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Reprint from: Handbook of Molecular Cytology, edited by A. Lima-de-Faria, 1969.
North-Holland Publishing Company, Amsterdam · London

CHAPTER 23

Chromosome movements during cell-division

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1. Introduction

This chapter is concerned with chromosome movements during anaphase of cell-division. It deals with such problems as the morphological identification of the material(s) which transmit force to the chromosomes, and the composition and function of this material. At the present time very little is known about such problems, but I hope that this survey will point out some of the questions which need be, and can now be, asked.

The material will be discussed in the following order: (a) the time course of cell-division as seen in living and fixed cells, defining the terms to be used, and defining the problem: chromosome movements during anaphase; (b) the morphological and chemical nature of the materials controlling chromosome movement as deduced from light microscopic observations and experiments; (c) electron microscopic observations and their relation to the light microscopic observations; and finally, (d) biochemical analyses of the spindle as studied by isolation techniques.

In all four sections I touch on many problems which are discussed in more detail in the following general reviews: Wilson; Cornman; Hughes; Schrader; and Mazia (1961).

2. Description of normal cell-division

The course of cell-division is as follows (see general reviews, above, for details and references).

Chromosomes are not seen within interphase nuclei, but become distinguishable in prophase nuclei. After nuclear membrane breakdown the chromosomes are seen inside a spindle-shaped region called the spindle. All or some of the chromosomes move within the spindle (toward and away from the poles) during prometaphase, and generally all chromosomes are at the equator during metaphase. The mitotic chromosomes divide (or meiotic chromosomes disjoin into half-bivalents) and move poleward during anaphase. This poleward movement can be primarily a shortening of the chromosome-to-pole distance while the pole-to-pole distance is constant, or it can be primarily an increase in the pole-to-pole distance while the chromosome-to-pole distance is constant, or it can be a mixture of the two. In this article I shall be concerned primarily with the first case.

Generally, all the chromosomes in one cell begin anaphase at the same time, and move poleward with about the same velocity. But in some cells various chromosomes regularly move poleward at different times; in some cells different chromosomes regularly have different velocities.

Chromosome velocities during anaphase range from 0.2 to 5.0 μ per minute, and each chromosome's velocity is approximately constant throughout anaphase. Pole-to-pole distances are of the order of 10–30 μ, and, as expected, the time between chromosome

separation (start of anaphase) and chromosome arrival at the poles (end of anaphase) is in the range 2–60 min, depending on the conditions and the cell in question.

The chromosomes reach the poles (telophase), and cell cleavage then ensues.

Cell-division is illustrated in Figs. 1 and 2, which show living crane fly spermatocytes photographed using a phase-contrast microscope. These cells are particularly favorable for studies of cell-division, for the pole-to-pole distances are of the order of 20–30 μ and there are only five chromosomes, usually less than 4–5 μ long; further, the chromosomal velocities in anaphase are of the order of 0.5 μ per minute, and anaphase lasts 30–40 min. As is seen in the photographs, the 2 sex-chromosomes do not form a bivalent, and do not move poleward at the same time as the autosomes.

The spindle area is not structureless in fixed and stained cells, but contains spindle fibres. The spindle fibres are of two kinds: *chromosomal spindle fibres* (or, for short, chromosomal fibres), which extend between individual chromosomes and the poles, and *continuous* (spindle) *fibres*, which extend from pole-to-pole, without connection with chromosomes. The chromosomal fibres can be from 0.2 to 1.5 μ in diameter, and often appear to be made up of many units of smaller diameter. The continuous fibres are of the order of 0.2 μ in diameter.

The chromosomal fibres attach to the chromosomes at specialised regions called kinetochores, chromosomes either having one localized kinetochore, or having a diffuse kinetochore spread throughout the length of the chromosome. During anaphase, chromosomes with localized kinetochores move poleward with the kinetochore in front and with the chromosomal arms trailing, while chromosomes with diffuse kinetochores move poleward as one straight unit.

In animal cells astral fibres extend radially from each pole, forming the asters.

The region between the chromosomes and the pole is the half-spindle, and the region between the separating anaphase chromosomes is the interzonal region. Ex-

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The photographs are all of *Nephrotoma suturalis* (Loew) primary spermatocytes. Fig. 1–7 are of living cells; Figs. 1 and 2 were taken through a phase-contrast microscope (objective-lens NA = 1.25), and Figs. 3–7 through a polarizing microscope (objective-lens NA = 0.6). Figs. 8–13 are electron micrographs, of which Figs. 8, 9, and 11 were generously provided by Dr. O. Behnke. Details of the techniques are given elsewhere (Forer 1964, 1965, 1966; Behnke and Forer 1966, 1967).

In Figs. 1–5 the times are given relative to the time of nuclear membrane breakdown, and in Figs. 6 and 7 the times are given relative to the time at which the irradiation took place.

In the phase-contrast microscope micrographs the chromosomes are dark (e.g., *b* in Fig. 1) and the spindle appears in large part structureless. In the polarizing microscope micrographs the spindle fibres appear bright (e.g., *sf* in Fig. 4), and the chromosomes (*b*) have little contrast; for those not familiar with polarizing microscope images, the amount of birefringence is roughly proportional to the brightness relative to the background.

Fig. 1 (overleaf) shows one cell progressing from before nuclear membrane breakdown to late anaphase. At 90 min after nuclear membrane breakdown 2 bivalents are illustrated, and one is labelled *b*; the third bivalent is in a different plane of focus. The sex-chromosomes (one of which is labelled *u*) do not move poleward until the autosomal half-bivalents have reached the poles. × 2,050.

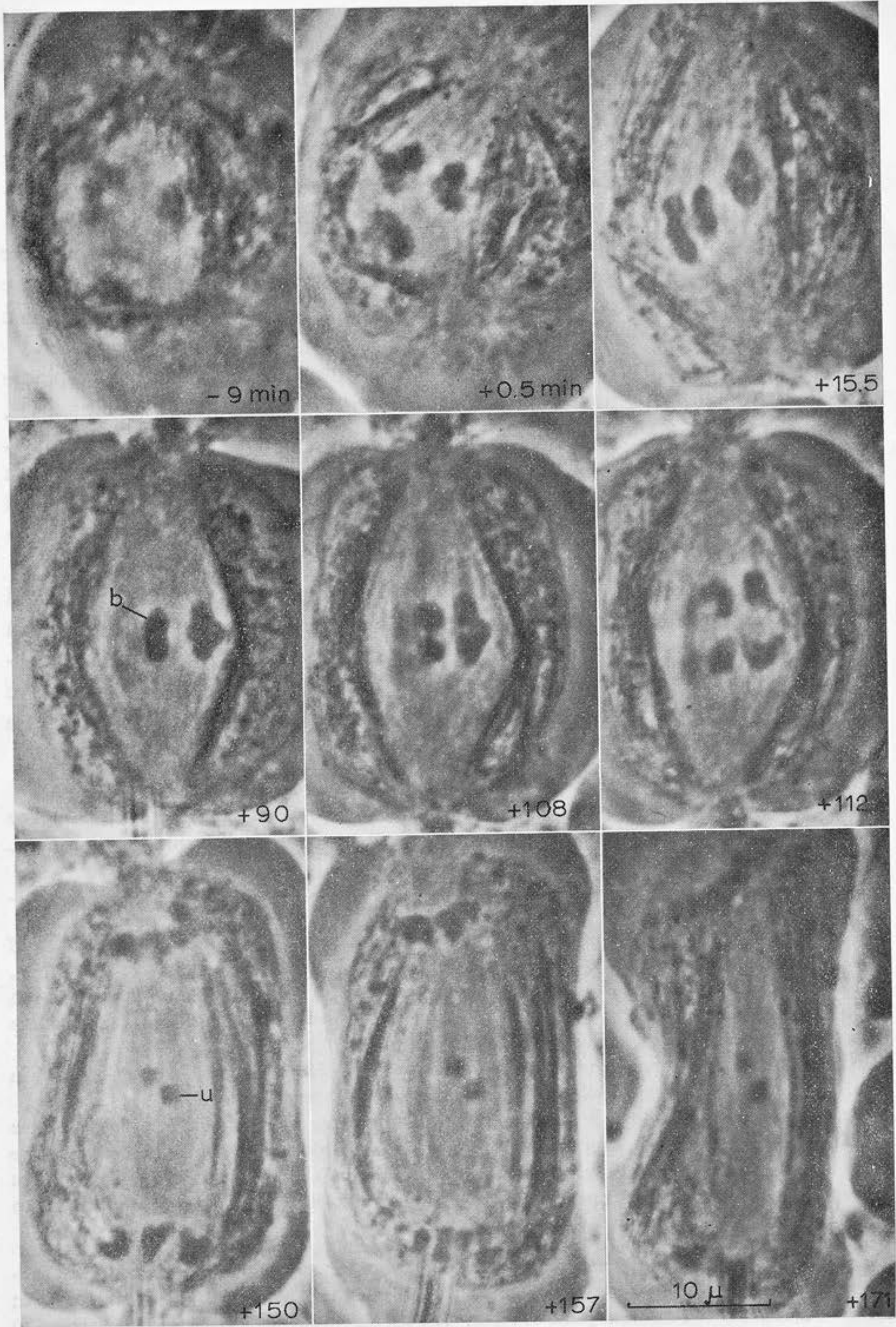
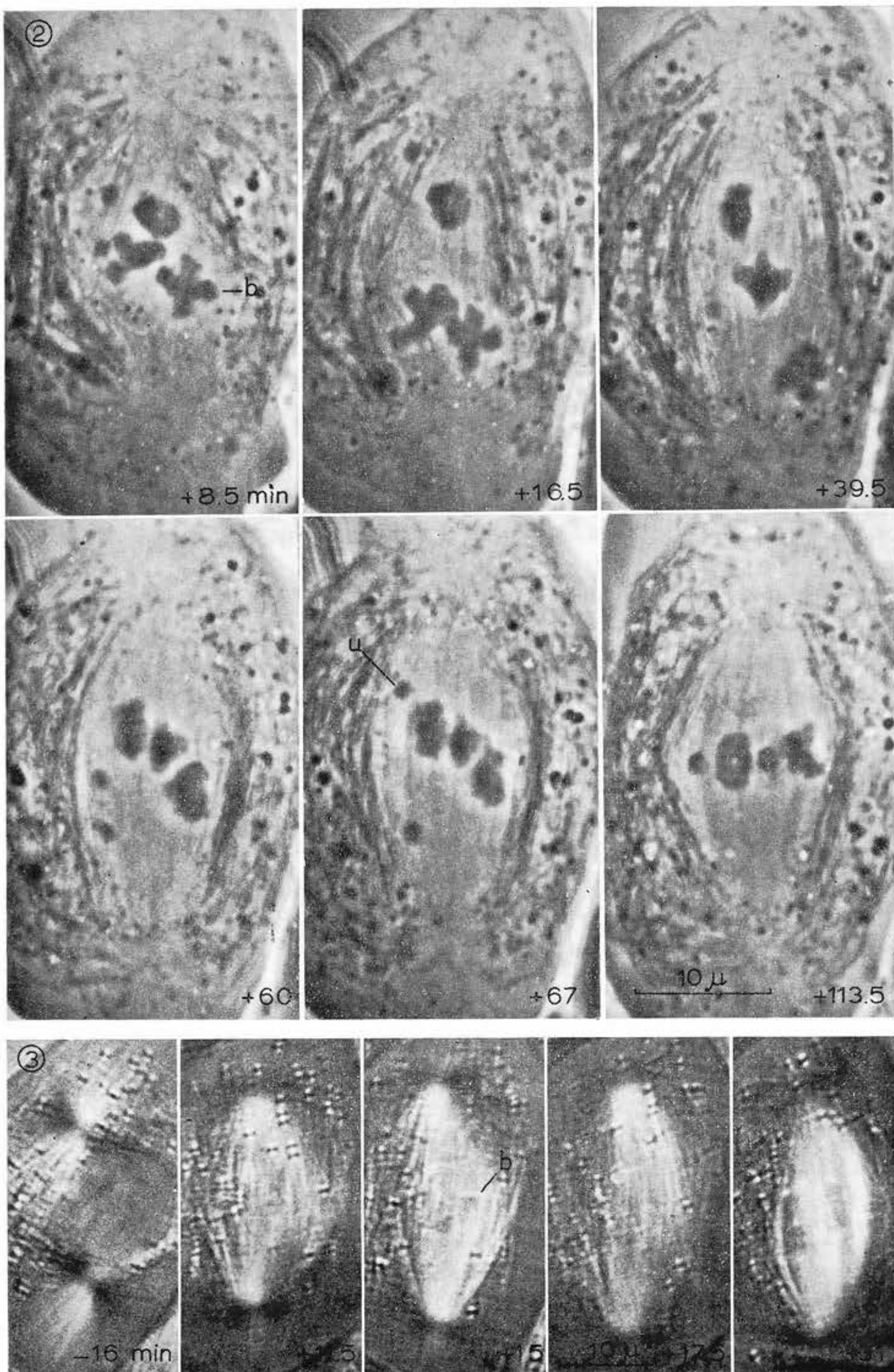


Fig. 1. *(Caption on p. 555.)*



Figs. 2 and 3. (*Capitions on p. 558.*)

tending across the interzonal region are often found fibres which stain differently from spindle fibres; since these fibres appear to connect the separating chromosomes, they are called interzonal connections.

