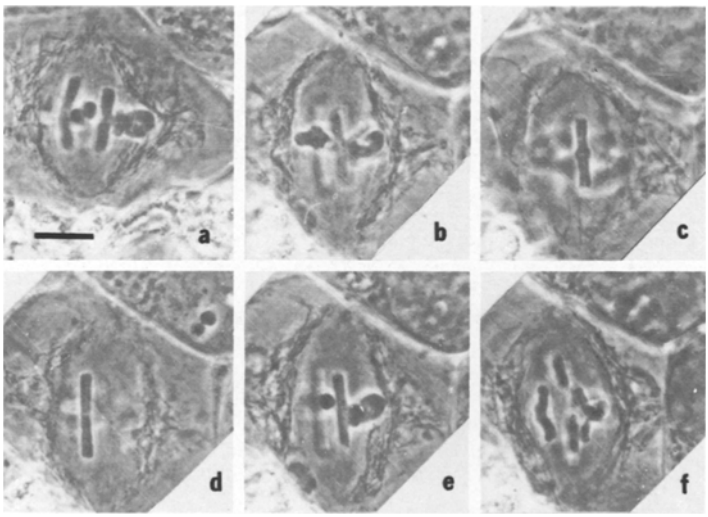

Fig. 1a–f.[1] *Arphia* spindle with six bivalents removed. Picture A shows the remaining bivalents lying at the bottom of the cell immediately after the operation. **b–e** Different focal levels through the cell some 50 min after the operation. Several of the chromosome have moved up in the spindle. These redistributions lead to decreased spindle width and increased spindle thickness. The cell finally divides normally (**f**)

The operations were simple. Chromosomes were detached from the spindle and moved to the edge of the cell. Usually several chromosomes were placed together at the edge. They were then moved away from the cell en masse. These chromosomes were surrounded by the cell membrane which stretched in a narrow strand from the point of removal to the chromosomes. The chromosomes could be moved many (five or more) cell diameters away without rupturing the membrane. Five or six chromosomes could be removed in a few minutes. In most operations, the membrane finally broke in the narrow strand, resealed, and pulled back to the original surface of the cell, leaving the extracted chromosomes separated from the cell and surrounded by their own membrane. If the cell membrane did not reseal, the cell quickly died. The membrane usually surrounded the removed chromosomes tightly, although occasionally some cytoplasm was removed with them. Even in the most extreme cases, the amount of cytoplasm removed was less than or equal to the volume of the removed chromosomes.

The cell shown in Figure 1 is an *Arphia* spermatocyte with six chromosomes removed. The first picture shows the remaining five bivalents lying in the bottom focal level of the cell immediately after the operation. The six chromosomes which were removed came from the upper levels of the spindle. The next four pictures (b–e) are a focal series through the spindle from bottom to top some 50 min after the operation. The remaining bivalents have repositioned themselves vertically in the spindle and the original spindle width has decreased. The final picture shows the cell dividing normally.

[1] Scales in Figs. 1, 2, 8, 11, and 15 indicate 10 μm

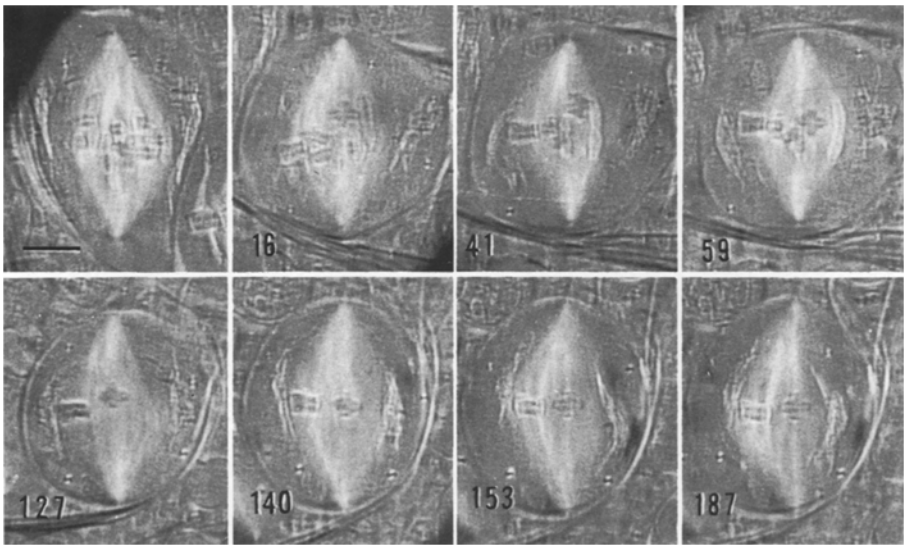

Fig. 2. Melanoplus spindle with only two bivalents. The first picture is a control cell next to the experimental cell. Five bivalents were initially removed from the spindle at zero minutes. The cell response from 16 to 59 min is shown. After 125 min, four more bivalents were removed. At first the chromosomal fibers are indistinct, but by 187 min show increased birefringence. The interpolar fibers also show increased birefringence. The cell later divided as illustrated in Figure 15

Figure 2 shows the spindle reaction in a *Melanoplus* cell in which five bivalents were removed, and the spindle changes monitored for 115 min. Then four more bivalents were removed – the final spindle had only two bivalents. The cell was followed in polarized light for 1 h after the operation, then placed on the phase microscope and followed with time-lapse video tape. About 45 min after the final polarized light observations, the cell – and the adjacent control cells – divided normally. The division is shown in Figure 15 (p. 384) and is discussed in the section on chromosome velocities. The major change in the spindle was the decreased width (38%). Spindle length increased 4% and spindle retardation decreased 15%. Overall, the spindle organization, as measured by the volume-birefringence, decreased 49%. Analysis of variance on measurements of the adjacent control cell versus those for the two states of the operated-upon cell (six and two chromosomes remaining) showed that the differences in average width and volume-birefringence were significant at the 5% level, whereas the average length and retardation were not.

Data for All Cells

During these studies it was apparent that spindle size could vary as much as threefold from one culture to the next. The variability was evident for all the parameters measured. Consequently, data for each experimental cell were compared to data from control cells in the same culture or to the experimental cell prior to the operation. The experimental data were divided by the control

Table 1. Summary of regression lines for normalized data: cells with chromosomes permanently removed

	Arphia xanthoptera	*Melanoplus differentialis*
Length		
Slope	−0.005	−0.0003
Intercept	1.05	1.002
r^2	0.023	0.001
F (d.f.)	0.2 (1,9)	0.03 (1,28)
P	N.S.	N.S.
Width		
Slope	0.046	0.039
Intercept	0.53	0.57
r^2	0.52	0.82
F	9.6	126
P	<0.01	<0.001
Retardation		
Slope	0.036	0.012
Intercept	0.58	0.86
r^2	0.55	0.30
F	11.2	12.0
P	<0.01	<0.005
Volume-birefringence		
Slope	0.079	0.049
Intercept	0.13	0.46
r^2	0.91	0.92
F	94	345
P	<0.001	<0.001

data and the resulting, normalized values used for analysis. These values were plotted against the number of chromosomes remaining in the spindle and linear regression lines calculated for each set of data. By this method, the control data had normalized values equal to 1.0.

