NUCLEAR DIVISION IN THE FUNGI

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CHROMOSOME MOVEMENTS DURING CELL-DIVISION: POSSIBLE INVOLVEMENT OF ACTIN FILAMENTS

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I. INTRODUCTION

In this chapter I concentrate on the question of whether or not actin is involved in producing force for chromosome movements during cell-division. The data to date concern whether or not actin is a genuine spindle component: these data will be considered in detail. By way of introduction, I first review some of the basic and generally agreed upon aspects of mitosis, such as the forces involved, the role of chromosomal spindle fibres, and so on. Then I briefly summarize the main hypotheses in which microtubules are considered to be the force producing agents, and I briefly summarize my view of the status of these hypotheses. Finally, I discuss critically and in detail the electron microscopic and light microscopic data which suggest that actin is a component of chromosomal spindle fibres. I discuss also the negative evidences, and I discuss the counter-arguments that are used to argue that actin is not a component of spindles. I conclude that actin is probably a spindle component, and is probably involved in chromosome movement. However, none of the evidences for actin being a spindle component are unequivocal, and I discuss the kinds of data that need to be obtained to substantiate that actin is a spindle fibre component and that actin is involved in producing the force for chromosome movement.

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II. BASIC POINTS ABOUT MITOSIS

The basic points reviewed here are discussed in various reviews (Schrader, 1953; Mazia, 1961; Inoué, 1964; Inoué & Sato, 1967; Forer, 1969, 1974; Nicklas, 1971, 1975; Inoué & Ritter, 1975; McIntosh et al., 1975), to which readers are referred for detailed discussion.

In anaphase, chromosomes move from the equator towards the poles; this is accomplished either by shortening of the chromosome to pole distance, as the poles remain a constant distance apart, or by separation of the poles as the chromosome to pole distance remains constant, or by simultaneous shortening of chromosome to pole distances and lengthening of pole to pole distances. Different situations apply in different cells, or at different times in the same cells (e.g. Ris, 1943, 1949; Jacquez & Biesele, 1954; Forer, 1966; Fuseler, 1975). These motions may be more than just descriptively different, for, as discussed by Ris (1943, 1949) and Jacquez & Biesele (1954), and as emphasized by McIntosh et al. (1975) and by McDonald et al. (1977), for example, the different motions may have different mechanisms (e.g., chromosome-to-pole motion might be caused by microtubule cross-bridges interacting with microtubules to cause sliding, whilst the poles might move apart because of force produced by actin filaments, as mentioned by McIntosh et al., 1975; or the converse might apply). The discussion in this chapter deals exclusively with movements which occur by shortening of the chromosome-to-pole distances.

Chromosomes move polewards with velocities in the range of 0.2 - 5.0 μm/min., much slower than protoplasmic movements, which are in the range of μm/sec. (see Wolpert, 1965; Nicklas, 1975); chromosome movements are at very slow velocities indeed, for they are very close to the velocities of tectonic plate movements! The forces for the movements are also very small: 1 actin filament together with 1 myosin filament, producing force at the efficiency of actin-myosin filament interactions in skeletal muscle, produce 3,000 times more force than necessary in order to move a chromosome to its pole (see Forer, 1969; Nicklas, 1971, 1975; Gruzdev, 1972). Likewise, one ATPase molecule (e.g., one arm on a ciliary doublet) working for one second produces more than enough energy to move a chromosome polewards (see Forer, 1969; Nicklas, 1975). Taking considerations like these into account, several authors have suggested that there is a rate limiting step, a 'governor', separate from the force production step, and that microtubule depolymerisation might be that rate limiting step: the slow depolymerisation of microtubules limits the rate of chromosome movement, which, without this impedance, would be much faster (e.g., McIntosh et al., 1969; Nicklas, 1971, 1975; Forer, 1974).