Collectively all of these structures constitute the 'mitotic apparatus', abbreviated MA, which, as defined by Mazia and Dan, is "the ensemble of structures constituting the 'chromatic' and 'achromatic' figures in the classical descriptions of mitosis".

Generally, spindle fibres can not be seen in *living cells* with bright-field microscopy, phase-contrast microscopy, dark-field (dark-ground) microscopy, ultraviolet absorption microscopy, or interference microscopy. Spindle fibres can be seen with these microscopes in fixed cells, in moribund cells, or in cells treated with acid (in the latter case the effect is reversible), but spindle fibres are not seen with these microscopes in healthy, living cells.

Spindle fibres can be demonstrated in living cells, however, with a sensitive polarizing microscope, and these fibres appear morphologically the same as in fixed and stained cells (reviewed by Inoué and Sato). This is illustrated in Figs. 3–5, in crane fly spermatocytes. Spindle fibres are weakly birefringent (generally of the order of 1 mμ) and the birefringence is positive with respect to the length of the fibre. (I use the term 'birefringence' to mean (thickness) *times* (difference of refractive indices), and use the term 'coefficient of birefringence' to refer to the difference of refractive indices, both referring to measurements with light of wavelength 5460 Å.) In animal cells the chromosomal spindle fibre birefringence is strongest near the kinetochores and near the poles, and weakest in the region in between. This condition applies in metaphase, and prevails throughout most of anaphase. In plant cells the chromosomal fibre birefringence is strongest near the kinetochores and gradually becomes weaker toward the poles, being weakest at the poles. This, too, applies to metaphase and most of anaphase.

Continuous fibre birefringence generally is strongest in early prometaphase, and is very weak during metaphase and early and middle anaphase. Continuous fibre birefringence generally increases during late anaphase.

Polarizing microscope photographs of living crane fly spermatocytes are presented in Figs. 3–5, for comparison with phase-contrast photographs of the same cell type (Figs. 1, 2). In these cells, at metaphase and anaphase each chromosomal spindle fibre is visible, there are 5 chromosomal fibres going to each pole, and each fibre is

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Fig. 2 (overleaf) shows one cell progressing from early prometaphase to metaphase, illustrating the prometaphase movements of the bivalents (one of which is labelled *b*) and the sex-chromosome univalents (one of which is labelled *u*). ×1,850.

Fig. 3 (overleaf) shows one cell progressing from prophase to early prometaphase. In these stages continuous fibre birefringence predominates and the chromosomes are difficult to see clearly (one bivalent is labelled *b*). ×1,350.

about 1 μ in diameter, with strongest birefringence (3–4 mμ) near the kinetochore and weakest birefringence near the pole. Continuous fibre birefringence predominates in early prometaphase (Fig. 3), but there is very little or no continuous fibre birefringence during metaphase and anaphase (Figs. 4, 5).

Fig. 4

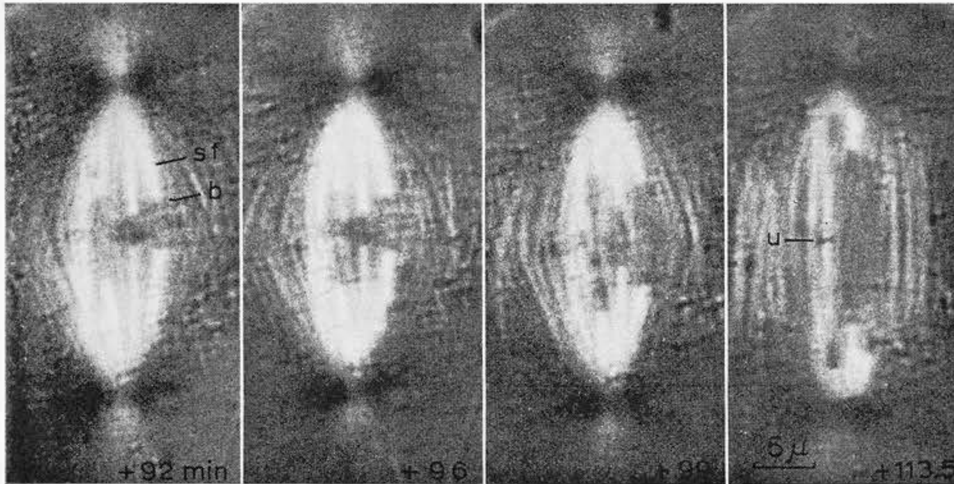
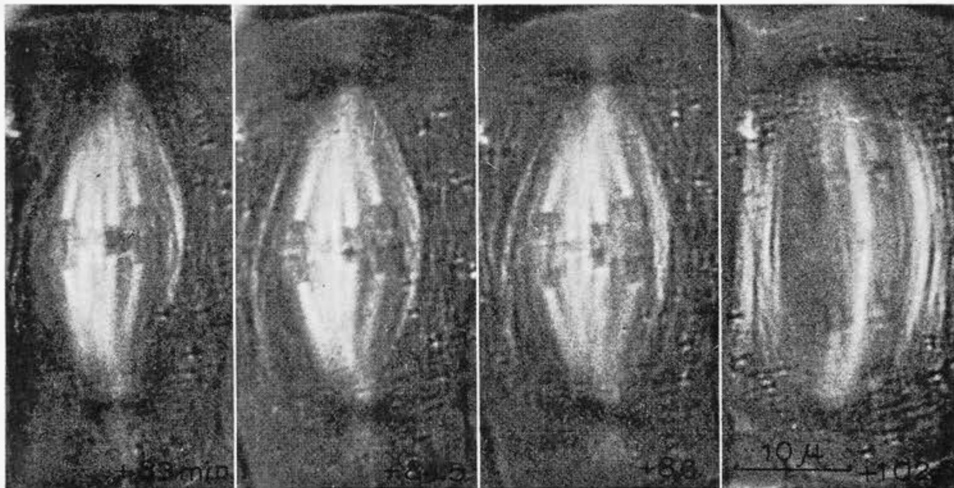


Fig. 5



Figs. 4 and 5 each illustrate individual cells progressing from metaphase to middle or late anaphase. In these stages there is very little continuous fibre birefringence, and though the bivalents are of low contrast (one is labelled *b*), they are easily identified at the termination of the clearly visible chromosomal fibres (one of which is labelled *sf*). Each univalent (one is labelled *u*) has a chromosomal spindle fibre extending to each pole. Fig. 4: $\times 1{,}590$; Fig. 5: $\times 1{,}380$.

TABLE 1

Chemicals present in half-spindle region

Cell	Protein	RNA	Lipid
Various cell types	Present, and constitutes most of the material in isolated MA	Present (cytochemically); about 5% by weight of isolated MA	Present in isolated MA
Primary spermatocytes of grasshopper (*Podisma sapporense*)	Present, even after RNAase removes RNA staining. Strongest staining near poles	Strongly staining and strongest near chromosomes	Present
Lilium pollen mother cells *Allium* root tip cells *Lilium*, *Pisum*, *Zea*, and *Vigna* root tips	Present; strongly staining	Strongly staining	—
Tradescantia pollen mother cells, and *Spirogyra* cells	Present. Spindle fibres stain differently from 'ground substance of the spindle'	—	—
Vicia faba root tip cells. *Tradescentia* pollen mother cells	—	—	Present in spindle fibres
HeLa cells; Ehrlich ascites tumor cells	Present. Spindle fibres stain strongly for tyrosine	— —	Present in spindle 'background'; stains most strongly near chromosomes
HeLa	—	Present	—
Various sea urchin eggs	Present	—	—
Various plant cells, meiotic and mitotic	—	—	Present in spindle
Meiosis and mitosis in *Artemia salina* eggs	—	—	—
Sea urchin eggs *Acanthocidaris crassispina* and *Pseudocentrotus depressus*		—	—

Carbohydrate	ATPase	Remarks	References
Present, cytochemically. Strongest staining near poles and equator	Present in isolated MA	ATPase is Mg^{2+} activated. Zinc and SH groups are also present. Phosphatase is not present	Mazia (1961)
Present	—	Studied cytochemically	Kobayashi and Makino
Strongly staining Faintly staining Very faintly staining – almost none	— — —	Studied cytochemically	Shimamura and Ôta
—	—	Studied cytochemically	Shinke and Shigenage
—	—		
—	—	Studied cytochemically	Love and Suskind
—	—	Detected by incorporation of radioactive RNA precursors	Errera and Brunfaut
—	—	Detected by incorporation of radioactive amino acids	Stafford and Iverson; Gross and Cousineau; Bibring and Cousineau
—	—	Detected cytochemically	Serra and Seixas
Present. More intensely staining near the chromosomes and the poles; weaker in between. Spindle fibres stain	—	Detected cytochemically	Fautrez and Fautrez-Firlefijn
—	Present, functional at pH 6.2 and pH 8.2	Detected cytochemically. Neither acid phosphatase nor alkaline phosphatase are present in spindle	Miki (1963); Miki (1964)

TABLE 2

Dry mass of *in vivo* spindles and cytoplasm

Cell	g dry mass per 100 cm^3 of spindle	g dry mass per 100 cm^3 of cytoplasm	Method	Remarks	References
Endosperm cells: *Leucojum aestiveum*	$\geqslant 11$ (metaphase) $\geqslant 22$ (anaphase)	$\geqslant 8$–9	Interference microscopy	These values are calculated from the stated values of phase retardations, and are *minimum* estimates, assuming spindle refractive index increment of 0.20, and assuming that the spindle occupies the entire 5 μ thickness of the cell	Ambrose and Bajer
Sea urchin eggs: *Psammechinus miliaris*	23	25.5	Interference microscopy	The spindle concentration is calculated from the stated cytoplasm concentration, and the statement that spindle concentration is 0.89 times cytoplasm concentration	Mitchison and Swann
Grasshopper spermatocytes: *Chortippus parallelus*	$\geqslant 11$	~ 11	Immersion refractometry with phase contrast and interference microscopes	The spindle value is deduced from the stated cytoplasm concentration and the appearance of spindle fibres as slightly darker than the cytoplasm in the published photographs	Barer and Joseph
Fixed eggs of *Cyclops strenuus*	50	?	X-ray absorption	This value is calculated from the stated dry mass and volume of spindle	Stich and McIntyre

Chick fibroblasts	Optical density is 0.28	Optical density is 0.3	Ultraviolet microscopy at wavelength 2650 Å	—	Davies
Grasshopper spermatocyte: *Podisma sapporense*	Spindle proteins stain more strongly than cytoplasmic proteins		Cytochemical staining for protein	—	Kobayashi and Makino
Various plant root tip cells, and pollen mother cells	Spindle proteins stain with about the same intensity as cytoplasmic proteins		Cytochemical staining for protein	—	Shimamura and Ôta
Various animal cells	The spindle absorbs ultraviolet light to about the same extent as does the cytoplasm		Ultraviolet microscopy at wavelength 2537, 2650 or 2750 Å	This is stated by authors, or is inferred from the lack of contrast between spindle and cytoplasm, in published micrographs	Bradfield; Lucas and Stark; Wyckoff, Ebeling and TerLouw; Wyckoff; Harvey and Lavin;
Carnoy fixed microsporocytes of *Zea mays*	The spindle has a higher concentration of dry matter than the cytoplasm		Interference microscopy	—	Longwell and Mota

In summary, spindle fibres seen in fixed and stained preparations can be seen in living cells only with a sensitive polarizing microscope. In general, the birefringent spindle fibres have their strongest birefringence near the kinetochores and near the poles, and this pattern is maintained throughout anaphase. During anaphase, chromosomes move poleward with a constant velocity, of the order of 0.2–5.0 μ per minute, moving with the kinetochores leading the way to the poles.

3. *Studies using light microscopy*

I have briefly described the course of cell-division as seen using light microscopes. The remainder of this article is concerned mainly with identifying the cause of chromosome movement, and discerning how this movement is controlled. This section deals with evidence from light microscopy. I approach the problem by discussing: (a) the chemical nature of spindles (kinds and amounts of materials); (b) the organization and physical nature of spindles; (c) the forces and energies needed for chromosome movement; and (d) characteristics of the force system which can be specified independently of any particular theory of chromosome movement. Using this information as background, I then discuss (e) the identity and function of the component(s) causing chromosomes to move.

(a) *The chemical nature of spindles*

Chemicals which have been found in spindles include: protein, RNA, lipid, carbohydrate, and zinc; enzymes present include ATPase (Table 1). Phosphatase has not been found (Table 1). This information derives mostly from cytochemical studies, and, in a few cases, from autoradiographic detection of precursor incorporation.

Various reports agree that there are differences in stainability within individual spindles: proteins, RNA, carbohydrate, and -SH groups stain strongly near the kinetochores and near the poles, and less strongly in between (Table 1). This is similar to the birefringence pattern discussed in the previous section.

These techniques give only qualitative results, however, of strong or weak staining, and of presence or absence. Quantitative data have been obtained as well, using a variety of techniques, and some results are listed in Table 2.

These results show, firstly, that the concentration of dry matter in the spindle is about the same as that in the cytoplasm. This is true for both quantitative and qualitative estimates, using a variety of techniques. Thus, the fraction of the cell's dry mass which is in the spindle is about the same as the fraction of the cell's volume which is occupied by the spindle. If, e.g., MAS in *Arbacia* eggs are 10% of the volume of the egg (Kane 1967), then these MAS are 10% of the mass of the egg.