Figures 3–6 summarize the results of permanently removing chromosomes from the spindles of *Melanoplus* and *Arphia* spermatocytes. In all cases, only the bivalents were counted in the chromosome number of the spindle. In most cells the sex univalent lay near a pole and did not contribute to the volume birefringence of the spindle. The parameters for the linear regression lines drawn through each set of data are listed in Table 1. Also listed are the coefficients of determination (r^2) and the F-values for each regression line. The coefficient of determination measures the proportion of variance of the normalized data which can be explained by the chromosomes in the spindle: in other words, the goodness-of-fit of the data to the calculated regressions (Remington and Schork, 1970). The F-values are calculated from the coefficient of determination and the number of data points. The formula is

$$F = \frac{r^2(n-2)}{1-r^2} \quad \text{(Li, 1964).} \tag{2}$$

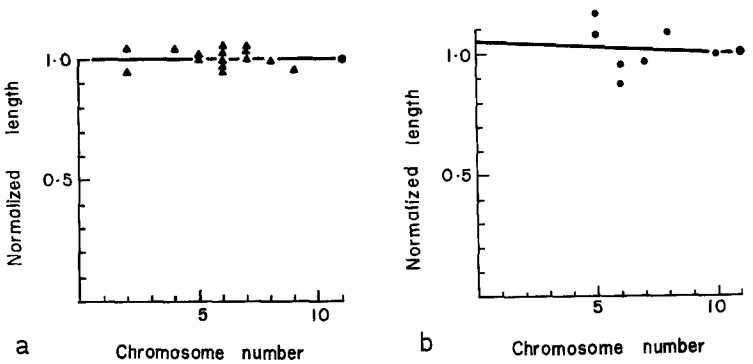

Fig. 3a and b. Normalized length changes. Neither *Melanoplus* spindles (**a**) nor *Arphia* spindles (**b**) show any change in spindle length after chromosomes are removed from the cell. Each point is an average of several measurements taken over a period of at least 1 h after the operation. The experimental data are normalized to a control cell next to the experimental cell or to the experimental cell before the operation. The parameters of the regression lines drawn through each set of data are listed in Table 1

The F-value, when applied to regression analysis, has one and (n-2) degrees of freedom. The F-value tests the hypothesis that the slope of the regression line is zero. The larger the sample size, the lower the r^2 may be and still produce a slope significantly different from zero. For example, the *Melanoplus* retardation data show a low r^2 ($=0.30$), but the sample size is large enough so that the regression line is different from zero (p less than 0.005) (Table 1).

The cells of both species showed no significant changes in spindle length as a result of chromosome removal (Fig. 3). In contrast, spindle width (Fig. 4) in both species changed more than any other parameter measured. This change results from the redistribution of the remaining chromosomes within the spindle (see Fig. 1). The two species differ, however, in their retardation responses (Fig. 5). *Arphia* shows a greater decrease in retardation than *Melanoplus*. Consequently, *Arphia* shows the greater change in volume-birefringence with decreasing chromosome number (Fig. 6).

Since the data in Figures 3–6 are averages of several measurements, the means of the experimental measurements were compared to the means of the control measurements by analysis of variance. The results of these tests for the *Melanoplus* data are given in Table 2. Only width and volume-birefringence show consistently significant differences between the experimental and control means.

A large number of *Melanoplus* cells were studied without suitable control cells and their data could not be normalized. Figure 7 is a compilation of the data for all the *Melanoplus* cells studied. The average for each set of experimental cells is plotted. The regression line (solid line) is calculated from the values of all the cells and not the means of the cells. The dashed regression line represents the normalized regression line from Figure 6. The unnormalized data are presented simply to show that the normalized data faithfully represent

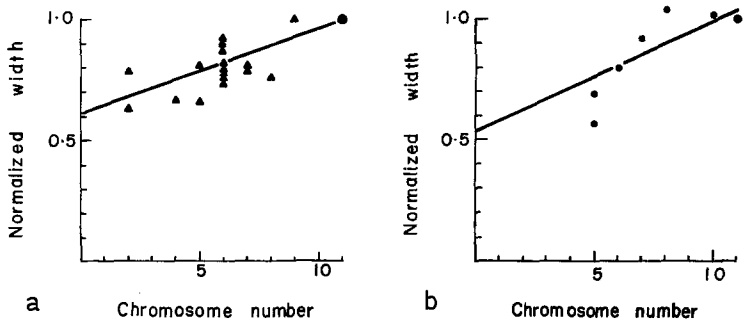

Fig. 4a and b. Normalized width changes. Both *Melanoplus* spindles (**a**) and *Arphia* spindles (**b**) show significant changes in width after chromosomes are permanently removed from the spindle. Regression line parameters listed in Table 1

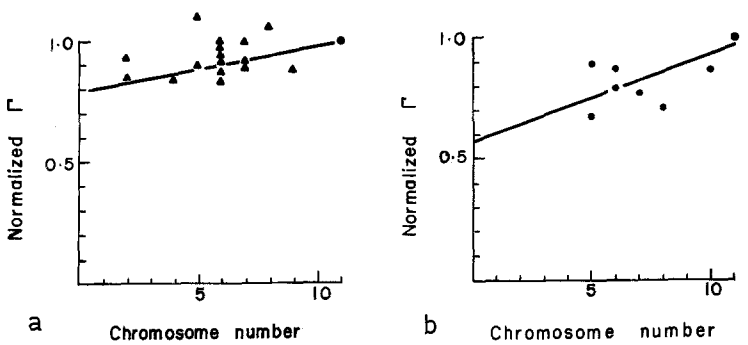

Fig. 5a and b. Normalized retardation changes. *Melanoplus* spindles (**a**) show a slight, but significant decrease in spindle retardation with decreasing chromosome number. *Arphia* spindles (**b**) show a more pronounced reduction in retardation as chromosomes are removed. Regression line parameters listed in Table 1

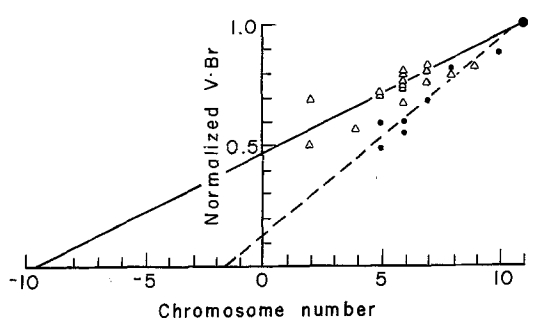

Fig. 6. Normalized volume-birefringence changes. The *Melanoplus* spindles (△——△) and *Arphia spindles* (●---●) are graphed together. *Arphia* spindles show a greater loss of spindle material with decreased chromosome number, because of their greater loss of retardation (Fig. 5). Extrapolation of the regression lines to the y-axis indicates a residual volume-birefringence which is the contribution of the polar organizers to spindle organization. Regression line parameters listed in Table 1

Table 2. Analysis of variance of *Melanoplus* cells with chromosomes permanently removed

Cell number	Chromosome number	Source of control cell[a]	Probability level			
			Length	Width	Retardation	Volume-birefringence
1	9	C	N.S.	N.S.	0.05	0.05
2	8	C	N.S.	0.001	N.S.	0.05
3	7	C	N.S.	0.01	N.S.	0.10
4	7	C	N.S.	0.05	N.S.	0.10
5	7	P[b]	N.S.	N.S.	N.S.	N.S.
6	6	P	N.S.	0.001	N.S.	0.01
7	6	C	N.S.	0.01	N.S.	0.05
8	6	P	N.S.	0.05	N.S.	N.S.
9	6	C	N.S.	0.001	N.S.	0.001
10	5	C	N.S.	0.001	N.S.	0.001
11	5	C	N.S.	0.01	N.S.	0.01
12	6,4	P	0.10	0.001	N.S.	0.001
13	6,2	C	0.05	0.1	N.S.	0.001
14	2	P	0.05	N.S.	N.S.	N.S.

[a] C = Control Cell is near experimental cell
[b] P = Control Cell is experimental cell before operation

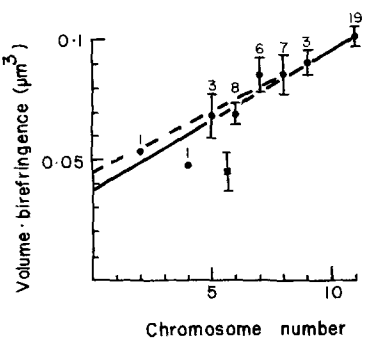

Fig. 7. Actual volume-birefringence measurements of *Melanoplus* spindles. All the actual volume-birefringence measurements, including cells without suitable controls, are averaged and plotted for *Melanoplus* first divisions (circles). The error bars represent the standard error of the mean. The numbers above the bars are the number of cells in each group. The solid regression line is calculated from all the data points, not just the averaged points. The r^2 of the regression line is 0.44, giving an F-value of 36.1 with 1 and 46 degrees of freedom. – The dashed regression line is taken from the normalized data (Fig. 6) and redrawn to fit. This figure demonstrates that the normalized data are a faithful representation of the population of actual experimental data. Normalization only reduces variance because of biological differences from culture to culture. – In addition, the average volume-birefringence of 5 second division spindles is plotted (square) (details in text)

the population of experimental cells: normalization only decreases the variance caused by biological differences from culture to culture.