As a chromosome moves poleward the chromosome's kinetochore (spindle-fibre-attachment region) generally leads the way. As seen in fixed and stained preparations or as seen in the polarising microscope, some spindle fibres extend from kinetochores to poles while other spindle fibres do not end at kinetochores: these two kinds of light-microscopically visible spindle fibres are called chromosomal spindle fibres and continuous spindle fibres, respectively, using terminology of Schrader (1953). As a chromosome moves polewards the associated chromosomal spindle fibre shortens without measurable change in width.

The force to move a chromosome appears to be applied at the kinetochore, is directed towards the pole, and is transmitted to the chromosome by the chromosomal spindle fibre (Cornman, 1944; Mazia, 1961; Nicklas, 1971). The evidences for this are in part negative, in that all other hypotheses seem to be ruled out (Cornman, 1944; Taylor, 1965; Gruzdev, 1972), and are in part positive, in that there is indeed a mechanical link between chromosome and pole (Nicklas & Staehly, 1967) and in that ultraviolet microbeam irradiation of single chromosomal spindle fibres can stop chromosome movement whilst equivalent doses applied elsewhere in the cell do not stop chromosome movement (Forer, 1966). Hence chromosomal spindle fibres seem to be necessary for chromosome movements in anaphase; there is not enough data to say whether the chromosomal spindle fibres are both necessary and sufficient, but most workers agree that the forces for chromosome movement are transmitted to the chromosome by the chromosomal spindle fibre.

Spindle fibres are birefringent. Birefringent materials by definition have indices of refraction which are different for light polarised in different directions, and it is this optical property of spindle fibres which allows them to be seen and studied in living cells (Swann, 1951 a, b; Inoué, 1953, 1964). It should be emphasized that spindle fibres contain components which do not contribute to the birefringence,

but which could contribute to producing the force: spindle fibre birefringence does not arise from all the components in a light microscopically observed spindle fibre. Hence, when one says that the force for chromosome movement arises from (or is transmitted by) the chromosomal spindle fibre, this does not necessarily mean that the force for chromosome movement arises from (or is transmitted by) the birefringent component of the chromosomal spindle fibre (see, e.g., Forer, 1976). To restate this somewhat, the birefringence of the spindle arises from material which occupies only 2 - 3% of the volume of a spindle fibre (Sato et al., 1975; Forer, 1976); the question is, whether the force comes from this birefringent material or rather from some other component in the remaining 97 - 98% of the volume. There are data which support the hypothesis that the force arises from a component which contributes little to the birefringence (Forer, 1966, 1969), but Inoué and co-workers (e.g., Inoué & Ritter, 1975), on the other hand, assume that the birefringent component gives rise to the force. Be that as it may, such considerations automatically raise the issue of what exactly a spindle fibre is composed of, which I now discuss.

It is important to realize that the chemical composition of the mitotic spindle and of individual spindle fibres is not known, even though the authors of many text books and many research articles seem to use 'spindle microtubule' as a synonym for 'spindle fibre'. The reason that we do not know the chemical composition of the spindle is that about 90% of the dry matter of the spindle is lost during the isolation of spindles from cells (Forer, 1969; Forer & Goldman, 1972), as summarized in Table I. How much of the lost material could be microtubule protein (tubulin)? One can deduce that microtubules and tubulin, combined, make up only a small fraction of the dry matter in a spindle, as follows. In analyzing the isolated mitotic apparatus (MA, the chromosome-spindle-aster complex), it is found that microtubules make up only 10% of the mass (Cohen & Rebhun, 1970; Bibring & Baxandall, 1971). Since the isolated MA contains only 10% of the dry matter of MA in vivo, this means that microtubules are only about 1% of the MA mass, that 9% (the remainder of the 10%) is different from microtubules, and that about 90% of the MA mass is unknown. Could this 90% be soluble tubulin which is lost during the isolation? If so, one could say that spindles are composed primarily (90%) of soluble tubulin in equilibrium with the poly-