Secondly, spindles contain of the order of 10–20 g of dry matter per 100 cm^3 of spindle volume (Table 2). While the data give concentration of material, the exact composition of this material can not be specified, because interference microscopy does

not distinguish between proteins, nucleic acids, carbohydrates, etc. (see Davies and Wilkins).

Thus, spindles contain protein, RNA, carbohydrate, and lipid totalling to a concentration of 10–20 g of material per 100 cm^3 of spindle; this is about the same concentration of dry matter as the cytoplasm. Spindle material is not evenly distributed throughout the spindle, but stains more strongly near the kinetochores and near the poles.

(b) *The organization and physical nature of spindles*

Spindles are in some senses *stable*, for they remain intact as a unit after being taken out of cells (see last section), and they can be pushed *en masse* through the cytoplasm by applying a microneedle to any point on the spindle (Carlson). The mechanical rigidity of a spindle fibre is more than enough to support the stretching of attached chromosomes. This is shown by micromanipulation experiments (Nicklas and Staehly): metaphase meiotic chromosomes can be stretched to more than 3 or 4 times their original length while the attached spindle fibres do not change in length.

While in some respects stable, spindle fibres are nonetheless quite *labile*, as follows. A chromosome can be detached from the spindle by micromanipulation, and the chromosome reforms a spindle fibre; this operation can be repeated many times (Nicklas 1967). Similarly, some chromosomes regularly break their connection with one pole, turn around, and make a connection with the other pole; this too may occur several times during the course of one division (reviewed by Nicklas 1967). Many other factors can cause changes in spindle volume and birefringence (reviewed by Inoué and Sato). These include changes in external *p*H, temperature, and pressure; application of chemicals; substitution of D_2O for H_2O; and stretching of spindles. With many of these agents spindle fibre birefringence can be made to disappear reversibly, such that upon return to the original conditions the birefringent fibres reappear.

Because of this lability, and because of changes in spindle birefringence during the division cycle, Inoué suggested that spindle fibres are in 'dynamic equilibrium' between a non-birefringent state, (A), of non-oriented material, and a birefringent state, (B), of oriented material (reviewed in Inoué 1964). This is written as $(A) \rightleftarrows (B)$.

To further characterize this equilibrium, Inoué and co-workers subjected some of their data to thermodynamic analysis (reviewed in Inoué and Sato). Below a certain temperature the birefringence is zero, and as the temperature is raised the birefringence increases to a maximum value, and may decrease again with further increase in temperature. Values for enthalpy changes (ΔH), free energy changes (ΔF), and entropy changes (ΔS) were calculated from the data of birefringence vs. temperature. The calculated parameters seem similar to those measured during tobacco mosaic virus polymerization and other polymerizations involving water displacement, so Inoué and co-workers inferred that water displacement is involved in the polymerization of (A) to form the birefringent spindle, (B). I shall now consider the assumptions

made in performing the calculation, and suggest other possible interpretations.

Besides the basic assumption of one-to-one stoichiometry in the reaction (A) ⇄ (B), the following four assumptions are made:

(1) *The amount of oriented material (B) is directly proportional to the birefringence.* While this seems a reasonable assumption, it is also possible that some of the change in birefringence is due to change in other parameters, such as orientation, stress and intrinsic birefringence, etc. Further, I argue in the following section that more than one component contributes to spindle fibre birefringence. If this is true, the thermodynamic treatment might be greatly affected, depending on the relationships of the contributing materials, and the temperature sensitivities of their chemical reactions.

(2) '*Only the equilibrium constant between oriented and nonoriented material is influenced by temperature*' (Inoué 1959). But entire cells are treated, and the temperature change could affect all reactions in the cell. It is possible, for example, that the birefringence reaction, (A) ⇄ (B), is a temperature-insensitive link in a series of reactions (X) ⇄ (Y) ⇄ (A) ⇄ (B) ⇄ (C) ...; if so, the temperature could primarily affect another reaction, and birefringence would be changed via the effects on the other reactions in the series. Thus, while *some* temperature sensitive reaction might have ΔS, ΔF, and ΔH values as calculated, this would not be the reaction (A) ⇄ (B). The following results favor this possibility.

Temperature changes affect the birefringence of *in vivo* spindles within a period of seconds, or minutes, yet temperature changes do not affect the birefringence of isolated spindles (i.e., spindles released from cells) during such small time periods (e.g., Kane and Forer). Temperature changes do affect the birefringence of isolated spindles as measured over time periods of hours, but this effect is *opposite* to that found *in vivo*: with isolated MA, cold temperatures *retard* the loss of birefringence which occurs at higher temperatures (Kane and Forer; Rebhun and Sander). Though these data are open to more than one interpretation, one distinct possibility is the one under discussion. Thus, it seems quite possible that the effect of temperature *in vivo* is not directly on the equilibrium between oriented and unoriented states, but rather on another reaction, and birefringence is affected via a coupled reaction (or reaction series) which is not present in isolated spindles. (I argue in the last section that there is indeed loss of material when MA are isolated.)

(3) *The maximum amount of unoriented (pool) material is fixed.* Inoué and Sato give supporting evidence for this, using protein inhibitors.

(4) *The maximum amount of unoriented material, called (A_0), is given by the plateau of the curve of birefringence vs. temperature.* If this is true, the plateau birefringence should never be exceeded. However, in the same cells for which the calculations were made, birefringence does exceed (A_0) when spindles are stretched by flattening (Inoué 1952), or when spindles are treated with D_2O (Carolan, Sato, Inoué 1965, 1966). One might try to avoid this internal inconsistency by arguing that some (A) material is in equilibrium with (B) while other (A) material is in an inaccessible compartment, and that these treatments cause (A_0) to be exceeded merely be opening this compartment

and making *all* (A) material accessible. But it is difficult to imagine that two such different treatments would unlock the same compartment. Thus the plateau can be exceeded and assumption 4 is not valid.

The use of the original (A_0) produces a straight line of ($\log B/(A_0-B)$) versus (1/temperature) which is quite striking compared with curves obtained using other values for (A_0). Nonetheless since (A_0) can clearly be exceeded, (A_0) is not the maximum amount of material present, and the interpretation of the calculation is not clear.

I should make it quite explicit that this discussion of the above 4 assumptions does not affect the idea of a 'dynamic equilibrium', but rather affects only the interpretation of the thermodynamic analysis.

I should also point out that the thermodynamic analysis is based solely on data from non-functional spindles, i.e., spindles which are arrested in metaphase and which will not cause chromosome movement unless they are further activated. Low temperature does cause reversible disappearance of birefringence from both arrested and functional spindles (Inoué 1964), but neither temperature vs. birefringence curves nor thermodynamic analyses have yet been published on functional spindles. Thus non-functional spindles may be different from functional spindles.

Summarizing the conclusions to this point: Spindle fibres are attached to chromosomes, and are mechanically rigid enough to support the stretching of attached chromosomes. On the other hand spindle fibres are labile and can be reversibly made to disappear. Birefringent spindle component(s) are in equilibrium with non-oriented component(s), but it is not clear if the agents causing spindle disappearance affect the birefringence reaction directly, or through coupled reactions. The molecular interpretation of this equilibrium is as yet uncertain.

I now consider how the birefringent material becomes organized.

It has been argued that spindle fibres are organized by kinetochores and spindle poles (centrioles). Mazia (1955), for example, suggested this because isolated spindles were least soluble near the poles. Such an idea might also account for the manner of formation of spindles, and for the differences in staining properties within spindles.

The regions responsible for the organization of spindle fibres were termed 'orienting centres' by Inoué (1964), and Inoué (1959) suggested that the birefringence is stronger adjacent to an orienting centre than it is in the rest of the structure. Orienting centres, then, can be detected in living cells using this criterion, and for spindle fibres the orienting centres would be kinetochores (in all cases) and spindle poles (in some cases). This criterion is tested in the following way.

Consider a chromosomal spindle fibre with strongest birefringence near the chromosome, and weakest birefringence near the pole. The kinetochore, then, would be the sole orienting centre. If the birefringence were locally destroyed on a portion of the fibre poleward from the kinetochore, the repair of this fibre should occur in the poleward direction, i.e., away from the orienting centre (kinetochore). Thus if ultraviolet light were focused on a portion of a spindle fibre, the birefringence of that portion would be expected to disappear and the regeneration of new fibre should occur from

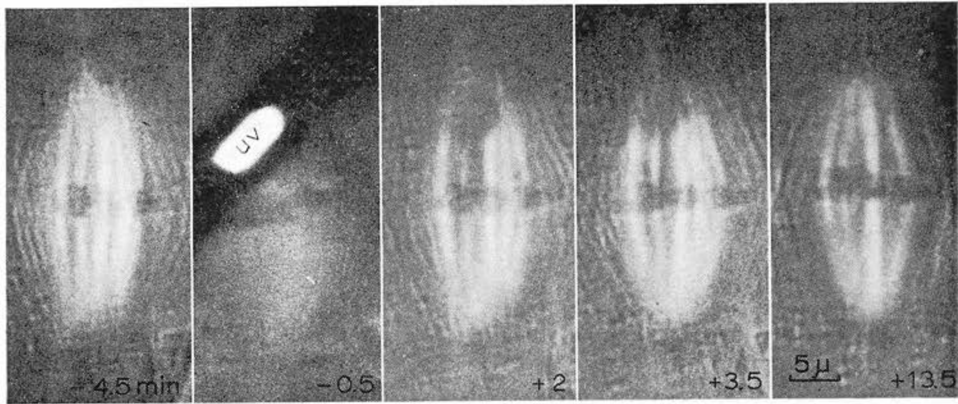


Fig. 6

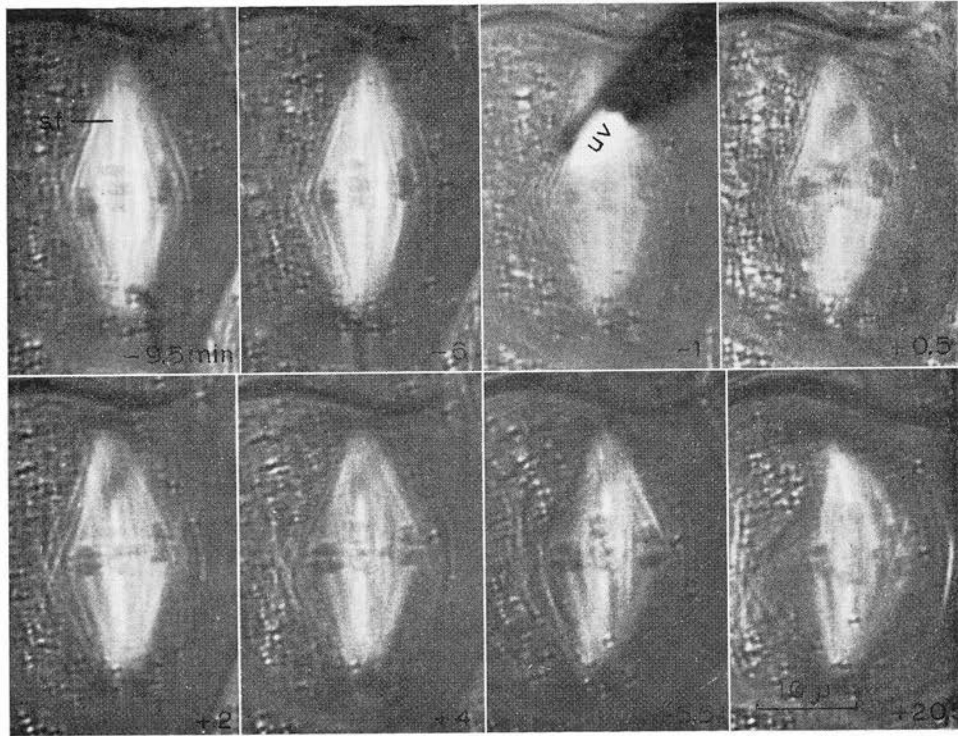


Fig. 7

Figs. 6 and 7 each illustrate the effect of ultraviolet microbeam irradiation on metaphase spindle fibres. The image of the ultraviolet source is labelled *UV*. Irradiation produces an area of reduced birefringence, and this area moves to the pole while the metaphase chromosomes remain at the equator. (Fig. 7 is the exceptional case cited in Forer (1966): all half-bivalents disjoined shortly after irradiation, and the half-bivalent associated with the irradiated chromosomal fibre moved poleward when the 'lesion' moved poleward, while the other 5 half-bivalents remained at the equator until the 'lesion' reached the pole.) Fig. 6: ×1,310; Fig. 7: ×1,300. (Fig. 6 from Forer 1965.)

the kinetochore poleward. (The portion of the fibre poleward from the irradiated region might be expected to retain or to lose birefringence, depending on what exactly the ultraviolet radiation does, and on how the orienting centre works.) In fact, that is what is found when the experiment is done.

When ultraviolet light is focused to a portion of a spindle fibre in metaphase crane fly spermatocytes, the birefringence disappears in the irradiated portion and is unaffected on both sides of this area (Figs. 6 and 7; Forer 1965). The irradiation-induced 'lesion' immediately moves poleward and a normal-looking spindle fibre is formed; the metaphase chromosomes remain at the equator during this process (Figs. 6 and 7; Forer 1965). Similar results are obtained with *Haemanthus* endosperm cells (Inoué 1964), in which spindle fibre birefringence is weak near the poles.

Thus, birefringent spindle fibres are organized by (and outward from) 'orienting centres' and stronger birefringence is diagnostic for 'orienting centres' in living cells.