Temporary Removal of Chromosomes from the Spindle

It could be argued that the change in volume-birefringence of the spindles was a result of the loss of chromosomal fibers removed with the chromosomes. This is not likely for two reasons: 1) If chromosomes are detached from the spindle while viewing with the polarized light microscope, one sees no evidence of a chromosomal fiber with the detached chromosome (Nicklas, 1967; Newton and Marek, unpublished observations). That is, the detachment of a chromosome seems to involve a separation between the kinetochore and the chromosomal fiber. 2) Brinkley and Nicklas (1968) found no ultrastructural evidence of chromosomal microtubules on newly detached chromosomes in *Melanoplus* spermatocytes. Nonetheless, experiments of two types were undertaken to avoid these objections. The first type is described in this section.

Chromosomes were temporarily removed from the spindles of *Melanoplus* spermatocytes by detaching and moving them to the periphery of the cell, as in the previous experiments; but the chromosomes were not moved out of the cell. They were repeatedly detached as they began to move back toward the spindle (see documentation of such movements in Nicklas, 1967). After varying periods of time (zero to sixty minutes after the initial detachment), the detached chromosomes were allowed to orient and reenter the spindle while being observed in the polarized light microscope. The subsequent, normal spindle was used as the control for the temporarily altered spindle.

The cell in Figure 8 shows a typical spindle response to the detachment of five bivalents for 20 min. In the first picture, the detached chromosomes are out-of-focus, but in the lower right portion of the cell. By 25 min after the last detachment, the chromosomes have reached the edge of the spindle and have begun to congress to the equator. As more of the chromosomes approach the equator, spindle width and volume-birefringence begin to increase (Fig. 9). Spindle length and retardation show no significant changes during the spindle recovery, although they decrease slightly during the chromosomal congression to the equator.

Bivalents were temporarily removed from the spindle in five cells. The normalized volume-birefringence values are plotted in Figure 10 and the parameters of the regression line are given in Table 3. The regression line for the spindles with temporarily removed chromosomes is virtually identical to that for the spindles with permanently removed chromosomes (cf. Table 1), although the variance is larger. Hence, spindles with a temporarily reduced number of chromosomes behave in the same way as spindles with a permanently reduced number of chromosomes.

Transfer of Chromosomes between Spindles

The third experimental series, like the second, was designed to alter chromosome number without removing either chromosomes or fiber material from the cell.

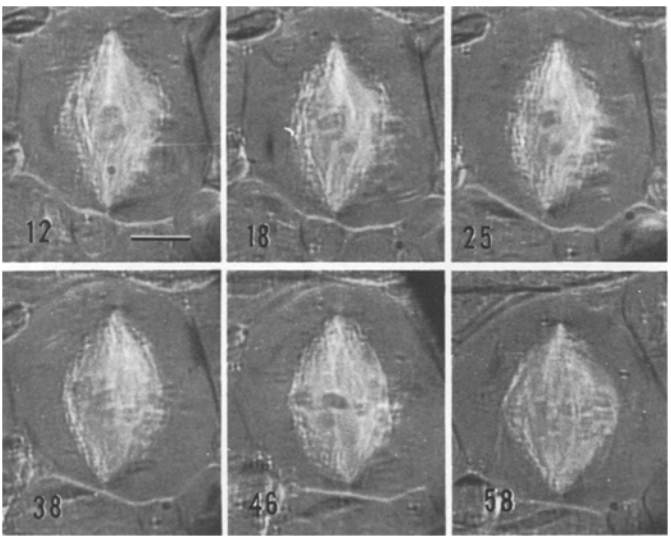

Fig. 8. *Melanoplus* spindle with five bivalents temporarily removed. Five bivalents were detached and held at the periphery of the cell. After 20 min the chromosomes were allowed to reenter the spindle. The first picture (12 min after last detachment) shows the cell with reduced width and volume-birefringence. By 18 min, the chromosomes are lying on the lower right edge of the spindle. By 58 min, spindle width has increased as the chromosomes have reached the equator of the spindle. The data on this cell are graphed in Fig. 9

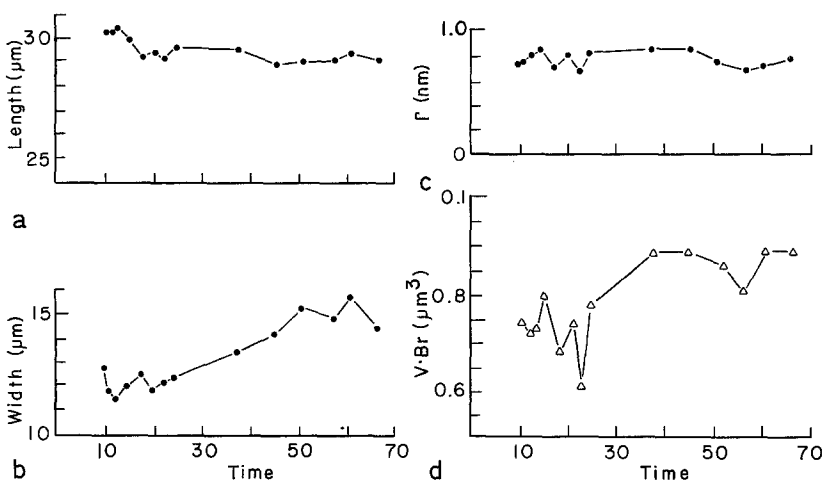

Fig. 9a–d. Graph of the cell in Figure 8. The three measureable parameters of length (**a**), width (**b**), retardation (**c**) and the volume-birefringence (**d**) calculated from them are graphed versus time (minutes). Length decreases slightly throughout the experiment. About 25 min after the last detachment, spindle width begins to increase as the chromosomes become associated with the spindle. An increased volume-birefringence follows the increased width

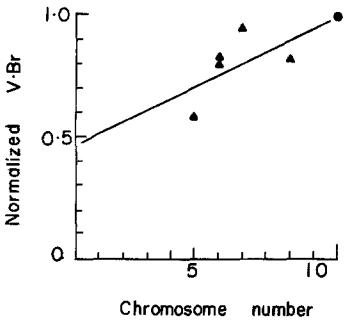

Fig. 10. Normalized volume-birefringence for cells with chromosomes temporarily removed. The five cells with temporarily removed chromosomes are plotted here along with the five control spindles (=1.0). The control spindle for each experiment is the experimental spindle after chromosomes have reentered the spindle. The regression line of these data almost exactly matches that of the cells with permanently removed chromosomes. Compare Table 1 and Table 3

Table 3. Summary of regression lines for normalized data of *Melanoplus differentialis*

	Chromosomes temporarily removed	Chromosomes transferred between spindles
Volume-birefringence		
Slope	0.046	0.084
Intercept	0.49	0.039
r^2	0.68	0.97
f(d.f.)	17 (1,8)	231 (1,4)

The preparation of spermatocyte cultures occasionally results in the spontaneous fusion of two or more cells. When two spermatocytes fuse during meiosis a cell with two spindles is formed. In three such cells, chromosomes were detached from one spindle and transferred to the other. The cell then had one spindle with more than a normal complement of chromosomes and the other spindle with fewer than a normal number of chromosomes. The two spindles were competing for the same subunit pool of the cell, but with differing numbers of chromosomes.

The cell in Figure 11 had four bivalents moved from the right spindle to the left spindle. The volume-birefringences of the two spindles were approximately equal before the operation (0 min). After the operation, the volume-birefringence increased in the left spindle with fifteen bivalents and decreased in the right spindle with seven bivalents. The progress of the shift is graphed in Figure 12. The larger spindle had increased width, but also increased length and retardation. All three parameters decreased in the smaller spindle.