TABLE I

Losses During Isolation of Mitotic Apparatus (MA) from Sea Urchin Zygotes

Material Studied	Dry Matter (gm/100 cm.^{3})	Reference
MA IN VIVO (INTERFERENCE MICROSCOPY)		
Zygotes of Psammechinus miliaris. This is measurement of asters.	21	Calculated from data of Mitchison & Swann (1953), as described in Forer & Goldman (1972) (see Table 6).
Zygotes of Psammechinus miliaris		
asters	21	Forer & Goldman (1972)
spindle	20	
Zygotes of Echinus esculentus		
asters	19	Forer & Goldman (1972)
spindle	18	
ISOLATED MA		
Material Studied	Dry Matter (gm/100 cm.^{3})	Reference
Various species, using various methods of isolation	0.75 - 1.5	Determined chemically by various authors, summarized in Forer (1969) (see Table 8).
From Psammechinus miliaris and Echinus esculentus, isolated using 1M hexylene glycol	2-6 (depending on the pH of the isolation)	Determined by interference microscopy (Forer & Goldman, 1972)

merised form, microtubules. The answer to this question, however, is 'no'. One argument which demonstrates this is based on birefringence measurements: one assumes that spindle birefringence (retardation) is a measure of the amount of polymerised tubulin (microtubules) relative to non-polymerised tubulin (Inoué & Ritter, 1975; Inoué et al., 1975), one assumes that the minimum amount of tubulin is polymerised into microtubules, one assumes that the microtubules are preserved during the isolation but that the soluble tubulin is lost during isolation of the MA, and one then calculates the amount of tubulin lost. One concludes from such an exercise that tubulin is at most 25% of the dry matter of the MA. [To present this in detail, Inoué and co-workers suggest that spindle birefringence (retardation) is a measure of the amount of microtubules relative to non-polymerised tubulin, and that when birefringence (retardation) is measured at different temperatures the maximum birefringence is attained when all the tubulin is polymerised into microtubules (e.g., Inoué, 1964; Inoué & Sato, 1967; Inoué & Ritter, 1975; Inoué et al., 1975). Data on MA birefringence relative to the maximum birefringence attainable can then be directly converted into relative amounts of microtubules vs non-polymerised tubulin. Accepting their hypotheses, for the purpose of this discussion, relevant data are given by Stephens (1973), who studied birefringence (retardation) of MA in vivo in zygotes of the sea urchin, Strongylocentrotus droebachiensus. When zygotes were grown at various temperatures, Stephens found a 50% increase in spindle birefringence (retardation) when the temperature was raised above the temperature that was optimum for growth; the birefringence at the lower limit of growth was 10% of the maximum birefringence. This means, then, that at the lower limit of growth there is 10 times more soluble tubulin than microtubules. In those studies in which microtubules were estimated as 10% of the mass of isolated MA (Cohen & Rebhun, 1970, for MA from zygotes of the sea urchin Arbacia punctulata; and Bibring & Baxandall, 1971, for MA from zygotes of the sea urchin Strongylocentrotus purpuratus), the zygotes were grown near the optimum temperature. But even if one were to argue that the zygotes were grown at the lower limit of temperature, then, assuming that there would be 10 times more monomer (tubulin) than polymer (microtubules) in the MA in vivo, and that all the monomer (tubulin) was lost during isolation, since the polymer (microtubules) is only 1% of the in vivo dry matter

(i.e., 10% of the 10% of the dry matter left in the isolated MA), the monomer plus polymer together comprise less than 20% of the in vivo dry matter (i.e., 11 times 1%).] Another argument which demonstrates that tubulin is less than 25% of the MA dry matter is based on measurements of the total amount of tubulin per cell: one uses chemical measurements of total tubulin per cell (or of fraction of the cell protein which is tubulin), interference microscope measurements of relative concentrations of dry matter in spindle vs cytoplasm, and cytological measurements of relative volumes of spindle vs cytoplasm; then, by assuming that all the cell's tubulin is in the volume occupied by the spindle, one calculates the maximum amount of the spindle dry matter which could be tubulin, which, too, is less than 25% (A. Forer, in preparation). Thus, tubulin is a minority fraction of the dry matter in a spindle, and one just does not know the chemical composition of $\geq$ 65% of the dry matter in spindles.