I should point out that in discussing the general properties of spindles and their organization, I have not distinguished between stages of cell-division; yet there are distinct differences between metaphase and anaphase spindles. While anaphase spindles are sensitive to some of the same agents as are metaphase spindles, and can be reformed after having been made to disappear (e.g., Inoué 1964; Forer 1965, 1966), distinct differences are seen with micromanipulation (Nicklas and Staehly), D_2O-induced volume changes (Inoué and Sato), spindle reformation after cold treatment (Inoué 1964), effects of ultraviolet microbeam irradiation (Forer 1966), and effects of chemical agents (Mazia 1961); many of the latter destroy spindles during metaphase but are no longer effective during anaphase, "as though the structure 'locks'" (Mazia 1961). These dramatic differences between metaphase and anaphase are detected only physiologically, however, and as of yet have not been seen as differences in staining, or in amount of birefringence, or in distribution of birefringence within the spindle. This might be because with the polarizing microscope and the staining techniques we do not observe the component(s) which change.

(c) *The forces and energies for chromosome movement*

The force needed to move a chromosome from the equator to the pole is estimated as 10^{-8} dynes, applied during the entire movement period (Table 3). This calculation is based on the measured chromosome velocities and sizes, and on an estimate of viscous drag on the chromosome. (The force needed to overcome chromosomal inertia is negligible compared to the force needed to overcome frictional resistance.) There is some uncertainty in the calculation because the spindle viscosity may be non-Newtonian (i.e. the viscosity coefficient may vary with velocity), but high values of viscosity were chosen, and thus the force estimates are upper limits, with order-of-magnitude accuracy (Nicklas 1965; Taylor).

A force of 10^{-8} dynes is very small relative to the capacity of a cell. The energy expenditure is (force) *times* (distance), and if this energy arises from terminal phosphates of ATP, at 7 kilocalories per mole, it is seen that dephosphorylation of around

TABLE 3

Forces and energies for various movements

Cell	Factor	Calculated value	Remarks	References
Grasshopper spermatocyte	Force per chromosome (applied throughout anaphase) to move the chromosome to the pole	$\sim 10^{-8}$ dynes	—	Nicklas (1965)
Newt fibroblast			This value is my extrapolation from the stated values of work and distance	Taylor
Grasshopper spermatocyte	Number of ATP molecules needed to move one chromosome to the pole	~ 13	—	Nicklas (1965)
Newt fibroblast		~ 36	This value is my extrapolation from the stated values of total energy, total chromosome number and kcal. per mole of ATP	Taylor
Pea root tip		~ 4	This value is my extrapolation from the stated values of chromosome velocity, number of chromosomes, and total power required, assuming a total distance of 20 μ	Amoore
Frog sartorius muscle	Force exerted by a muscle	$\sim 3 \times 10^{-2}$ dynes per μ^2 cross-sectional area	This is a measured value	Lowy et al.
Large abfrontal gill cilium of *Mytilus edilus*	Force exerted by one cilium	$\sim 2 \times 10^{-4}$ dynes	This is a measured value, for a cilium 1 μ in diameter	Yoneda
		$\sim 10^{-5}$ dynes	This value is for force per 9+2 group of tubules, assuming that the large abfrontal cilium is composed of 20 groups of 9+2 tubules	
Frog sartorius muscle	Force exerted by one thick filament (cross-sectional area 10^4 Å^2) and associated thin filaments	3–5 $\times 10^{-5}$ dynes	—	Lowy et al.; Wolpert

30 ATP molecules could suffice to move one chromosome to the pole (Table 3). This is a small number of ATP molecules: 30 ATP molecules could be dephosphorylated in *one second* by *one molecule* of dynein (the ATPase from cilia; see the data of Gibbons). In fact the number of ATP molecules required to move the entire set of chromosomes to the poles is of the order of 10^9 times smaller than is required to synthesize spindle protein, assuming one ATP per peptide bond (Taylor).

As further comparison, it is seen that the force for chromosome movement is much smaller than that exerted by one cilium or by one myofibril (Table 3). In skeletal muscle, one thick filament (and associated thin filaments) bears a force 10^3 larger than that required to move a chromosome to a pole (Table 3). Thus, even if the actual force required to move a chromosome were 100 times larger than estimated, the force generated by one thick filament (and associated thin filaments) is still 30 times larger than needed. It is concluded, then, that the force necessary to move a chromosome against viscous resistance is very small, and could be accounted for by one thick filament (and associated thin filaments) from skeletal muscle.

The force produced may be quite a bit larger than that calculated, however, for it is possible that the chromosomal frictional resistance is negligible compared to other factors (Taylor; Nicklas (1965) termed this model 'force insignificance'). If this were true, chromosome velocity would not decrease when there is an increase in frictional resistance to motion; indeed, chromosome velocity is unchanged when this resistance is increased (a) by a factor of 10, due to variations in spindle viscosity (Taylor), (b) by a factor of more than 2, due to variations in chromosome size (Nicklas 1965), and (c) by a factor of more than 2, due to late disjunction of meiotic chromosomes (Nicklas 1963). However, none of these demonstrations are in themselves foolproof; for example, Taylor assumed that the measured cytoplasmic viscosity was a direct measure of spindle viscosity, and, as pointed out by Nicklas (1965), this assumption needs not be valid. But while one might argue against each conclusion separately, the 3 different arguments taken together make a reasonably convincing case that velocity is independent of load, for each of the 3 relies on a different set of assumptions.

The independence of velocity and load does not prove that frictional resistance is negligible compared to other factors, however. It is possible, for example, that a constant velocity is obtained, independent of load, because the applied force changes as the load changes, that is, the force for chromosome movement is adjusted continuously to give exactly that which is needed to overcome viscous resistance (Nicklas (1965) termed this model 'force compensation'). There are arguments which favor force compensation (Forer 1966), but there is no conclusive evidence to rule out either force insignificance or force compensation.

It is worth noting that 'force insignificance' still requires relatively small forces. Even allowing a tolerance of 10^2 to account for inefficiency in spindle 'thick filaments' and errors in estimates of viscous resistance, 2 or 3 thick filaments still produce 100 times the necessary force. With forces this large, increases in viscous load by factors of 10 are still 'insignificant'. Since such a small amount of material *could* cause

chromosome movement, this suggests that much of the spindle material is not involved in force production *per se*, but has some other role(s).

In conclusion: the viscous resistance to chromosome movement is very small ($\sim 10^{-8}$ dynes). But chromosome velocity is independent of load and thus viscous resistance might be negligible compared to the force actually available (force insignificance). Alternatively, the force is continually adjusted to account for different loads (force-compensation). In either case only a few thick filaments could account for the force, suggesting that a large part of the spindle material has some role(s) other than force production.

(d) *General characteristics of the force system*

Other factors as well must be accounted for by theories of anaphase chromosome movement and Mazia (1961) has listed and given evidence for several 'criteria for a theory of chromosome movement'. I shall not repeat his evidence, but I shall give further evidence relating to some of these same points, and shall discuss additional criteria. Theories for anaphase chromosome movement can be rejected if they run counter to these criteria.

(1) The force for chromosome movement acts at the kinetochore, and directs the chromosome toward the pole (i.e., along the length of the chromosomal fibre) (see Mazia 1961).

(2) The force causes chromosomes to move with approximately constant velocities in the range 0.2–5.0 μ per minute.

(3) The initial separation of single metaphase chromosomes into two anaphase chromosomes is independent of subsequent anaphase movement (Mazia 1961, p. 267; Forer 1966).

(4) Of the chromosomes moving toward one pole, the forces act on each chromosome individually, and independently of others moving to the same pole.

The evidence for this is: (a) In some cells, different chromosomes regularly move at different times from others (e.g., Hughes-Schrader; Dietz 1956, 1958, 1959; Mazia 1961; Bauer et al.; Forer 1966). (b) In some cells some chromosomes regularly move poleward with a velocity different from other chromosomes (Hughes and Preston; Dietz 1956; Bauer et al.; Nicklas 1965). (c) Micromanipulation of one anaphase chromosome (half-bivalent) moving to a pole has no effect on the movement of other chromosomes going to the same pole (Nicklas and Staehly). (d) Individual metaphase and anaphase chromosomes can be moved laterally across the spindle (i.e., perpendicular to its length) yet the movements of other chromosomes are not affected (Nicklas and Staehly). (e) Chromosomes moving to one pole are affected independently by ultraviolet microbeam irradiation of spindle fibres: some half-bivalents continue to move normally while others temporarily stop moving; and of those that stop moving, different chromosomes resume movement at different times, and with different velocities relative to the pre-irradiation velocities (Forer 1966).

(5) The forces act *individually* on separating chromosomes, but under some circum-

stances the force on one chromosome is *not independent* of the partner moving to the opposite pole.

The evidence for this is: (a) *Individuality.* Chromosomes moving to opposite poles do not necessarily move with the same velocity (Mazia 1961; Dietz 1956; Bauer et al.; Izutsu; Forer 1964). (b) *Not independence.* When irradiation of a chromosomal fibre stops the anaphase movement of the associated chromosome (half-bivalent), the partner chromosome moving to the opposite pole also stops moving; both partners resume movement at the same time (Forer 1966).

(6) In meiosis, pairing is not a prerequisite for coordinated movement to opposite poles (Schrader; Mazia 1961; Dietz 1956).

(7) No theory can be incompatible with the chemical and physical nature of the spindle and the independence of velocity and load described in the previous three sections.

With these criteria in mind, I now discuss the morphological entities which cause chromosomes to move during anaphase.

(e) *The identity and function of the elements causing chromosome movement*

What causes the chromosomes to move poleward during anaphase? It is thought that each chromosome is somehow pulled to a pole by its chromosomal spindle fibre; the chromosomal fibre transmits the force to the kinetochore, in any case, even if the force is produced elsewhere. Detailed arguments for this conclusion are presented in the general reviews cited in the Introduction, in Inoué (1964) and in Inoué and Sato. These arguments are all indirect ones, however, being based either on treatments of whole cells or on the elimination of other theories.

More direct evidence is supplied by ultraviolet microbeam irradiation of various parts of the cell: irradiation with a given dose stops movement only when chromosomal fibres are irradiated, but not when the interzonal region or cytoplasm are irradiated (Forer 1966). While this does not prove that chromosomal fibres *cause* movement, it at least shows that chromosomal fibres are *necessary* elements for chromosome movement.

The experiments also provide evidence about the *length* of chromosomal fibre that is necessary for normal movement. This is shown by irradiating at different positions between the kinetochore and the pole and seeing if chromosome movement is blocked. The results are for positions up to 8 μ out of a fibre length of 10 μ, and they indicate that at least 80% of the fibre length is necessary for chromosome movement (Forer 1966, and unpublished).

Though these results directly implicate *something* associated with the chromosomal fibre, this something is not the birefringent material. This conclusion is based on the following observations (Forer, 1966): (1) Chromosomes can move normally after irradiation of the chromosomal spindle fibre adjacent to the kinetochore has caused spindle fibre birefringence to be greatly reduced or to disappear; (2) the chromosome and the irradiated fibre region move poleward with velocities which can differ by as

much as a factor of 3, the chromosomes moving faster, at the same rate as, or slower than the irradiated fibre region; (3) when irradiation of the fibre causes the associated chromosome to stop moving, the irradiated region on the fibre nonetheless moves to the pole; (4) chromosomes which have stopped moving may resume movement before the irradiated region reaches the pole; and (5) ultraviolet microbeam irradiation can block chromosome movement without affecting spindle fibre birefringence.

These results suggest that chromosomal spindle fibres contain: (a) a force-transmitting component and (b) a birefringent component, and that these components are independent and can be affected independently (detailed arguments are presented in Forer (1966).

This argument and these 5 points refer solely to irradiations during anaphase. None of the first 4 points is valid for irradiations prior to anaphase. After irradiation of spindle fibres during metaphase (Forer 1966), (1) chromosomes do not move poleward when the irradiated region is present on a spindle fibre: even if the half-bivalents disjoin before the irradiated region reaches the pole, poleward movement is delayed until normal-looking chromosomal fibres are formed; and (2) when the half-bivalents finally move poleward, their velocity is related to the previous movement of the irradiated region, always being less than the velocity of the irradiated region. Using the same argument as above, one arrives at the opposite conclusion, namely that the birefringent material *is* necessary for chromosome movement. Thus, *before anaphase* the birefringent material is necessary for something related to anaphase chromosome movement, but *during anaphase* the birefringent material is *not* necessary for chromosome movement. It might be that before anaphase the birefringent fibre is necessary because it helps to form and make functional the force transmitting element, i.e., it helps in the completion of Mazia's 'motor mechanism' (Mazia 1961, p. 268).

Thus, my explanation involves two hypotheses, (1) there are two components, and (2) the two components become independent at anaphase (Forer 1966). These hypotheses are testable, they are consistent with the various components in spindles, with the various criteria for theories of movement, and with the idea that only a small portion of the fibre produces force for movement while most of the fibre has some other role (see above). Furthermore, the hypotheses suggest that we do not observe the 'locking' of the spindle at anaphase because we do not as yet observe the force transmitting component. As pointed out above, the temperature effect on spindle birefringence might be indirect, and due to its effect on the force element (the 'motor mechanism'). Little can be said about possible mechanisms until these two components are further identified.