The normalized volume-birefringences of the spindles with the larger number of chromosomes increased in all three cells studied (Fig. 13, cells 1–3) and decreased in the spindles with the smaller number of chromosomes. The differences between the two spindles were greater than would be predicted from the first two experimental series. At least some of this unexpected divergence was due to chromosomes in the smaller spindle reorienting spontaneously to the larger spindle. Such reorientations could not be detected with certainty in the polarized light microscope because of the low contrast of the chromosomes. Two of the three cells finally divided as single, large spindles.

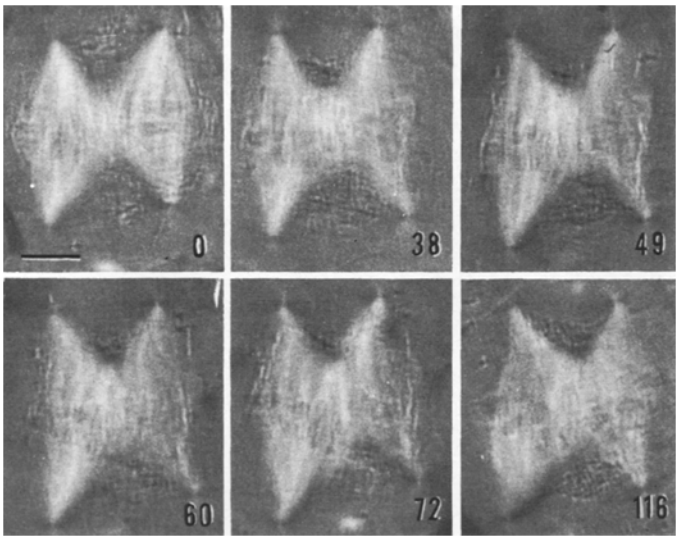

Fig. 11. Fused cell with chromosomes transferred from one spindle to the other. The first photo (0 min) shows the two spindles before the operation. Four bivalents were transferred from one spindle to the other. The time of the last transfer was 8.6 min. By 38 min, the spindle with the greater number of chromosomes (left spindle) is larger, while the other spindle is smaller. In the pictures shown here the left spindle is oriented 45° to the polarizer-analyzer axes. In the last picture (116 min) the smaller spindle axis is oriented 45° to the polarizer-analyzer axes. By this time, the spindle with fewer chromosomes is much smaller than its partner spindle. Retardation measurements were made photographically with each spindle oriented properly. The changes in volume-birefringence with time are graphed in Figure 12

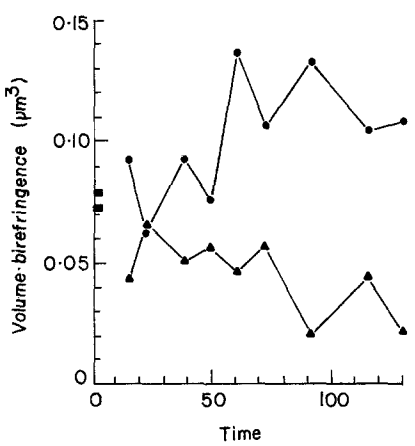

Fig. 12. Changes in volume-birefringence of the spindles in Figure 11. The spindle with the larger number of chromosomes gradually increases in volume-birefringence (circles), while the spindle with the smaller number of chromosomes (triangles) decreases gradually in volume-birefringence. The original spindles (squares) are plotted before the operation, but their identity was lost during the operation. For normalization (Fig. 13) the two control spindles were averaged and used as the control value.

One striking example of spindle fusion was seen in which two spindles were fusing at the poles (i.e., in an end-to-end fashion). As the fusion progressed, the two poles which were the original points of contact between the spindles were eliminated from the final spindle. The final normalized volume-birefringence of this cell (1.63) was very close to the value predicted for a cell with 22 bivalents, but only one set of poles (1.55) (Fig. 13, cell 5).

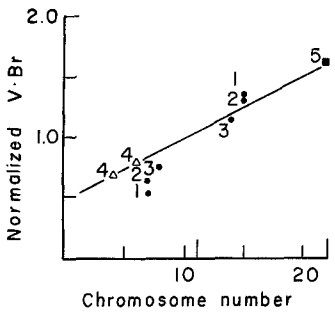

Fig. 13. Normalized volume-birefringence data for *Melanoplus* cells with two spindles. The regression line is taken from Figure 6 and Table 1. – Cells 1, 2, and 3 are cells where chromosomes were transferred from one spindle to another. – Cell 4 is a cell in which five chromosomes were removed permanently from one spindle and seven chromosomes from the other. The spindles act as if they were in normal, single cells. – Cell 5 shows a cell in which two spindles fused at the poles. During the fusion, the two polar organizers at the point of fusion were excluded from the main spindle. They formed no spindle between themselves. The volume-birefringence of the final spindle was reduced by an amount equal to that expected for a set of polar organizers (Fig. 6)

In another fused cell, seven chromosomes were permanently removed from one spindle and five from the other. The two spindles behaved as if they were in single cells with permanently removed chromosomes (Fig. 13, cell 4).

Volume-Birefringence in the Second Meiotic Division

The first meiotic division distributes the birefringent material and the number of organizing centers equally into the two secondary spermatocytes. After an interphase period of several hours, these cells complete the second meiotic division and form spermatids.

The volume-birefringences of spindles in the second meiotic division were calculated and compared to values for first division spindles. In one instance, a first division spindle was available as a control cell. The second division cell had a normalized volume-birefringence of 0.53. For all five second division cells measured, the volume-birefringence average was about 45% of the average volume-birefringence of the first division control cells. The average for the second division cells is plotted in Figure 7 as having 5.75 chromosomes (bivalent equivalents). Some second division cells had eleven chromosomes (5.5 bivalent equivalents) and others twelve chromosomes (6 bivalent equivalents) depending on which cell received the X-univalent in the first division. Second division spindles were 20–25% shorter and narrower than first division spindles, but had retardations only slightly less (10%) than first division values.

Chromosome Movement and Spindle Elongation

The anaphase separation of chromosomes has been described as consisting of two separate processes: the movement of the chromosomes closer to the poles and an increase in the pole-to-pole distance.

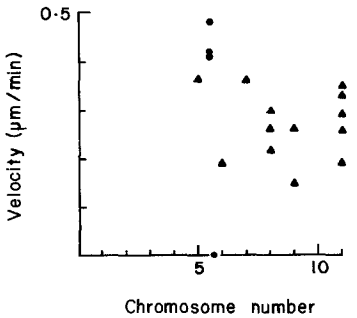

Fig. 14. Chromosome velocities. The rate of chromosome motion to a pole during anaphase is graphed versus the number of chromosomes remaining in the spindle. No correlation between chromosome number and anaphase rate is apparent. The triangles are the first division cells and the circles are the control, second division cells. Details in text

Table 4. Anaphase data of *Melanoplus* cells with chromosomes permanently removed

Cell number	Chromosome number	Volume-birefringence (μm^3)	Maximal spindle elongation rate (μm/min)	Chromosome separation rate[c] (μm/min)	Chromosome velocity[d] (μm/min)
First division cells					
1	11	–[a]	0.20	0.55	0.35
2	11	0.088	0.21	0.49	0.19
3	11	–	0.03	0.66	0.33
4	11	–	0.53	0.58	0.29
5	11	0.117	0.46	0.52	0.26
6	11	0.088	–0.07	0.42	0.21
7	11	0.116	0.20	0.77	0.28
8	9	0.091	0.21	0.27	0.15
9	9	0.085	0.07	0.55	0.26
10	8	0.096	0.30	0.76	0.30
11	8	0.119	0.26	0.26	0.26
12	7	–	–0.22	0.36	0.36
13	6	0.064	0.31	0.70	0.19
14	5	0.081	0.21	0.84	0.36
15[b]	11	0.105	–	0.94	–[a]
16[b]	2	0.053	–	0.94	–[a]
Second division cells					
1	11	0.047	0.24	0.92	0.41
2	11	0.056	1.38	1.38	0.0
3	11	0.040	–0.14	0.67	0.41
4	11	0.050	0.96	1.9	0.48

[a] Not measured.