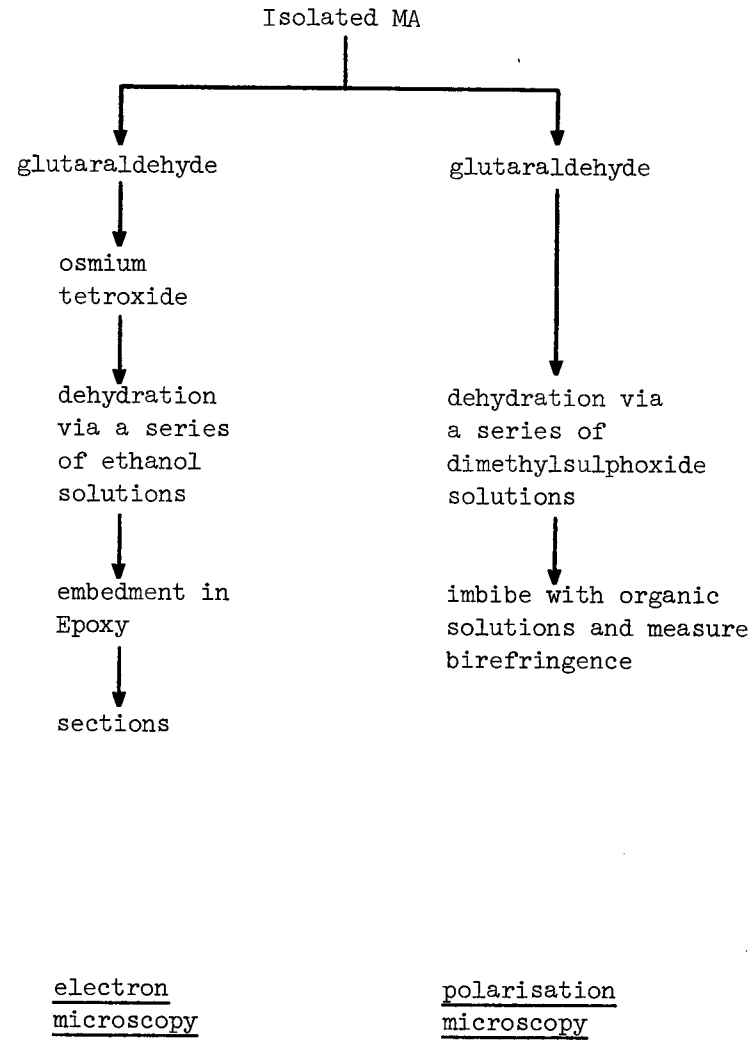
To summarize, spindle fibres produce (or, at least, transmit) force for chromosome movement. Spindle fibres are birefringent, but the birefringence arises from a small volume fraction of the spindle and, in my view, it is not known whether the birefringent fraction or a different fraction gives rise to the force. The chemical composition of the spindle is unknown, because most of the material in in vivo spindles is lost during isolation. Microtubules and tubulin, combined, make up a minority fraction of the spindle, and the composition of the remainder is largely unknown.

One last point to be considered is the source of spindle birefringence. I have argued previously (Forer, 1976) that chromosomal spindle fibre birefringence is most likely due to the birefringence of microtubules plus birefringence due to other components, and that spindle birefringence in other parts of the spindle is due to the birefringence of microtubules minus birefringence due to yet other components. Some other workers disagree, however; Inoué (1976), for example, considers that the "problem is now pretty much resolved", and that, without doubt, spindle birefringence is due solely to microtubules. Inoué & Ritter (1975) reach similar conclusions. I will discuss the evidences on this point in detail elsewhere (Forer, Int. Rev. Cytology, in preparation). I here reiterate that there are strong evidences suggesting that several components contribute to spindle birefringence (e.g. Forer et al., 1976) and that the evidences adduced by Inoué