Because of its relevance to theories of anaphase chromosome movement I should point out that removal of material from chromosomal fibres must take place at the pole; the evidence for this is that the irradiated region on the fibre moves to the pole without changing shape (Forer 1965; Inoué and Sato). This is true for *Nephrotoma suturalis* spermatocytes, but it is not yet tested for other materials. It will be recalled that spindle fibres in crane fly spermatocytes have weakest birefringence at the poles,

and strongest birefringence at the kinetochores, so it is not surprising that the poles act as 'disorienting centres'. But if stronger birefringence is a valid criterion for orienting centres, the poles would be orienting centres in cells in which the chromosomal fibre birefringence was strong at the poles. In such cells the repair of the irradiation-induced lesion should be away from both orienting centres, and thus the irradiated spindle fibre region should not move poleward, as in crane fly spermatocytes, but rather the two ends should grow together. This experiment has not yet been done, however.

In summary, some material associated with the chromosomal spindle fibre is necessary for anaphase chromosome movement, and the necessary length of this material is at least 80% of the length of the chromosomal spindle fibre, both conclusions being deduced from ultraviolet microbeam irradiations of spindle fibres. The results strongly suggest that in anaphase the spindle fibre component necessary for chromosome movement functions independently of the birefringent material, though in metaphase this is not true. It is suggested that the 'locking' of the spindle at anaphase is due to completion of the functional form of the non-birefringent, force-producing material.

4. *Studies using electron microscopy*

I have discussed several aspects of cell-division as viewed with light microscopes, and have presented evidence that the force for chromosome movement comes from some chromosomal spindle fibre component separate from the birefringent component. Studies of cell-division using the electron microscope should in theory provide evidence at a different level of organization, near the molecular. I shall now discuss some electron microscope studies, firstly to see which structures in the electron microscope correspond to the structures seen with the light microscope, and secondly to consider the functions of these structures.

(a) *Electron microscopy compared with light microscopy*

The term 'microtubule' (Slautterback; Ledbetter and Porter) is used to designate cellular structures which with usual fixation and staining procedures appear cylindrical, of outer diameter in the range 180–300 Å. Microtubules have electron-dense walls about 50–60 Å thick, and are present in spindles fixed with glutaraldehyde, and, in some cases, fixed with osmium (e.g., Harris 1965).

It is generally assumed that the light microscopic chromosomal and continuous fibres are groups of microtubules. This idea is based on (1) the observations that spindle microtubules are oriented in the pole-to-pole direction, and are often found in 'bundles', (2) the idea that protein oriented in this way would give rise to positive birefringence, and hence would give rise to the observed birefringence of spindle fibres,

and (3) the observations that some microtubules appear to be attached to chromosomes.

The arrangement and number of spindle microtubules has not been compared quantitatively with the birefringence and morphology of *in vivo* spindle fibres, however, and fixation for electron microscopy seems to alter the spindle fibre birefringence. Spindle birefringence is reduced by more than 50% after glutaraldehyde fixation and osmium tetroxide post-fixation of *Pectinaria* eggs (Inoué and Sato), and spindle fibre birefringence is reduced by about 30% after osmium tetroxide fixation of isolated sea urchin egg MA and isolated clam egg MA (Rebhun and Sander). Dehydration, as well, can cause loss of birefringence (Forer, unpublished). In most cases birefringence has not been measured, but if this loss of birefringence is a general phenomenon with glutaraldehyde and osmium fixations, it is not clear how the electron microscopic image of spindles corresponds to the *in vivo* situation. Quantitative comparison needs be made between the amount of birefringence and the electron microscopic image at various portions of the same spindle, under conditions where the birefringence is preserved. Until something like this is done, uncertainty must attend any presumed correlation of spindle microtubules with spindle fibre birefringence.

Furthermore, microtubules might not necessarily give rise to form birefringence. It is possible, for example, that the microtubule 'wall' is not protein at all, but rather a phase boundary which is selectively stained; or the wall could be lipoprotein, or other

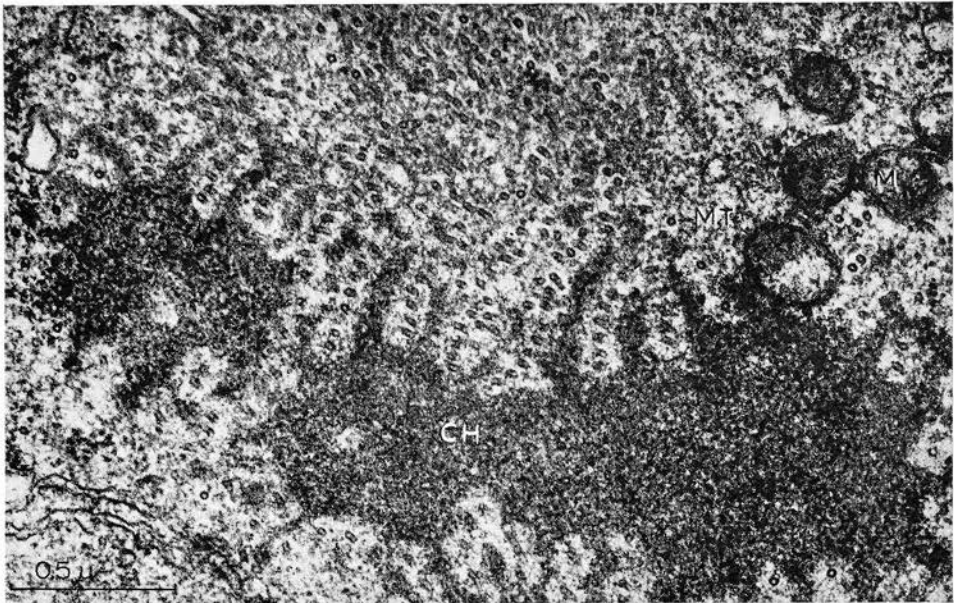


Fig. 8 illustrates an individual chromosome (CH) in a primary spermatocyte. This figure shows primarily cross-sectioned microtubules (MT). M: mitochondria. × 42,500.

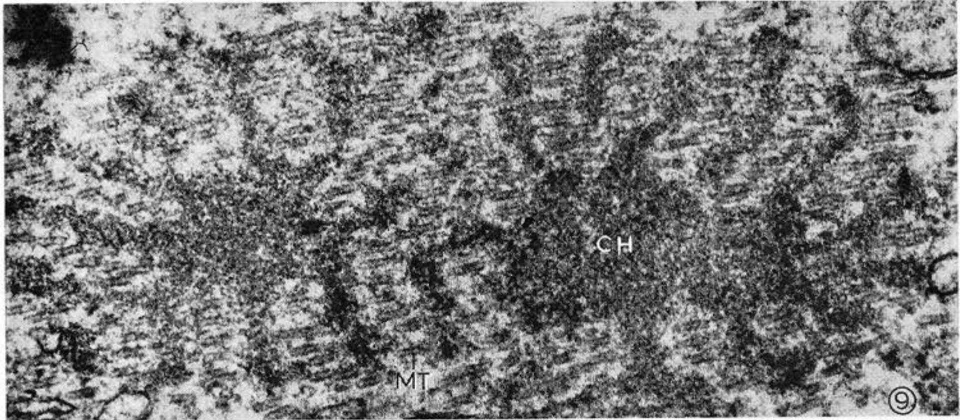
substances; or even if microtubules were protein, a microtubule could be of the same refractive index as its surrounds, and hence cause no form birefringence; or, if microtubules are protein, in a medium of different refractive index, the form birefringence of microtubules might not be measurable, or might be masked or added to by the intrinsic and form birefringence of other materials which are present and which are oriented by the microtubules. While there is some evidence that microtubules give rise to at least some birefringence (discussed in Rebhun and Sander; and Inoué and Sato), this evidence is open to other interpretations as well. Thus, it is not clear how much, if any, of the spindle birefringence is due to microtubules.

Despite the uncertainties stressed above, there are some positive conclusions which can be drawn, as follows.

(1) *Spindle fibre birefringence is probably not due solely to microtubules.* This conclusion is based on the observation that no microtubules are seen when 30–50 % of the spindle birefringence remains (and is preserved through fixation). This occurs after isolated MA are stored at *p*H 6.1, or are washed several times (Goldman; Rebhun, Sander, Goldman and Bernstein). Using the same techniques, microtubules are present in abundance in freshly isolated MA. In this case, then, at least 30–50 % of the birefringence of isolated MA is due to non-microtubular material. The converse experiment implies the same conclusion, as follows: addition of basic protein to glutaraldehyde-fixed isolated MA causes the birefringence to double, without a change in the number of microtubules (Goldman).

These experiments do not *conclusively* demonstrate the proposition stated above, for, in the absence of further data, counter arguments are possible. Furthermore, extrapolation to *in vivo* MA is uncertain, since it is not known how the birefringence of isolated MA is related to that of *in vivo* MA (see last section, and discussion of Rebhun and Sander). Nonetheless, these data are at least as convincing as those from which the opposite conclusion is drawn and thus I state the conclusion as above and reiterate that we need careful experimental analysis of microtubules and other spindle components and how they contribute to the observed spindle birefringence.

(2) *Spindles contain more than microtubules.* Published reports and electron micrographs show that besides microtubules, spindles contain vesicles (smooth and granular); membranes; ribosome-like particles; endoplasmic reticulum; amorphous or greyish 'background' material; filamentous material associated with microtubules; lamellae; dense material associated with microtubules; 'small circular bodies' (Whaley et al.); and non-staining 'clear areas' around microtubules. Figs. 11–13 show microtubules in spindles in crane fly spermatocytes, illustrating 'clear areas' around many of the spindle microtubules, and illustrating the large amount of non-microtubule material. Other illustrations of this latter point can be seen in papers of Robbins and Gonatas; George et al.; Roth et al.; Krishan and Buck (1965a, b); de Thé; Jokelainen; and Brinkley et al. These articles and the discussion below provide references to other studies.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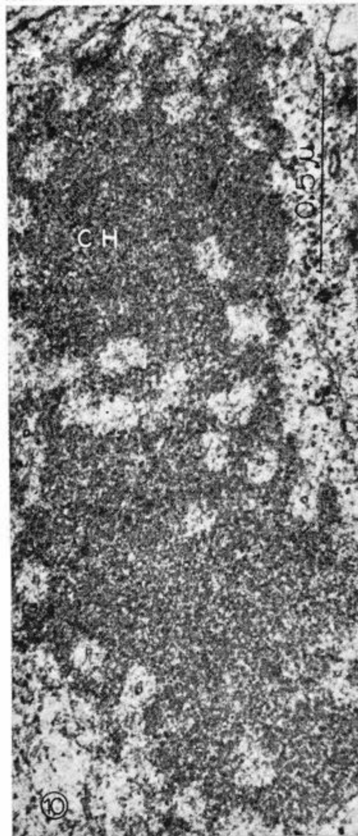
9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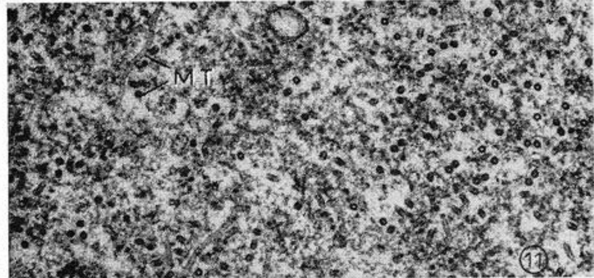
10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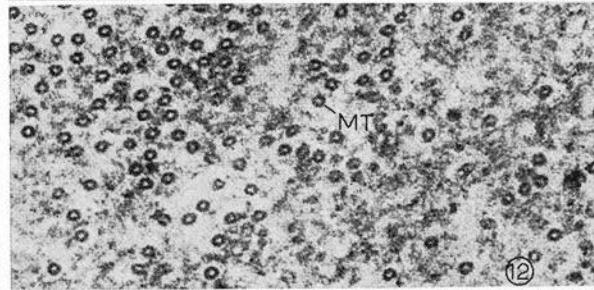
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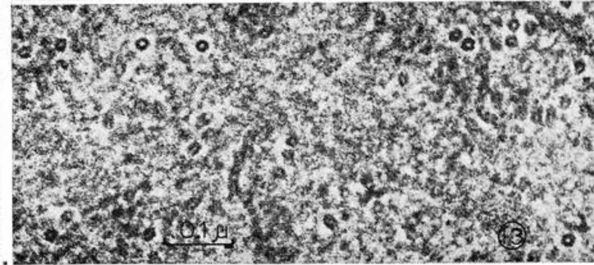
0.1 μ
13

That the non-microtubule components might have an important role in spindle structure is suggested by the finding that isolated MAs are stable even when the micro-tubules have been extracted (Kane and Forer; Goldman; Borisy and Taylor; Bibring and Baxandall).

(3) *A minority portion of the spindle mass and volume is in the form of microtubules.* There are of the order of 0.1–2.0 ml of microtubules per 100 ml of spindles (i.e. 0.1–2.0% by volume). These are maximum values estimated from published counts of microtubules per cross-section of spindle, and estimated from published micrographs by direct measurement over regions of at least 1 μ^2 (Table 4). As seen in Table 5, one can estimate the mass fraction of microtubules as about 9 times the volume fraction, and thus volume fractions of 0.1–2.0% correspond to 0.9–18.0 g of microtubules per 100 g of spindle (i.e., 0.9–18.0% by weight). In this calculation I assume that a microtubule is a solid cylinder of protein, of density 1.37 g/cm^3 (Mahler and Cordes), and that the spindle is 15% dry matter (Table 2). For 'hollow' cylinders of such protein, the corresponding factor is seven and the mass fractions are 0.7–14.0%. The mass estimates, too, are estimates of maximum values.