[b] Control cell and experimental cell illustrated in Figures 2 and 15

[c] The total separation divided by time – the given rates should be divided by two to give rates for individual chromosomes

[d] Average rate of approach of individual chromosomes to a pole. The chromosome-to-pole distance in each frame was calculated by subtracting the distance between chromosomes from the pole-to-pole distance and dividing by two. The velocity is the slope of the linear regression line calculated when the chromosomes were nearing the poles. This velocity cannot be calculated from the preceding two columns because maximal spindle elongation occurred before, during, or after chromosome movement in different cells

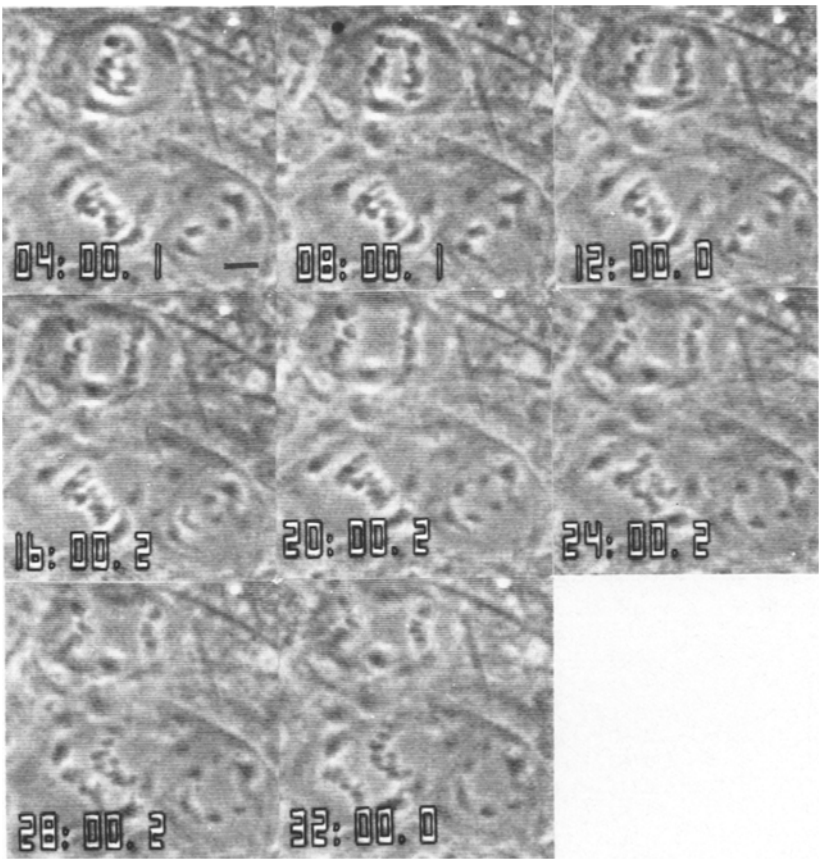

Fig. 15. Division of the cell in Figure 2. The cell with only two bivalents (lower right in each print) is shown dividing normally and at the same rate as a nearby control cell (upper center). The time scale (lower number) (minutes: seconds. tenths) has no relation to the time scale of the experiments. The time was added after the experiment in order to measure the rate of chromosome separation during anaphase. The control cell begins division at 4 min and the experimental cell is dividing at 12 min. Both cells separated their chromosomes at a rate of 0.9 μm/min

The rate at which chromosomes approached the poles varied greatly from cell to cell and was not related in any way to the number of chromosomes remaining in the spindle (Fig. 14). Interestingly, chromosomes in three of the four second division cells moved more rapidly than in any of the first division cells. The fourth cell separated its chromosomes entirely by spindle elongation – no decrease in chromosome-to-pole distance was seen.

The chromosome velocities are listed in Table 4, along with the maximal rates of spindle elongation seen during the division, the rates of chromosome separation, the number of chromosomes left in the spindles and the average volume-birefringence of the cells before division. No correlation existed among any of the parameters listed, with the exception of volume-birefringence and chromosome number.

The most striking instance of the lack of any effect on chromosome motion was the division of the *Melanoplus* cell with only two bivalents (Fig. 2). The division of this cell and a control cell is illustrated in Figure 15. The control cell (top center of pictures) divides first (4 min) and the experimentally ravished cell (lower right of pictures) is dividing by 12 min. The location of the spindle poles was uncertain in the phase microscope, so only a comparison of the rate of chromosome separation could be made between the control and the experimental cell. Both cells separated their chromosomes at about 0.9 micrometers/min. Since the operated cell had only two chromosomes and half the control volume-birefringence, any effect on anaphase dynamics by altering chromosome number should be obvious. No effects were seen.

Discussion

In the following, spindle organization will be discussed in terms of birefringent material (polymerized and regularly aligned) and the subunits of this (these) material(s). Much of the birefringence of the spindle is undoubtedly of microtubular origin, but recent demonstrations that materials such as actin may be in the spindle (reviewed by Forer, 1976; Cande et al., 1977) preclude any discussion of spindle organization solely in terms of microtubule content.

Measurement of Spindle Organization

The mitotic spindle is weakly birefringent in polarized light. Most of this birefringence is form birefringence contributed by the parallel arrangement of microtubules in the spindle (Sato et al., 1975; Stephens, 1971, 1972), but additional, regularly arranged components could influence the birefringence (Cassim et al., 1968; Forer, 1976).

Spindle birefringence is measured as retardation (Bennett, 1950). Traditionally, spindle retardation has been used to measure the concentration of birefringent material in the spindle (Inoué, 1964), but retardation is not a direct measure of this organization. Retardation is the product of two variables: spindle thickness and the coefficient of birefringence. It is the coefficient of birefringence which depends on the concentration of birefringent materials (Cassim et al., 1968; Sato et al., 1975). Therefore, only in a spindle where the thickness is constant throughout an experiment does retardation reflect changes in the concentration of the birefringent material. Retardation ignores width and length changes as well (Forer, 1976). An example of this problem is seen in the glycol treatment of *Spisula solidissima* eggs (Rebhun et al., 1975). With treatment the spindles in these eggs increase over fifteen times in volume. Length doubles and width (and, presumably, thickness) triples. Retardation also triples, but this increase is caused by the increased spindle thickness, *not* by an increased concentration of birefringent material in the spindle. Since the coefficient of birefringence remains constant, a strict interpretation of the retardation would say that the concentration remains constant within the spindle. Use of volume-

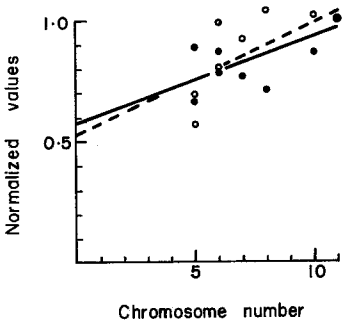

Fig. 16. Normalized width and retardation data for *Arphia*. The normalized width data (Fig. 4b) and the normalized retardation data (Fig. 5b) are plotted together. The width data are represented by the open circles and dashed line and the retardation data by the closed circles and the solid line

birefringence as a measure of spindle organization would show that the total amount of aligned material increased over fifteen times because of the overall increase in spindle volume. Obviously, retardation measures only one dimension of assembly or disassembly within a spindle and data based solely on retardation measurements should be reinterpreted to consider all changes in spindle dimensions and concentration.

An additional problem in using only retardation measurements in the experiments described in this paper is the uncertainty of spindle thickness at any given time. Cells cultured as described tend to have flattened spindles with an elliptical cross-section, i.e., a spindle wider than it is thick. The amount of flattening can vary from cell to cell, depending on the size of the cell and the packing of the cells in the culture. Thickness can be measured using the fine focus micrometer scale of the microscope, but such measurements are relatively inaccurate (Bennett, 1950; Ross, 1967). A more important consideration, however, concerns the method of removing chromosomes from the spindle in these experiments. In micromanipulation, the needle enters the cell from above and the uppermost chromosomes are detached first (see Fig. 1). Chromosomes lying on the bottom of the spindle are rarely manipulated with success; because the actual needle tip cannot be resolved in the microscope, the needle may be lower than expected – tearing the cell membrane by contact with the underlying coverslip. Removal of the uppermost chromosomes immediately decreases spindle thickness to an inknown value. Thickness again changes as the remaining chromosomes move upward in the spindle (Fig. 1). This change in thickness and its effect on retardation measurements can be seen in Figure 16, where the normalized width data for *Arphia* spermatocytes (Fig. 4) and the normalized retardation data (Fig. 5) are graphed together. The *Arphia* spindle decreases in width only after several bivalents are removed, and retardation decreases with the removal of only a few bivalents. The decrease in retardation without decrease in width and the leveling-off of the retardation as the width begins to decrease is an indication of the effect of spindle thickness on retardation measurements. Overall, spindle organization decreases steadily with decreased chromosome number (Fig. 6).