(1976) and Inoué & Ritter (1975) are equivocal. As one example of this, I consider the data of Sato et al. (1975) which are interpreted by them as proof that the birefringence of the isolated mitotic apparatus (MA) is due solely to microtubules. These authors isolated MA from oocytes of the sea star Pisaster, using hexylene glycol, and they measured the form birefringence of the MA by imbibing the MA with media of different refractive indices. From the measured form birefringence curve they calculated the volume fraction of rods which theoretically would account for the birefringence, and this was about 2%. Electron microscopic observations of isolated MA showed that microtubules occupied 2% of the volume, exactly the same fraction of the volume as predicted by the form birefringence measurement. They concluded that the birefringence of the isolated MA is due solely to microtubules. For several reasons, however, this argument is not conclusive. Consider, for example, the following argument concerning their experimental protocol. Sato et al. (1975) measured birefringences of MA that were fixed with glutaraldehyde, were dehydrated through a series of dimethylsulphoxide-hexylene glycol solutions, and then were imbibed with various organic solutions, each of which had a different refractive index. For electron microscopic observations, however, the MA were treated differently from those in which birefringence measurements were made: they made electron microscopic observations on MA which were fixed in glutaraldehyde, post-fixed in osmium-tetroxide, dehydrated through a series of ethanol solutions, and then embedded and sectioned. The two protocols, summarized in Table II, do indeed differ; but are the differences significant? Let us consider the one difference, the presence of osmium tetroxide in the electron microscopic protocol and its absence in the polarisation microscopic protocol. Forer & Blecher (1975) studied crane fly spermatids and found that microtubules are seen clearly when glutaraldehyde fixation is followed by osmium tetroxide post-fixation, but that microtubules are no longer seen when the osmium-tetroxide post-fixation is omitted. There were no electron microscopic data in Sato et al. (1975) on MA treated without osmium tetroxide post-fixation, so it is possible that in fact no microtubules as such were even present in the MA in which they measured form birefringence. To see if this might be true, I isolated MA from sea urchin zygotes, using hexylene glycol, and fixed the MA with glutaraldehyde; without osmium tetroxide post-fixation I

TABLE II

Difference Between Polarisation Microscopy and Electron Microscopy Protocols



dehydrated the MA via a series of dimethylsulphoxide-hexylene glycol solutions (using the protocol of Sato et al., 1975), embedded the MA in Araldite, and studied serial sections of several MA, in the electron microscope (Forer, in preparation): I saw no microtubules. Hence, even on these grounds alone, the arguments of Sato et al. (1975) concerning isolated MA are equivocal.

Regardless of the exact nature of the microtubule contribution to spindle fibre birefringence, in most eukaryotes microtubules are associated with chromosomes, at their kinetochores, and hence microtubules would seem to have some role in chromosome movement. The question is, what exactly is the role of spindle microtubules? I now consider several hypotheses in which it is assumed that microtubules produce (and transmit) force for chromosome movement.

III. MICROTUBULES AS FORCE PRODUCERS

Several hypotheses state that microtubules produce the force for chromosome movement. Some authors consider that forces arise by slow depolymerisation of microtubules (Dietz, 1969, 1972; Inoué & Ritter, 1975; Gruzdev, 1972). Other authors consider that forces arise by interactions between parallel microtubules, via cross-bridges extending between the tubules (McIntosh et al., 1969; Nicklas, 1971), and that, as in the sliding filaments of muscle, forces are exerted by microtubules which slide against each other. Yet others consider the forces to arise from interactions between microtubules, but that the forces arise by non-sliding ("zipping") interactions between non-parallel microtubules (Bajer, 1973; Bajer et al., 1975). I summarize these hypotheses in turn.