Spindle microtubules can occupy 4–5% of the spindle volume, however, in rather small regions of the spindle, of the order of 0.1 μ^2 (Table 4). In a few cases they occupy 10% of the volume (Table 4). These values are yet higher in protozoans. Again, this is based on a few published counts of numbers of microtubules, and on direct measurement from published micrographs. One can not have as much confidence in estimates of mass fractions over small areas, though, because the spindle masses have been measured as averages over the entire spindles, and it is not known if small regions may have smaller or larger mass than average. Nonetheless, applying the calculation as above, volume fractions of 5% correspond to mass fractions of no more than 45% (or 34%, for 'hollow' microtubules). Even in the few cases with 10% of microtubules by volume, the micrographs show that materials other than microtubules are present in the microtubule bundles.

It should be emphasized that conclusion (3) is solely for material in the form of microtubules. The estimates do not include microtubule precursors which might be present, for it is not known how much, if any, of the non-microtubule material is precursor. One can say, however, that at least *some* of the non-microtubule material

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Figs. 9 and 10 illustrate individual chromosomes (CH) in primary spermatocytes. Fig. 9 shows primarily obliquely sectioned microtubules and Fig. 10 shows primarily cross-sectioned microtubules (MT). Microtubules are associated with chromosomes over a large area. M: mitochondria. Fig. 9: × 61,500; Fig. 10: × 51,750.

Figs. 11–13 illustrate cross-sections of spindles, showing microtubules (MT), large amounts of non-microtubule material, and non-staining 'clear areas' surrounding many of the microtubules. Some regions show microtubules in both cross-section and longitudinal section (Fig. 11). Fig. 11: × 35,000; Fig. 12: × 87,000; Fig. 13: × 89,500.

TABLE 4

Volume fractions occupied by spindle microtubules

Cell	Volume fraction of microtubules	Type of section, and *cross-sectional areas* for measurement	Remarks	References
		A. Larger areas		
Haemanthus endosperm cells	$\leqslant$ 0.035 (metaphase)	Longitudinal sections, over areas of $\sim 1\ \mu^2$	The authors estimate 50–100 microtubules per metaphase kinetochore. The volume fraction is a maximum estimate, using the maximum number of microtubules, the minimum kinetochore size in light micrographs (1 μ) and maximum size of microtubules (200 Å)	Harris and Bajer
Nephrotoma spermatocytes	0.01	Cross-sections, over areas of $\sim 75\ \mu^2$	The authors count $\sim$1700 microtubules per spindle cross-section	Behnke and Forer (1966)
	0.02–0.05	Cross-sections over areas ranging from 1 μ^2 to 12 μ^2	These volume fractions are measured from unpublished photographs	Behnke and Forer (unpublished)
Isolated MA, from *S. purpuratus* eggs	0.001	Cross-sections, over areas of $\sim 200\ \mu^2$	The authors count on the average 465 microtubules per spindle cross-section (in metaphase)	Bibring and Baxandall (personal communication)
Isolated MA, from *A. punctulata* eggs	0.005	Cross-sections, over areas of $\sim 200\ \mu^2$	The authors count approximately 2000 microtubules per spindle cross-section (in metaphase)	Cohen and Rebhun (personal communication)
S. purpuratus eggs	0.02	Longitudinal section, over an area of $\sim 1\ \mu^2$	My estimate, from published micrographs	Harris and Mazia
	0.005–0.01	Longitudinal sections, over areas of ~ 4–$9\ \mu^2$	My estimate, from published micrographs	Harris (1962) and Harris (1965)
HeLa cells	0.025	Longitudinal section, over an area of $\sim 1\ \mu^2$	My estimate, from published micrographs	George et al.

Isolated MA, from *A. punctulata* eggs	0.005–0.015	Longitudinal sections and cross-section, over areas of $\sim 1\ \mu^2$–$36\ \mu^2$	My estimate, from published micrographs	Kane (1962b); Kane and Forer
Isolated MA, from *Spisula* eggs	0.01–0.02	Longitudinal sections and cross-sections, over areas ranging from $\sim 7\ \mu^2$ to $16\ \mu^2$	My estimate, from published micrographs	Rebhun and Sander
Isolated MA, from *S. purpuratus* eggs	0.003–0.02	Longitudinal sections and cross-sections, over areas ranging from $1\ \mu^2$ to $15\ \mu^2$	My estimate, from published micrographs	

B. Smaller areas

Pelomyxa (Giant amebae)	0.025	Longitudinal section, over an area $\sim 0.2\ \mu^2$	My estimate, from published micrograph	Roth et al.
Ostrinia nubilalis spermatocytes	0.04–0.05	Cross-sections, over areas ~ 0.09–$0.13\ \mu^2$		
HeLa cells	0.045	Longitudinal sections, over areas of $\sim 0.3\ \mu^2$	My estimate, from published micrographs	Robbins and Gonatas
L-strain fibroblasts	0.05–0.055	Longitudinal sections, over areas of ~ 0.2–$0.5\ \mu^2$	My estimate, from published micrographs	Krishan and Buck (1965a)
Ascites tumor cells	0.055	Cross-section, over an area of $\sim 0.06\ \mu^2$	My estimate, from published micrograph	de Thé
Mitotic cells in fetal rats	0.055	Cross-section, over an area of $\sim 0.03\ \mu^2$	Jokelainen counted 7 microtubules per 2000 Å diameter kinetochore	Jokelainen
	0.025	Cross-section, over an area of $\sim 0.12\ \mu^2$	My estimate, from published micrographs	
S. purpuratus eggs	0.065	Longitudinal section, over an area of $\sim 0.05\ \mu^2$	My estimate, from published micrograph	Harris and Mazia
	0.035–0.10	Longitudinal sections, over areas of ~ 0.04–$0.09\ \mu^2$	My estimate, from published micrographs	Harris (1962, 1965)
Blatoides germanica spermatocytes	0.09	Longitudinal section, over an area of $\sim 0.2\ \mu^2$	My estimate, from published micrographs	Krishan and Buck (1965b)
Campanella umbellaria (macronuclei)	0.13–0.17	Cross-section, over areas of ~ 0.04–$0.06\ \mu^2$	My estimate, from published micrographs	Carasso and Favard
Nassula sp. (micronuclei)	0.12–0.13	Longitudinal sections and cross-sections, over areas of $\sim 0.8\ \mu^2$	My estimate, from published cross-sections of 'separation spindles'	Tucker

TABLE 5

Volume and mass fractions of microtubules

Diameter of microtubule	180 Å	200 Å	240 Å	250 Å
Number of microtubules per μ^2 cross-sectional area giving rise to the volume fraction below	4	3.2	2.2	2
Volume fraction of microtubules (ml of microtubules per ml of MA)		0.001		
Mass of microtubules (g of microtubules per ml of MA)		0.00137		
Expected g dry mass per ml of MA (Table 2)		0.15		
Mass fraction of microtubules in MA (g microtubules per g MA)		0.009		

is not microtubule precursor. The evidence for this is: (a) chemical analyses show at least three different components (discussed below), and (b) spindles remain structurally intact after microtubules have been chemically extracted (Borisy and Taylor; Bibring and Baxandal).

(4) *Spindle microtubules are not evenly distributed throughout the spindle area.* Inspection of micrographs and estimates of microtubule density show that in some areas of the spindles microtubules are closer together than in other areas of the same spindles.

Taken together the evidence leads us to conclude that a considerable portion of the spindle birefringence is probably due to non-microtubule material, and that more than 50% of the spindle mass and more than 95% of the spindle volume is not in the form microtubule. Non-microtubule components are seen in all regions of the spindle.

These conclusions are relevant to observations of crane fly spermatocytes (Behnke and Forer 1966), which will now be briefly discussed.

Behnke and Forer found no correlation between the positions of microtubules and the positions of birefringent spindle fibres. (The birefringent spindle fibres of these cells are illustrated in Figs. 3–5, and discussed above.) This statement is based on the following: we do not see 5 bundles of microtubules in cross-sectioned spindles, but rather microtubules throughout the entire spindle, and even outside the spindle area; we do not see microtubules attached to the chromosomes, but rather microtubules passing through and around the chromosomes; spindle microtubules are not all oriented in the same direction (e.g., Fig. 11); in anaphase we see microtubules in the not birefringent interzonal region as well as between the chromosomes and the pole; and, finally, spindle microtubules are associated with individual chromosomes over a region of up to 3–4 μ in length and 1.5–2 μ in width (Figs. 8–10), while the kinetochore region as seen with the light microscope is only 1 μ in diameter (compare the electron micrographs of Figs. 8–10 with the light micrographs of Figs. 4–7).

Why are these results different from the results reported by others? We have not yet sectioned cells in predetermined regions, so it is possible that we never have seen a proper cross-section of an anaphase spindle. However, we have looked at many cells, and have used secondary criteria for identifying stages, so this is probably not the main difference. In line with the previous discussion, I suggest the following as more likely possibilities.

(A) It is possible that there are different kinds of spindle microtubules, different either in themselves, or by virtue of some intimately associated material, some microtubules giving rise to birefringent spindle fibres and other microtubules not. In crane fly spermatocytes 'bundles' of 'birefringent microtubules' are masked by the presence of the other, morphologically identical microtubules, while in other cells the fixative preserves only the microtubules in the birefringent bundle. This explanation implies that these different microtubules exhibit differences in fixation and other properties; indeed, when spermatids are treated in various ways, different microtubules respond differently to the same treatment, and 4 classes of microtubules can be distinguished in individual cells (Behnke and Forer 1967). This lends credence to the postulated different kinds of spindle microtubules. Furthermore, chromosome-associated microtubules respond differently from other spindle microtubules when crane fly spermatocytes are treated experimentally (Behnke and Forer, in preparation), suggesting that there may indeed be different kinds of spindle microtubules.

(B) It is possible that the main contribution to spindle fibre birefringence is not from microtubules, but from some as yet unidentified material, which the microtubules might help to orient (see conclusion 1, above).

(C) It is possible that the birefringence was not preserved throughout the preparatory procedures for the electron microscope. While there was no change of birefringence during the first 2–3 min in glutaraldehyde (Behnke and Forer 1966), longer fixation causes loss of birefringence in other cells (discussed above), so on this point our results may be in doubt.

In summary: spindles contain various components, including microtubules; microtubule-associated filaments, clear areas, and dense material; amorphous or greyish 'background' material; membranes; endoplasmic reticulum; ribosome-like particles; and smooth and granular vesicles. The exact contribution of spindle microtubules to spindle birefringence is unknown, but it is probable that at least 30–50 % of the spindle birefringence is due to non-microtubule material. More than 50 % of the spindle mass and 94 % of the spindle volume is non-microtubule in form, and at least some of this non-microtubule material is not microtubule precursor. It is suggested that there may be different kinds of spindle microtubules. Because of these considerations and because in crane fly spermatocytes spindle microtubules are associated with chromosomes over a larger area than represented by the light microscopic kinetochore, there is considerable uncertainty regarding the exact relationship between the light microscopic and electron microscopic components.

(b) *Functional aspects*
If one considers the functions of the various components seen in the electron microscope, it seems clear from the previous uncertainties and discussion that many problems need be clarified before one can begin to speculate on what these components might be doing. Furthermore, while it is known that the spindle properties change dramatically between metaphase and anaphase, electron microscopy has not yet provided indications of any corresponding morphological changes between metaphase and anaphase. This fact, plus the previous conclusion that only a few thick filaments could provide 100 times enough force for chromosome movements, suggest that as of yet we might not be looking at the material responsible for transmitting the force to the chromosomes.

5. *Biochemical analyses of isolated spindles*

We have pointed out that chromosomes are pulled to the pole by a spindle fibre component different from the birefringent component. From electron microscopic observations it is not clear which components give rise to birefringence, or to the force for chromosome movement, but the observations do show that spindle microtubules are a minority component of spindles, by volume and by mass. We will now consider biochemical analyses of isolated MAS; firstly the correspondence between isolated MAS and *in vivo* MAS, and secondly biochemical studies on isolated MAS. The methods for isolation of MAS will not be considered, for these can be obtained from the references discussed below. There are several different methods for isolating MAS from sea urchin eggs (references below), and methods for isolating MAS from clam eggs (Rebhun and Sander) and from HeLa cells (Sisken et al.). The biochemical data derive primarily from MA isolated from sea urchin eggs.

(a) *Isolated MA compared with in vivo MA*
There are some *similarities* between isolated and *in vivo* MA. Firstly, the effect of *p*H is similar (Kane 1962a), acid *p*H accentuating the fibrillar structure. Secondly, the polar regions of isolated MA are least soluble (Table 6), reminiscent of the *in vivo* staining and birefringence patterns. Thirdly, direct chemical analyses of isolated MA (Table 7) reveal most kinds of material detected cytochemically (Table 1). Finally, isolated MA contain microtubules and other structures seen electron microscopically in *in situ* MA (cf. Kane 1962b; Harris 1962, 1965).

Some points are not yet clear. Isolated MA are birefringent (Kane and Forer), but these birefringence values have not been compared quantitatively with those of *in vivo* MA. And there have been no reports of differences between isolated metaphase and anaphase MA, though perhaps this has not been looked for carefully.