Measurements of dry mass in interference microscopy are based on the product of optical path difference (which has a thickness component) and area (Ross, 1967). Nicklas and the present author have developed a measure of

the total amount of birefringent material in the spindle – *volume-birefringence* – a measure analogous to dry mass measurements in interference microscopy. This measure does not require knowledge of the exact spindle thickness. Volume-birefringence is the product of spindle volume times the concentration of birefringent material within the spindle, where concentration is expressed as the volume fraction of birefringent material in the spindle (Cassim et al., 1968; Sato et al., 1975). The net result measures the total birefringent material in the spindle. It must be stressed that volume-birefringence is not a measure of concentration, but a measure of total anisotropic material.

The formula for volume-birefringence is derived as follows. Spindle volume is proportional to the product of its linear dimensions: length (L, measured from pole to pole), width (W, measured at the equator), and thickness (T, measured at the equator). Retardation (Γ) is thickness times the coefficient of birefringence (C_{br}). The coefficient of birefringence is linearly proportional to the concentration of birefringent material in the spindle (Cassim et al., 1968; Forer, 1969; Sato et al., 1975). Therefore, we can measure spindle length and width and take the product of thickness and concentration from retardation measurements to give the parameters necessary to calculate volume-birefringence.

In particular, if we assume a grasshopper spindle has an elliptical cross-section at the equator and straight sides from equator to the poles, then the volume of the spindle (V_{sp}) is given by the formula:

$$V_{sp} = (\pi/12) \times L \times W \times T. \tag{3}$$

This formula is derived from the integration of the area of an ellipse along the length of the spindle. The length of the major axis of the ellipse is the width and the length of the minor axis is the thickness. If one assumes a straight-sided spindle, the ratio of the major axis to the minor axis is constant anywhere between the spindle and the pole, which facilitates the integration.

Since

$$\Gamma_{sp} = T \times C_{br}, \tag{4}$$

then V-Br = volume $\times$ volume fraction

$$\begin{aligned} &= (\pi/12) \times L \times W \times T \times C_{br} \\ &= (\pi/12) \times L \times W \times \Gamma_{sp}, \end{aligned} \tag{5}$$

where length, width, and retardation are readily measurable and $\pi/12$ is the shape constant for a grasshopper spindle, assuming thickness is measured at the equator. This constant will change for spindles with different geometries. If the shape of a spindle is hard to define mathematically, it would be more appropriate to normalize the results of experimental spindles to control spindles. In such cases, the shape factor is eliminated and the ratio of the products of length, width, and retardation is calculated. By normalizing the results in this way, one assumes that spindle shape is similar for both experimental and control cells. In addition, thickness (as a part of the retardation measurement)

does not have to be measured at the equator so long as the point of measurement is constant in both experimental and control cells. As seen in this study, normalization may be useful if a large amount of biological variation exists in the system studied and the sample size is small. In the present study, normalized and actual volume-birefringence data give similar results (Fig. 7), although the actual measurements have a greater variance.

The Dynamic Equilibrium of Spindle Formation

The formation and disassembly of the mitotic spindle is a continuing, dynamic process in cell division. Even as the spindle reaches its maximally organized form at metaphase, the birefringent materials persist in a dynamic equilibrium with the subunits in the cell (Inoué, 1964; Inoué and Sato, 1967; Inoué et al., 1975; Inoué and Ritter, 1975). Studies of this dynamic equilibrium have centered on changing the equilibrium value by changing the kinetics of the assembly reactions and by changing the subunit pool size within the cell.

These studies and those discussed below use retardation to measure the concentration of anisotropic material in the spindle (Inoué and Sato, 1967). As discussed previously, retardation contains a thickness factor which must be considered in determining concentration changes in spindles. Therefore, quantitative interpretations based solely upon retardation measurements should be reinterpreted to consider all changes in spindle dimensions and concentrations. The qualitative interpretations, however, are still valid.

Spindle organization is sensitive to temperature (Inoué et al., 1975 and earlier; Fuseler, 1975) and pressure (Salmon, 1975a, b, c). Chemical inhibitors of mitosis, most notably colchicine and Colcemid, also act in decreasing spindle size (e.g. Sluder, 1976). In contrast, the spindles in eggs can be enlarged using D_2O (Inoué and Sato, 1967; Stephens, 1973) and several six-carbon glycols (Rebhun et al., 1974, 1975). The temperature and pressure experiments indicate the spindle behaves like a self-assembly system similar to tobacco mosaic virus or actin. In general, the system shows a large volume change, a high positive energy and enthalpy change, and a low, negative standard free energy change. The positive entropy and volume changes indicate the release of water normally bound to tubulin (and other?) subunits (Inoué et al., 1975). Changes in the thermodynamic parameters with D_2O treatment confirm this interpretation. D_2O may also affect spindle organization by changing the available tubulin pool within the cell (Stephens, 1973). Colchicine, glycols, and environmental temperature (Stephens, 1972, 1973) can affect spindle size by changing the number of subunits available for assembly.

Control of Spindle Organization by Organizing Centers

While the agents discussed above are used to study the spindle in living cells, they are not the agents which the cell uses to assemble or disassemble its spindle. Furthermore, most of these agents affect the whole cell and organizing center activity could be altered as well. In vitro assembly studies show that

isolated chromosomes and poles are competent to bind microtubules and/or nucleate the polymerization of tubulin subunits (Weisenberg and Rosenfeld, 1975; Telzer et al., 1975; McGill and Brinkley, 1975; Snyder and McIntosh, 1975). In addition, the microtubule-organizing ability of these centers depends on the time of the cell cycle and requires a minimum concentration of tubulin for assembly (Snyder and McIntosh, 1975).

In studying microtubule elongation in vitro, Bryan (1977) shows an interesting result which is applicable to the work discussed here. In a system where both the concentration of tubulin subunits and the number of nucleation sites (microtubule fragments) can be controlled, the number of nucleating fragments has not effect on the final equilibrium value attained by the system. If these biochemical studies model the dynamic system of the mitotic spindle, then spindle equilibrium would be controlled by subunit pool size and not by the number or activity of nucleating (organizing) centers in the cell. The experiments presented here suggest that the organizing centers of the cell (both kinetochores and poles) play a more complicated role in spindle assembly than can be deduced from current biochemical studies (Dietz, 1972).

Several important deductions about spindle organization can be made from the volume-birefringence data in Figures 6, 7, 10, and 13. These deductions, however, require certain assumptions to be made about the nature of the detachment of chromosomes from the spindle. The assumptions are 1) the point of detachment of a chromosome is at the junction of the kinetochore and the chromosomal fiber, 2) the birefringent components of the fiber depolymerize after detachment, and (3) the released subunits are then added to the pool of available subunits and are free to participate in the dynamic equilibrium of the spindle. Preliminary observations from electron microscopy (Brinkley and Nicklas, 1968) find no microtubules at the kinetochores of freshly detached chromosomes. Detached chromosomes show no evidence of having birefringent fibers (or portions of the fiber) in the polarized light microscope (Nicklas, 1967; Newton and Marek, unpublished observations). The polarized light observations could be influenced by two possibilities. The first is that the chromosomal fiber (still attached to the kinetochore) could splay apart, which would mean that the concentration of material decreases along the fiber and, more importantly, the fiber loses its 45° orientation to the polarizer-analyzer axes. Either situation would decrease the birefringence of the fiber. The second possibility is that some material is below the limits of detectability of the polarized light microscope and, therefore, only a small fraction of the total fiber material. While the preliminary observations from polarized light and electron microscopy indicate that the first assumption is reasonable, hard evidence to support or deny this assumption is not available.