Slow depolymerisation of microtubules is considered by Dietz (1969, 1972) to be the motive force for chromosome movement: microtubules extend between chromosomes and poles, anchored at both ends; some microtubule subunits (tubulin) leave each microtubule at various (random) places along the entire length of the microtubule, and immediately the remainder of the microtubule subunits rearrange and fill in the gaps. This results in shortening of the microtubules, at constant microtubule width, and this shortening of the microtubules by slow depolymerisation and rearrangement provides the force to move the chromosomes. Inoué (1964) and Inoué & Ritter (1975) also

suggest that slow depolymerisation of microtubules provides the force for chromosome movements and that the subunits which remain in the microtubule rearrange (move) and fill in the gaps after subunits leave the microtubule; the difference is that in the Inoué & Ritter (1975) hypothesis this slow depolymerisation is considered to take place exclusively at the poles or near the poles, rather than along the entire microtubule length as Dietz (1969, 1972) postulates. Gruzdev (1972) suggests a similar hypothesis, but in this case the slow depolymerisation takes place primarily at (or near) the kinetochores.

Kinetochore-associated microtubules undoubtedly shorten as chromosomes move polewards, as in the previously described hypotheses, but the question is whether this shortening causes chromosome movement, or whether something else causes movement and shortening is a consequence of movement. In hypotheses of McIntosh et al. (1969) and of Nicklas (1971), bridges between parallel microtubules exert the forces which cause chromosomes to move, and microtubule shortening is a consequence and not the cause of movement. In the hypothesis of McIntosh et al.(1969) some of the spindle microtubules arise (grow) from spindle poles and others arise (grow) from kinetochores. The former become interpolar ('continuous') microtubules, and do not attach to chromosomes, whilst the latter become kinetochore microtubules and extend from kinetochore to pole. According to the hypothesis, each microtubule has an inherent polarity which is a function of the direction of growth of the microtubule, and this polarity causes force to be exerted towards the origin of growth of each microtubule (either kinetochore or pole). The force is exerted by microtubule-associated cross-bridges, and net force, which produces sliding, arises only when cross-bridges on any given microtubule interact with cross-bridges on microtubules with opposite polarity. Thus, if one considers a kinetochore facing a pole, the microtubules from that kinetochore slide polewards (and pull the attached chromosome polewards) by interacting with microtubules which arose (grew) from that pole; no net sliding force is exerted if the kinetochore microtubules interact with microtubules which arose from the opposite pole. According to the hypothesis shortening of kinetochore-to-pole microtubules is due to disassembly of microtubules at the pole, and, again by hypothesis, the kinetics of this microtubule disassembly in part defines the rate of chromosome

movement. Similar arguments explain spindle elongation: microtubules from one pole will interact with microtubules from the other pole to produce force (sliding) which pushes the poles apart; but microtubules from the same pole will not interact with each other to produce force (nor will microtubules from the same kinetochore). Nicklas (1971) critically evaluated this hypothesis and modified it to better account for various experiments which did not fit the model. Nicklas (1971) proposed that only microtubules arising from poles have associated cross-bridges, that is to say, that microtubules arising from kinetochores do not have cross-bridges. In the Nicklas model the cross-bridges produce force towards the pole to which they are attached, but the sliding forces which they exert are exerted only when activated by kinetochore microtubules of opposite polarity; the resulting forces cause sliding between microtubules such that a chromosome is moved towards the pole to which its kinetochore faces, but no force is exerted towards the opposite pole.