There are some *clear differences* between isolated and *in vivo* MA. Firstly, chromosome movement has not yet occurred in isolated MA. Secondly, with isolated MA

TABLE 6

Solubility of regions of isolated MA

Isolation method	Solubility	Solvent	Reference
Ethanol-digitonin	First the peripheral asters and the equator dissolve, and then dissolution progresses toward the poles	Thioglycollate, *p*H 11	Mazia (1955)
		Salyrgan	Zimmerman
	The same as above, but the regions around the poles (centrospheres) remain undissolved	Parachloro-mercuri-benzoate or sulfite	Sakai
Dithiodiglycol or dithiodipropanol	First the spindles dissolve, then the asters. The centrospheres remain undissolved	0.53 M KCl, *p*H 7–8.5	Sakai; Kiefer et al.
Hexylene glycol, *p*H 6.4	First the spindles dissolve, and then the asters	H_2O, at *p*H 7.5	Kane and Forer

neither colchicine nor low temperature cause loss of spindle fibre birefringence or dissolution of structure (e.g., Borisy and Taylor; Inoué and Sato; Kane and Forer). In fact, the temperature effect on isolated MA is the reverse of that on *in vivo* MA, as has been discussed previously.

Finally, there is quite a big difference in the amount of material present. Sea urchin egg MA *in vivo* contain about 23 g of dry matter per 100 ml of MA (Table 2), while sea urchin egg MA when isolated contain about 1 g protein per 100 ml of MA (Table 8), i.e., 95% *less than expected.* (As seen in Table 8, this is true for all isolation methods for which this measurement is available.) It is unlikely that interference microscope measurements on *in vivo* MA are wrong by more than a few per cent in dry mass, and indeed interference microscope estimates of cytoplasmic dry mass agree reasonably well with chemical analyses: interference microscopy of *Psammechinus miliaris* egg cytoplasm (Table 2) shows 25 g of dry matter per 100 ml of cytoplasm, while biochemical analysis of *Arbacia punctulata* eggs shows about 26 g dry matter per 100 ml of eggs (Harvey). Furthermore, interference microscope measurements in various cells and by various workers all give *in vivo* concentrations in the range of 10–20 g of dry matter per 100 ml of MA (Table 2). Therefore I conclude that the discrepancy between the masses of *in vivo* and isolated MA represents a real difference, and that MAs lose of the order of 95% of their mass during the isolation procedure.

There is one important qualification, however: since interference microscopy does not distinguish between different materials, it is in theory possible that the entire MA dry mass is indeed isolated, but that 95% of it is not protein, and has not been analyzed. This is not the case for ethanol-digitonin isolated MA, for measurements of dry weight (Zimmerman) indicate that isolated MA contain 1.55 g of dry matter per 100 ml

TABLE 7

Materials present in MA isolated from sea urchin eggs (as % of total weight)

Isolation method	Protein	RNA	Lipid	Carbo-hydrate	ATPase	Species	Remarks	Reference
Ethanol-digitonin	> 95%	2–3%	—	Some	—	*Strongylocentrotus purpuratus*	These are the percentages of materials present in the thyo-glycollate soluble fraction	Mazia (1955)
Ethanol-digitonin	94–95%	5–6%	—	—	—	*Strongylocentrotus purpuratus*	These are the materials present in the Salyrgan soluble fraction	Zimmerman
Dithiodiglycol	—	—	—	—	Present	*S. purpuratus*	ATPase was present in KCl soluble fraction	Mazia et al.
Dithiodipropanol	—	4–5%	Present	—	—	*S. purpuratus*	These are materials present in the KCl soluble fraction. The lipid is noted, and discarded	Sakai
Ethanol-sonication	—	—	—	—	Present	*Anthocidaris crassispina*	—	Miki (1963)
20% Hexylene glycol-sonication	—	—	—	—	Present	*Pseudocentrotus depressus*	ATPase is present, in 2.3S material found after solubilization with KCl, and treatment with NaOH	Miki-Noumara
Hexylene glycol (*p*H 6.4)	90%	5–10%	None	6%	None	*S. purpuratus*, *S. droebachiensis* and *Arbacia punctulata*	These are the percentages of material present in the 22S fraction of KCl soluble material	Stephens (1967)

TABLE 8

Amount of protein in MA isolated from sea urchin eggs

Isolation method	g protein per 100 ml of MA	ml per MA	g protein per MA	Species	Remarks	References
Ethanol-duponol	1.1	—	$\sim 2.4 \times 10^{-9}$	*Strongylocentrotus franciscanus*	—	Mazia and Dan
Ethanol-digitonin	<1.46	—	<1.2–3.1×10^{-10}	*Strongylocentrotus purpuratus*	The value 1.46 is an upper limit, obtained by subtracting the measured amount of RNA from the measured dry weight. The other value was calculated from the value 1.46, and the volume of the MA, using the 2 different volume estimates of Kane (1967) and Bibring and Baxandall	Zimmerman
Hexanediol, *p*H 6.3	~ 1.0	—	2×10^{-10}	*Arbacia punctulata*	The value 2×10^{-10} was calculated from the measured value 1.0 using the volume of the MA given by Kane (1967)	Mangan et al.
Hexylene glycol, *p*H 6.4	0.75	2.7×10^{-8}	2×10^{-10}	*S. purpuratus*	The ml per MA was calculated from the stated yield of MA (0.1 ml) from 1 ml of eggs, and from the volume of the eggs (Harvey)	Kane (1967)
		2.1×10^{-8}	1.6×10^{-10}	*A. punctulata*		
Hexylene glycol, *p*H 6.4	0.9	—	7.5×10^{-11}–2.4×10^{-10}	*S. purpuratus*	These values were calculated from data given in authors' Table III (protein in various fractions of MA), and methods given on p. 541 (MA gave 'about 0.1 ml packed volume'), assuming these methods were used to obtain the data in Table III	Borisy and Taylor
Hexylene glycol *p*H 6.4	0.9	8.3×10^{-9}	7.5×10^{-11}	*S. purpuratus*	The authors calculated MA volume from measurements of dimensions	Bibring and Baxandall (personal communication)

TABLE 9

Protein components solubilized from sea urchin egg MA after isolation (as % of total weight in the soluble fraction)

Isolation method	Solubility	Components in soluble fraction			
		2.3–2.5S	3.2–5S	6S	8.6S
Ethanol-duponol	Not soluble in salts. Soluble in 0.5 N NaOH	—	All	—	—
Ethanol-digitonin	Soluble in thioglycollate, Salyrgan, performic acid, and parachloro-mercurobenzoate	—	83%	—	17%
Dithiodipropanol	85% soluble in 0.53 M KCl	—	50%	—	—
Dithiodipropanol	70% soluble in 0.6 M KCl	75%	—	—	—
Dithiodipropanol	70% soluble in 0.6 M KCl	—	60%	—	—
Hexylene glycol, pH 6.4	60% soluble in 0.6 M KCl, pH 7.5	—	20%	—	—
Hexylene glycol, pH 6.4	80% soluble in 0.6 M KCl	—	Some	Some	—
Hexylene glycol pH 6.4	8% soluble after 75 min in HCl, pH 3	—	85%	—	—

11–13S	22S	28S	S > 28	Species	Remarks	References
—	—	—	—	*Strongylocentrotus purpuratus* and *S. franciscanus*	—	Mazia and Dan
—	—	—	—	*S. purpuratus*	Data are for material soluble in Salyrgan, using Salyrgan at a concentration of $>10^3$ moles Salyrgan per 6×10^5 g of MA protein (cf. Stephens 1968), and are my estimates of areas under published curves	Zimmerman
11%	13%	10%	—	*S. purpuratus*	The percentages of the various S components are my estimates of areas under published optical density curves	Wilt et al.
21%	5%	—	—	*S. purpuratus*	The data are of sulfite treated material, and are my estimates of areas under published curves	Sakai
35%	5%	—	—	*S. purpuratus*	The value 60% is calculated from the stated KCl solubility and the statement that the 3.5S component of Sakai is 40% of all the protein in the MA. The other values are calculated from this 60% value and the data of Sakai, for sulfite-treated material, knowing that sulfite does not affect the amount of 22S material (Sakai)	Kiefer et al.
—	80%	—	—	*S. purpuratus* and *A. punctulata*	The 20% fraction is 'heterogenous 4–5S material'	Kane (1967)
—	Most	—	—	*S. purpuratus*	The solubility is deduced from their Table 3	Borisy and Taylor
—	15%	—	—	*S. purpuratus*	—	Bibring and Baxandall

of isolated MA, i.e., still 93 % less than expected. This possibility should be tested for the other isolation methods as well.

In conclusion, while there are some similarities between *in vivo* and isolated MA, there are striking differences as well, notably in the effect of temperature, and in the loss of 95 % of the *in vivo* mass.

I now discuss analyses of the components present in isolated MA, and try to relate these components to structural and functional properties of spindles in cells, realizing that this latter correlation is severely restricted by the loss of material during isolation.

(b) *Components present in isolated MA*

Biochemical analyses of isolated MA are summarized in Table 9. It is seen that different workers obtain different results.

Chemical studies have been performed on various of the components, studying their behaviour at different *p*H, their dissociation and reassociation reactions, etc. (e.g., Zimmermann; Sakai; Stephens 1967). Stephens purified 22S material, and carefully studied its dissociation into subunits, and reassociation into various larger units; on the basis of the conditions under which units of certain S values were stable, Stephens concluded that 'the 4S component of Mazia (1955), the 8.6S material of Zimmerman, and the 13–14S particles of Sakai correspond to subunits obtained from the 22S protein' while the 2.5S and 3.5S components of Sakai and the 3.7S component of Zimmerman 'may more likely correspond to some component in the 4–5S heterogeneous mixture reported by Kane (1967)'. However, as Stephens admits, the arguments are somewhat circumstantial. Also, there are still unresolved differences. Nonetheless, it is tentatively concluded that there are at least two chemically different MA components in the soluble fraction, plus at least one other component in the insoluble fraction.

Other chemical data support this conclusion. Bibring and Baxandall show that the 22S material is antigenically different from the 4–5S material (in hexylene glycol isolated MA) and the 4–5S material is precipitated by calcium whereas the 22S material is not; furthermore, the 4–5S material is similar to the 3.5S material of Zimmerman, in that at *p*H 7 the 4–5S units do not associate to form 13S or 22S units, as do subunits of 22S material. Finally, Stephens (1968) reports that the molecular weight of the 22S subunit is significantly different from that of the 4–5S material, and, preliminarily, that the amino acid composition of 'sub-optimal amounts' of 4–5S material (from hexylene glycol preparations) is different from the amino acid composition of 22S material.

Cumulatively, then, this is reasonably strong evidence that isolated MA contain at least two different components in the soluble fraction. The possibility of a third component is suggested by the work of Borisy and Taylor, who showed that colchicine binds to only a small fraction of the 4–5S material in hexylene glycol preparations. But at present it is too early to be certain of more than two components in the soluble fraction.

While isolated MAs contain at least two different components in the soluble fraction, MAs isolated by different methods have different proportions of the two components. For the purposes of discussion, I consider the 13.5S and 21S components of Sakai, and the 8.6S component of Zimmerman as 22S material; and I consider the 4–5S material of Kane and Stephens as 3.5S material. It is seen in Table 10 that the proportions of the two components are reversed when one compares hexylene glycol MA *vs.* dithiodipropanol MA or ethanol-digitonin MA. But MA prepared with different methods all have about the same total mass (Table 8). One might account for the different proportions of the same material found in about the same total mass by assuming that there is an MA 'skeleton', which comprises a small fraction of the MA mass; if this skeleton can bind only a limited amount of material the total mass would remain constant, but the bound material would be different depending on how the skeleton and various bound components change with different isolation conditions. Such an

TABLE 10

(derived from Table 9)

Soluble components present in MA isolated using different isolation procedures

Method of isolation	Amount of component 22S	3.5S	Ratio of amounts 22S:3.5S
Hexylene glycol	80%	20%	4:1
Dithiodipropanol	25%	75%	1:3
Ethanol-digitonin	17%	83%	1:5

idea might also explain the observation of Goldman, cited above, that the birefringence of glutaraldehyde-fixed isolated MA can be doubled by the addition of basic protein.

Summarizing, I conclude that isolated MAs contain at least two different components in the soluble fraction, plus insoluble component(s), and that under different conditions of isolation MAs have the same total mass but different proportions of the soluble components. This may arise by differential binding to a limited number of sites on an MA 'skeleton'.

But what do these different components do?

MAs exist as stable structures with only 5% of their *in vivo* mass. Thus it is reasonable to think that this 5% contains skeletal elements responsible for holding the MA together. While it is tempting to consider that the spindle microtubules are these skeletal elements, one should remember that MAs stay together even when microtubules have broken down (Kane and Forer; Goldman) or when microtubules have been selectively extracted (Goldman; Borisy and Taylor; Bibring and Baxandall). But perhaps the microtubules are the initial supports on which the skeletal structure is cast.

Even though it is not clear what the spindle microtubules do, there is some evidence that the 3.5S material derives from microtubules. This evidence is that microtubules

disappear when 8 % of the MA mass is extracted, and this extracted material is mostly 3.5S material with properties similar to those of material extracted from the outer doublet tubules of sperm tails (Bibring and Baxandall).

Other than structural aspects, it is difficult to discuss the biochemistry of MAs in functional terms, because isolated MAs are not functional, and because most of the MA material is lost during isolation. Because of this loss of material it is not even certain that isolated MAs contain the force producing machinery.