The second assumption, that a detached fiber would depolymerize, is very likely true. Certainly in the present study, a lack of depolymerization would lead to no change in spindle volume-birefringence after chromosomes are removed. In addition, Nicklas and Koch (1969) have demonstrated that spindle fiber stability requires tension between the kinetochore and the pole. A detached fiber, therefore, is unlikely to be stable in the absence of tension. This argument holds for a fiber detached *from* a chromosome or *with* a chromosome.

The third assumption, that released subunits are capable of participating in further assembly, follows from acceptance of the first two assumptions and data presented here on 1) cells with chromosomes removed temporarily from the spindle, and 2) fused cells where chromosomes are transferred from one spindle to the other. In these experiments the subunits must remain in the cell and are incorporated into the spindle, but only as a chromosome interacts with the spindle. In many experiments where chromosomes are permanently removed, the volume-birefringence drops and then recovers to a reduced level. For example, the cell with two chromosomes left shows a reduced volume-birefringence (0.04 μm^3) and chromosomal fiber birefringence (Fig. 2, 127 min). After 1 h (187 min) the volume-birefringence has increased (to 0.06 μm^3) and chromosomal fibers are distinct, indicating the further organization of subunits in the cell.

For the present discussion, the assumptions are considered to be reasonable in the absence of any conflicting data. Nonetheless, the assumptions, and the conclusions drawn from these assumptions, must necessarily await firm proof of their validity.

Given the assumptions above, the experiments described here show that the amount of birefringent material in a spindle depends on the number of chromosomes in the spindle. Furthermore, extrapolation of the regression lines (Fig. 6) to the Y-axis (i.e., "no chromosomes") and then to the X-axis indicates a contribution of the polar organizers to overall spindle structure. *Arphia* poles can account for about 13% of the total spindle volume-birefringence and *Melanoplus* poles about 47%; the *Arphia* poles are equivalent to one *Arphia* bivalent in the amount of material they organize and in *Melanoplus* the poles equal about nine bivalents. These results are mirrored in the living cells. *Melanoplus* spindles have chromosomal fibers barely distinct from the background, interpolar fibers. In *Arphia,* however, the chromosomal fibers are much more prominent than the interpolar fibers. Removal of a bivalent therefore causes a greater loss of birefringent material in the *Arphia* spindle than in the *Melanoplus* spindle.

A significant polar contribution in *Melanoplus* spindle organization is supported by the volume-birefringence of the cell which eliminated two polar organizers during fusion (Fig. 13, Cell 5). The normalized volume-birefringence compares favorably with the value calculated for a spindle with 22 bivalents and only one set of polar organizers. The two eliminated asters of this spindle are also interesting. They formed no detectable spindle between themselves, even though they were adjacent to one another.

Hence, spindle organization in grasshopper spermatocytes is under both chromosomal and polar control. The polar contribution, however, seems to depend on interactions between the pole and the chromosomes, as just noted. The extent of the polar contribution, relative to the chromosomal contribution, varies greatly in different organisms (reviewed by Nicklas, 1971). In hypermastigotes a central spindle derived from the polar organizers provides virtually all the spindle structure (Cleveland et al., 1934; Kubai, 1973; Inoué and Ritter, 1975), but the nuclear membrane remains intact throughout division in these organisms. The kinetochores interact with the spindle through the nuclear membrane (Kubai, 1973).

In multi-astral eggs, asters without associated chromosomes do not normally form spindles between themselves (Wilson, 1925). Occasional exceptions may exist, however (cf. Wilson, 1925, Fig. 79G; Goldman and Pollack, 1974, Fig. 12). The separation of centrioles before nuclear membrane breakdown in many organisms might also be considered a non-chromosomal spindle. In this case, the ability to assemble microtubules may depend on the stage of the mitotic cycle for each type of organizing center (Snyder and McIntosh, 1975).

Chromosomal control of spindle formation and organization has been shown in earlier works on fixed materials. Galeotti (1893; see Fig. 73 in Wilson, 1925) showed differences in half-spindle size in human cancer cells with differing numbers of chromosomes oriented to the two poles. Juel (1897, plate 7, Fig. 17) demonstrated that spindle size is proportional to chromosome number in the pollenmother cells of *Hemerocallis fulva*. The most striking examples of the chromosomal organization of the spindle were seen in *Acroschismus wheelerii* and male llaveiine coccids (Hughes-Schrader, 1924, 1942), where individual chromosomal spindles form prior to their aligning to form the final spindle.

Chromosome Velocity, Spindle Elongation, and Tubulin Pool Size

As noted in the Results, the process of chromosome separation in anaphase is a combination of chromosome migration to the poles and spindle elongation. The removal of chromosomes presumably releases subunits of the birefringent fibers and yet this does not influence the dynamic equilibrium of the metaphase spindle. It is of interest to see if these additional subunits influence the two components of chromosome motion in anaphase.

Chromosome movement to the pole requires a shortening (disassembly) of the chromosomal fiber. The force used to move the chromosomes can be provided by the disassembly of only one microtubule (Nicklas, 1971; Inoué and Ritter, 1975). Alternatively, the microtubules may control and orient (Rickards, 1975) a force producer such as actin. The rate of disassembly of the microtubules would limit the rate of chromosome motion (Forer, 1974; Nicklas, 1975; Rickards, 1975; Salmon, 1975c). Fuseler (1975) finds that chromosome velocity is proportional to the decay of birefringent material in the spindle. The absolute spindle retardation value at the beginning of anaphase does not influence the subsequent chromosome velocity. The results of the present study confirm the independence of chromosome velocity and initial spindle organization (Fig. 14 and Table 4) as seen by Fuseler. Chromosome velocity is unrelated to the number of chromosomes in the spindle and, therefore, to the volume-birefringence of the spindle. These results also support Fuseler's conclusion that the disassembly process is irreversible and unpolymerized subunits no longer participate in a dynamic equilibrium with the spindle fibers during anaphase.

Spindle elongation might require a sliding of interpolar microtubules which overlap in the interzone of the spindle (McIntosh et al., 1969) or a pushing apart of the poles by growth of interpolar fibers (reviewed in Nicklas, 1971). Microtubule counts of interpolar microtubules at the interzone during anaphase give conflicting data. McIntosh and Landis (1971) favor a sliding hypothesis

to explain their observations of human tissue culture cells (WI-38 and HeLa). Brinkley and Cartwright (1971) find that microtubule growth is sufficient to explain their results in cultured rat kangaroo (PTK_1) and Chinese hamster (Don-C) cells. These authors postulate that the subunits involved in the growth are those subunits released from the chromosomal fibers during anaphase. This could not happen in hypermastigotes (Kubai, 1973; Inoué and Ritter, 1975) where spindle elongation may reach five times the original pole to pole distance and few, if any, chromosomal fibers shorten during anaphase. In grasshopper spermatocytes, the shortening of chromosomal fibers could provide new subunits which could participate in microtubule growth during spindle elongation. Excess subunits in the cell produced by micromanipulation do not affect spindle elongation in grasshopper spermatocytes, and it seems unlikely, therefore, that chromosomal fiber subunits directly cause microtubule growth and spindle elongation.

The ineffectiveness of excess subunit pools in changing chromosome velocity or the rate of spindle elongation, coupled with the dependence of volume-birefringence on chromosome number before division, are consistent with the hypothesis that the organization and function of the birefringent material during cell division are controlled by the organizing centers – the kinetochores and the polar organizers. Determination of the precise mechanism of this control awaits further study.

Estimates of Microtubule Content

The use of volume-birefringence to measure spindle organization requires that spindle retardation reflect the amount of polymerized material in the spindle. Whether or not the retardation of a spindle is caused completely by microtubule arrangement is unclear. Sato et al. (1975) argue that microtubules can account for all the spindle birefringence in the isolated oocyte spindle of the sea star *Pisaster ochraceus*. These spindles show no loss of retardation during the isolation procedures and contain only microtubules when viewed with the electron microscope. Forer (1976), on the other hand, argues that the microtubules alone cannot account for the birefringence seen in the chromosomal fibers of living spermatocytes of the crane fly – there must be additional contributions to the fiber birefringence.