It should be emphasized that in these studies, the solubility properties, the birefringence, and the fine-structure of isolated MAs are not invariant. The solubility decreases with storage (Zimmerman; Kane and Forer; Sakai), and both birefringence and fine-structure change with storage (Kane and Forer; Rebhun and Sander; Goldman), with conditions of isolation (Kane 1962b), and with washing of isolated MAs in the absence of salts (Goldman; Rebhun, Sander, Goldman and Bernstein). Since small and perhaps yet unknown differences do matter, solubility, birefringence, and fine-structure should not be taken for granted, but should be measured as controls during the isolation and subsequent experimental procedures.

In summary, MAs isolated by various techniques are mostly protein, with some RNA (about 6 % by weight), and some carbohydrate and lipid. There are at least two different protein components in the soluble fraction of isolated MAs, though the concentration of material in isolated MAs is about 95 % lower than the concentration *in vivo*. It is suggested that the 3.5S material derives from microtubules, and it is argued that isolated MAs consist of various proteins bound to a 'skeleton', the proportion of different proteins being different with different isolation methods. But it is not known which chemical elements comprise the structural element which holds MAs together.

6. *Summary*

This chapter deals with the movements of chromosomes during cell-division, and the role of the spindle in these movements. It is concluded that some component associated with the chromosomal spindle fibre causes the chromosome to move. The force for chromosome movement is either continually adjusted or is large compared to the viscous drag on the chromosome, the latter being $\sim 10^{-8}$ dynes. In either case a few thick filaments from skeletal muscle produce enough force to account for chromosome movement; this suggests that much of the spindle fibre material is not involved in force production but has some other role. Similar conclusions are drawn from experiments in which spindle fibres are irradiated with an ultraviolet microbeam. These experiments suggest (a) that spindle fibres contain two separate components, a birefringent component and a force-producing component; (b) that during anaphase the birefringent component is independent of the force-producing component; and (c) that during metaphase the birefringent component is not independent of the force-producing component, and may even be necessary for the formation of the force-producing component.

These conclusions are compatible with the physical and chemical nature of the spindle. There are about 15 g of dry matter per 100 ml of spindle (*in vivo*), and this dry matter includes protein, nucleic acid, carbohydrate, lipid, ATPase, free -SH groups, and zinc, but not phosphatase. The staining and birefringence are different in different regions of the spindle, being strongest near the chromosomes and the poles, and weakest in between. The birefringent spindle fibres are labile throughout all stages of cell-division; they are probably in equilibrium with non-birefringent material; and they are probably organized by kinetochores (and, in some cases, poles). Physiologically, there are striking differences between metaphase and anaphase spindles, though both appear identical morphologically.

Electron microscopic observations agree with the light microscopic observations in that they show several spindle components. It is not certain how these components relate to the birefringent chromosomal and continuous fibres of light microscopy because spindle fibre birefringence may not be preserved by electron microscope fixatives, and because spindle microtubules are associated with chromosomes over an area much larger than that of the light microscopic kinetochore. It is concluded that 30–50% of the spindle fibre birefringence is probably due to some non-microtubule component; that spindle microtubules as seen to date are a minority fraction of the spindle fibre volume and mass; and that spindle microtubules are not distributed evenly throughout the spindle region. It is not known which, if any, of the described components causes chromosome movement.

Spindles (MA) lose 95% of their mass when they are isolated from sea urchin eggs with present techniques. There is about 1 g of protein per 100 ml of isolated MA, and this corresponds to about 5% of the material present in *in vivo* MA.

Isolated MAs contain at least two components in the soluble fraction, plus insoluble material. The relative proportions of the soluble components are different with different isolation techniques, but the total mass is about the same with all techniques. To account for this it is suggested that there is an MA 'skeleton', comprising a small fraction of the mass of the isolated MA, and that only a limited amount of material can adhere to this skeleton.

Acknowledgements

I would like to thank Drs. T. Bibring and J. Baxandall, R. Goldman, J. Mangan, and R. Stephens for allowing me to see reports of their work prior to publication; and I would like to thank Drs. Bibring and Baxandall, and W. Cohen and L. Rebhun for allowing me to cite their unpublished results. I am especially grateful to Dr. O. Behnke for the unpublished electron micrographs in Figures 8, 9, and 11, and for invaluable discussion and collaboration (some of it in conjunction with F. B. T. Birkes-Børge). I am grateful to Dr. V. A. Parsegian for discussion of various portions of this review, and I would like to thank Drs. A. Mullinger and R. Bruce Nicklas for valuable critical comments on a draft manuscript. This work was supported by a Helen Hay Whitney

Foundation research fellowship. Portions were supported by the European Office of Aerospace Research contract F6 1052 67 C0012 to Prof. T. Weis-Fogh.

Note added in proof

Light microscopic cytochemical studies on spindle components relevant to Table 1 of this review can be found in the articles by Fautrez-Firlefyn and Roels; Shifrin and Levine; and Goldman and Rebhun; and in that of Hartmann which I had overlooked. No change is required in Table 1 except that the exact localization of spindle ATPase is not clear (discussed by Shifrin and Levine).

Electron microscopically, several spindle components are seen in *Haemanthus* endosperm cells (Bajer 1968a, b, c) and isolated sea urchin MA (Goldman and Rebhun). The volume fractions of microtubules seen in the electron micrographs are all in the same range as summarized in Table 4 of this review.

In section 4a I discussed birefringence with no microtubules present, and loss of microtubules during the washing of isolated MA; Goldman and Rebhun present these data and detailed discussion of them. I should point out one discrepancy not discussed by them: whereas Goldman and Rebhun found that all microtubules were lost aftei isolated MA were washed in Mg^{++}-free hexylene glycol at *p*H 6.4, electron micrographs of Bibring and Baxandall, and Kane and Forer show similarly washed MA which do indeed contain microtubules. Some technical differences which might account for this discrepancy include speed of centrifugation, cation of the phosphate buffer, amount of shaking, etc., and since Goldman and Rebhun found loss of microtubules only at *p*H 6.4, and not at *p*H 6.3, *p*H might need to be controlled to within several hundredths of a *p*H unit. This emphasizes the point made at the end of section 5, above, that the fine-structure (and other parameters) of isolated MA should not be taken for granted but should be measured routinely.

Comparison of the electron microscopic results of Bajer (1968a, b, c) with previous studies of birefringence in the same endosperm cells (Inoué and Bajer; Inoué 1964) substantiates the discussion in section 4a on the lack of one-to-one correspondence between microtubules and spindle birefringence. For example, Bajer found microtubules associated with many regions of the chromosomes and not just the kinetochores. Photographs in Inoué and Bajer, and in Inoué (1964) show that the birefringent chromosomal fibres are at least 1.0–1.5 μ in diameter at the kinetochores, whereas electron microscopically the microtubule bundles are only 0.5–0.6 μ in diameter at the 'kinetochores'. The continuous fibres are very weakly birefringent at metaphase and anaphase while the chromosomal fibres are strongly birefringent, yet, when the same stages are studied electron microscopically, the bundles of 'continuous fibre microtubules' in both the half-spindle and interzonal regions are of the same volume fraction of microtubules and overall diameter as the bundles of 'chromosomal fibre microtubules'. Furthermore, Bajer observed that 'continuous fibre microtubules' and 'chromosomal fibre microtubules' *intermingle* a short distance from the 'kinetochore'. If microtubules were the sole contributor to birefringence, such intermingling

would obscure the chromosomal fibre birefringence. But since it is not obscured (Inoué and Bajer; Inoué 1964), the microtubules are not the sole contributors to birefringence.

These results are quite relevant to my discussion of Behnke and Forer (1966) at the end of section 4a, and they support the possibilities suggested there. But I must restate the point made in section 4, that any correlation of electron microscopic structure and birefringent spindle fibres will be tentative until one measures birefringence throughout the entire electron microscopic preparation procedure, and quantitatively compares this birefringence with the electron microscopic image. In this regard, Cassim et al. discuss several theoretical difficulties inherent in predicting birefringence from known orientations of macromolecules and filaments.

Burton gives further evidence that all microtubules are not the same (discussed in section 4). Comparison of Burton's data with those of Behnke and Forer (1967) suggests the possibility of even more than 4 classes of microtubules.

With regard to the functional aspects of ultrastructural components (section 4b), Girbardt gives convincing evidence that microtubules are not responsible for nuclear oscillations and stretching in the basidiomycete *Polystictus versicolor*. In these cells, nuclear stretchings and oscillations arise from forces applied to 'activity centres', or 'kinetochore equivalents', found at the two ends of the nucleus. Microtubules are associated with the nuclear 'kinetochore equivalents' much as they are with chromosomal 'kinetochores', but because of their specific arrangement and changes of arrangement during the movements (see Girbardt's Fig. 2) microtubules can not be responsible for the force. Microtubules are also not responsible for the polyelectrolyte-gel properties of isolated MA. Cohen demonstrated that isolated MA which were stored for up to 2 months swelled and shrank like polyelectrolyte gels. This property is not due to microtubules, for such stored MA do not contain visible microtubules (Goldman and Rebhun). The stored MA are birefringent (Goldman and Rebhun), though, and this non-microtubular birefringent material may be responsible for, or at least necessary for, the polyelectrolyte-gel properties.

Data on isolated MA relevant to Table 6 are given by Hartmann and Zimmerman, and Miki-Noumara. Data relevant to Table 9 are given by Miki-Noumara, who studied *Hemicentrotus pulcherrimus* egg MA isolated using 1 M hexylene glycol at *p*H 6.3, and found that such MA were 60% soluble in 0.5 M KCl, and that the soluble material was at least 25% 2.3S material and contained no 22S component. The discrepancy between these results and those in Table 9 is probably methodological, for there are many differences in methods. Further, there are differences in species, and, as discussed below, MA isolated from eggs of different sea urchin species sometimes have different properties at the same *p*H and equivalent properties only at different *p*Hs (Forer and Goldman).

Sakai provides further evidence that the 4–5S material of hexylene glycol isolated MA is different than the 22S material (section 5b). He used hexanediol isolated MA, and showed that sulfite converted 4S material to 2.8S material while having no effect

on the 22S component. Data of Hartmann and Zimmerman show *four* different soluble components, all with sedimentation values $\geqslant$74S, when hexylene glycol isolated MA are dissolved in solutions of KCl plus Mg^{++}. This is different from the 22S and 4–5S material found after solubilization with KCl only (Table 9), so it is likely that some of these heavier components are polymerized from 22S and 4–5S material. While one can not exclude the possibility that three of these compounds arise from such polymerization, the 74S material can not have arisen simply in this way, for it has much more RNA than either the 4–5S or 22S material. This suggests that there is at least one more soluble component besides those discussed in section 5b.

Cohen showed that isolated MA contain divalent cation. This ties in with the results of Goldman and Rebhun that addition of divalent cation retards ultrastructural changes during washing and storage of isolated MA. Both these results, together with the result that solubilization in KCl plus Mg^{++} yields different components than solubilization in KCl alone (discussed above), suggest that the chemical components in *in vivo* or in intact MA exist as polymers stabilized (or induced) by divalent cations.

Finally, I summarize some recent results which bear on several sections of this review (Forer and Goldman; and Goldman and Forer, in preparation; and Forer and Goldman, in preparation).

Goldman and I studied *in vivo* and isolated MA of eggs of the British sea urchins *Echinus esculentus* and *Psammechinus miliaris*. We compared these isolated MA with previous studies of MA isolated from the eggs of the American sea urchin *Arbacia punctulata*, and found that the MA of the different species have different properties with the same *p*H hexylene glycol isolation medium. They had equivalent properties only at different *p*Hs, and we concluded that *Arbacia* egg MA isolated at *p*H 6.4 are equivalent to *Echinus* egg MA isolated at *p*Hs > 6.8 and to *Psammechinus* egg MA isolated at *p*Hs > 7.0 (the data are summarized in Forer and Goldman). Thus one can not directly compare MA isolated from eggs of different species until one is certain of the properties ot the different MA at the *p*Hs in question.

We measured MA mass and birefringence, both *in vivo* and after isolation, and found that at the usual *p*H (equivalent to *Arbacia* egg MA at *p*H 6.4) MAs lost 85–90% of their mass during the isolation (as deduced in section 5a), and lost about 40% of their birefringence. At lower *p*Hs 100% of the birefringence and up to 60% of the mass were preserved during the isolation (summarized in Forer and Goldman). Chemical analyses showed that more than half of the 60% was non-protein.

Though there was 5 times more mass in high-mass MA than in low-mass MA, preliminary electron microscopic observations showed no differences (Goldman), and thus most of the material is either lost during the electron microscopic preparatory procedure or is not stained.

Analyses of the isolated MA gave strong evidence that the birefringence derives from at least 2 components. One reason is that the birefringence was fractionated both by varying the *p*H of the isolation medium and by adding different solvents. Low *p*Hs preserved 100% of the birefringence, and high *p*Hs 60%, implying that there might be

two components of different solubilities. This was substantiated by taking MAs with 100% of the *in vivo* birefringence, adding a solvent which removed 40% of the birefringence, adding a second solvent which removed the rest of the birefringence (without dissolving the MA), and seeing that different chemicals were extracted with the two different solvents. This substantiates the discussion in section 4a, and provides strong evidence that the thermodynamic treatment of spindle birefringence (section 3b) is based on a faulty assumption.

It might be of interest to note that when the MA birefringence was extracted with the two solvents, one of the extracted proteins had bound nucleotide.

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