If microtubules alone cause birefringence in the spindle, then the amount of birefringence can be estimated by Wiener's equation for rodlet birefringence. In particular, Bear et al. (1937) have approximated Wiener's equation in terms of measureable cell parameters: the volume fraction of rods in a structure (d_1), the remaining volume fraction of the surrounding medium ($d_2 = 1 - d_1$), the index of refraction of the rods (n_1) and the index of refraction of the medium (n_2). The formula is

$$C_{br} = \frac{d_1 d_2 (n_1^2 - n_2^2)^2}{2[(d_1 + 1) n_2^2 + d_2 n_1^2](d_1 n_1 + d_2 n_2)}.$$

Sato et al. have demonstrated that $n_1 = 1.512$ and $n_2 = 1.352$ in the isolated *Pisaster* spindles. If these values are used in the above formula, then the theoreti-

cal form birefringence is linearly proportional to the volume fraction of rods up to 0.20. Forer (1969) estimates the volume fraction of microtubules in spindle reported in the literature to range from 0.001 to 0.17, well within the linear portion of the curve. The slope of this curve is equal to 1.8×10^{-2} units of the coefficient of birefringence per unit volume fraction. Cassim et al. (1968) estimate the slope to be $(1.7 \pm 0.3) \times 10^{-2}$ for polymerized actin filaments. This estimate is empirically derived and avoids calculation dependent on Wiener's equation. The close agreement between the value obtained theoretically with the empirical data indicates that other proteins aligned with the microtubules would contribute to spindle birefringence. Using the above value and the data presented in this paper, we can explore the hypothesis that microtubules cause all the form birefringence of the spindle.

In order to estimate the amount of microtubules in a living cell, we also need to know the volume of the optically active microtubule subunit. Sato et al. (1975) have calculated this to be $119.6\,\mathrm{nm}^3$.

We can now predict the theoretical, maximal microtubule content of a spindle from the volume-birefringence, the volume of the subunit, the molecular weight of the subunit (110,000 Daltons), and the coefficient of birefringence per unit volume fraction (1.8×10^{-2}). These calculations are as follows:

1. to calculate the total weight of spindle microtubules:

a. $$\frac{\text{volume-birefringence}}{C_{br} \text{ per volume fraction}} = \text{volume} \times \text{volume fraction} = \text{total volume of microtubules}$$

b. $$\frac{\text{total volume of microtubules}}{\text{volume per subunit}} = \text{number of subunits}$$

c. $$\text{subunits} \times \frac{\text{mol.wt.}}{\text{Avogadro's No.}} = \text{grams microtubules}$$

For the control cells of *Melanoplus differentialis*, the calculations are

a. $$\frac{0.1\,\mu\mathrm{m}^3}{1.8 \times 10^{-2}} = 5.6\,\mu\mathrm{m}^3$$

b. $$\frac{5.6\,\mu\mathrm{m}^3}{119.6\,\mathrm{nm}^3} = 4.7 \times 10^7 \text{ subunits}$$

c. $$\frac{4.7 \times 10^7 \times 110{,}000}{6 \times 10^{23}} = 8.6 \times 10^{-12} \text{ grams of microtubules.}$$

For *Arphia xanthoptera,* with an average volume-birefringence of $0.3\,\mu\mathrm{m}^3$, the estimate is 2.6×10^{-11} gm. Cohen and Rebhun (1970) estimate the total amount of microtubules in the isolated spindles of *Arbacia punctulata* to be $1\text{–}2 \times 10^{-11}$ gm. Fuge (1974b) estimates the total length of microtubules in *Pales* (=*Nephrotoma*) *ferruginea* to be 23,150 μm. This corresponds to 6.9×10^{-12} gm. (The conversion factor is given below.)

To examine Forer's contention that the birefringence of a chromosomal fiber is greater than can be explained by the number of microtubules attached

to the kinetochore, we can further calculate the number of microtubules per chromosomal fiber from the regression lines given in Table 1. We assume there are 13 protofilaments in a microtubule and 125 subunits per one micrometer length of protofilament (Sato et al., 1975). A microtubule one micrometer long would weigh 3×10^{-16} gm. With these values, a *Melanoplus* spindle has a maximum of 2.9×10^4 micrometers of microtubules. If we subtract the percentage of material contributed by the poles (47%), divide by the average spindle length (29.5 μm), and divide by the number of chromosomes (11), we estimate that a chromosomal fiber has about 45 microtubules. Sato et al. find that about 10% of the *Pisaster* birefringence is intrinsic birefringence and not the form birefringence of the microtubules. The values calculated above might require correction for intrinsic birefringence, but the exact correction factor is unknown. It is probably about 10%, as in *Pisaster*.

The calculated microtubule number per chromosomal fiber of *Melanoplus* spindles is about twice the number seen at the kinetochore (20) with the electron microscope (Brinkley and Nicklas, personal communication). This is only a preliminary estimate from materials fixed under conditions in which a loss of up to 50% of the original spindle retardation occurs. Hence, there may be a loss of microtubules (and other material) during fixation. The discrepancy between electron microscopic data and estimated numbers from volume-birefringence measurements might also reflect our ignorance of the microtubule distributions along a chromosomal fiber. Several investigators comment that kinetochore microtubules become indistinguishable from other spindle microtubules a few micrometers from the kinetochore (Brinkley and Cartwright 1971; Fuge, 1971, 1973, 1974a; Roos, 1973; McIntosh et al., 1975). Microtubules are sometimes seen extending from a kinetochore to a pole, but not often, and many microtubules lie between the chromosome and the poles without extending to either. These microtubules have been termed free microtubules and some are as short as 0.2 μm (Fuge, 1974a; McIntosh et al., 1975). In anaphase the intermingling of kinetochore microtubules with other microtubules is clear (Jensen and Bajer, 1973; Fuge, 1974a).

Forer (1976) states that he and Brinkley find a density of 52 microtubules per μm^2 at *Nephrotoma suturalis* kinetochores, whereas LaFountain's (1974) counts indicate a density of 110 microtubules per μm^2 [Forer's calculation]. LaFountain's counts are taken away from the kinetochore—that is, the cross-section of the spindle is along the fiber. The increased density in LaFountain's data could be due to an interdigitation between microtubules growing out of the kinetochore, microtubules growing from the poles toward the kinetochore, and the free microtubules. Such interdigitations would give a greater number of microtubules along the fiber than could be accounted for by counts at the kinetochore. In fact, Forer (1976) states that the birefringence of a kinetochore fiber is maximal three to four micrometers from the kinetochore.

Additional support for microtubule interdigitation can be found in the work of Cohen and Rebhun (1970). They counted spindle and astral microtubules in isolated spindles of *Arbacia punctulata*. The average half-spindle had 2,091 microtubules and the average aster 1,312 microtubules. Since the half-spindle occupied 5.6% of the astral volume, then about 73 microtubules in the half-spindle

should be contributed by the aster. This means that the remaining microtubules in the half-spindle should be contributed by the 36 to 38 kinetochore fibers, or about 55 microtubules per fiber. Cohen and Rebhun indicate that only 10–20 microtubules are seen at the kinetochore in their preparations. Hence, there are more microtubules in the half-spindle than can be explained by the number of microtubules attached to the kinetochores; this may well explain some of the discrepancy between the volume-birefringence and the number of microtubules per kinetochore, especially given the possibility of some microtubule loss during fixation.

Increased evidence implicates actin as a real component of the spindle (reviewed by Forer 1976; Cande et al., 1977). The alignment of actin filaments with spindle microtubules would add to the birefringence seen in living cells (Cassim et al., 1968).

Hence, predictions of microtubule number from volume-birefringence measurements, while feasible, emphasize our continuing uncertainty of the molecular components of the spindle. Certainly, volume-birefringence can estimate the maximal number of microtubules in a spindle which would be expected if microtubules cause all the form birefringence seen in polarized light.

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