In the final hypothesis to be considered, non-sliding ("zipping") interactions between non-parallel microtubules are assumed to provide the motive force for chromosome movement, in the "zipper hypothesis" (Bajer, 1973, and Bajer et al., 1975). "Zipping", defined by Bajer (1973), occurs between microtubules which are initially at an angle to each other (e.g., 30°): the end of one ('movable') microtubule initially bonds to another ('fixed') microtubule. The two microtubules then bond laterally, in increasing amounts, and eventually become parallel and tightly bound. The lateral bonding occurs "progressively along their length..." and as a result the non-bonded "free end..." of the 'movable' microtubule moves laterally towards (i.e. roughly perpendicular to) the 'fixed' microtubule. Thus, "zipping" is basically a lateral movement of one microtubule due to lateral interactions between it and an initially non-parallel microtubule. According to the hypothesis, such interactions can cause the free end of the 'movable' microtubule to move longitudinally (i.e., in the direction of the length of the 'fixed' microtubule) if there are constraints which prevent the region near the 'free end' of the 'movable' microtubule from moving laterally. In such a case the 'bound end' of the 'movable' microtubule would bond laterally; then the bonding progresses towards the 'free end' and in regions where no lateral movements can occur this constraint causes the 'movable' microtubule to bend, and the

bending, in turn, causes the 'free end' to move longitudinally. By attaching the chromosome's kinetochore to the 'free end', one thereby, by hypothesis, has "zipping" move the chromosome polewards. A constraint to lateral movement which would force longitudinal movement of "free" (kinetochore associated) ends of a microtubule could occur, according to Bajer (1973), if interpolar microtubules are adjacent to the 'free end' to prevent it, physically, from moving laterally; another constraint is if two microtubules which are attached to the same kinetochore 'zip' in opposite lateral directions. Lateral movement is prevented with either constraint and, according to the hypothesis, microtubules bend in the region of the lateral constraint, which in turn results in the kinetochore (and hence the chromosome) being transported polewards. Stated in another way, the hypothesis says, for example, that if all non-kinetochore microtubules are parallel, in a pole-to-pole direction, then kinetochore microtubules which are at an angle to the non-kinetochore microtubules interact with the non-kinetochore microtubules to "zip", and lateral restraints convert "zipping" into polewards movement. Complete movement of a chromosome to a pole (maximum shortening of kinetochore microtubules) is a result of a series of such "zippings", amounting to "hundreds, if not thousands..." (Bajer et al., 1975). In common with previous hypotheses (e.g. McIntosh et al., 1969; or Forer, 1974), Bajer et al. (1975) assume that "assembly-disassembly of MTs [microtubules] is a factor regulating ... the rate of movements..."

Several hypotheses for chromosome movement have been summarized in this section. Different hypotheses assume different roles for spindle microtubules, though in all hypotheses spindle microtubules (with or without associated cross-bridges) produce force for chromosome movement. How is one to decide which, if any, of these hypotheses is correct? One can look at the experimental bases of each hypothesis, or one can look at the phenomena explained by the hypothesis and the phenomena which are difficult to explain by each individual hypothesis. This is standard procedure, and I will elsewhere review these hypotheses in this manner (A. Forer, Int. Rev. Cytol., in preparation). For the purpose of this discussion, I assert that, in general, the hypotheses are very broad and are used to explain many facets of mitosis; furthermore, data which argue against some aspects of individual hypotheses do not necessarily rule out other aspects of the

same hypotheses. For example, there seem to be no interactions possible between kinetochore microtubules and interpolar microtubules in some spindles (e.g., Heath, 1974; McDonald et al., 1977); while this would seem to argue against zipping or cross-bridges causing chromosome-to-pole movements, the data do not rule out the idea that cross-bridge action causes pole-to-pole elongation. The difficulty is that the data do not allow one to rule out or rule in any of the hypotheses; nor, indeed, do any data demonstrate unequivocally that microtubules produce the force, or even transmit the force to the chromosomes. This is emphasized by the points, summarized above, illustrating that we do not really know what spindle fibres are composed of, or what the birefringence is due to. One would guess that until we get these data, or, more importantly, that until we can chemically dissect an in vitro model system or until we can genetically or otherwise rigorously dissect in vivo spindles, that we will not be able to accept or reject any of the above hypotheses.

I now discuss the hypothesis that actin produces the force for chromosome movement. In my view, the poleward movement of chromosomes is more likely to be due to processes involving actin than to microtubules functioning as predicted by any of the microtubule hypotheses summarized above.

IV. ACTIN AS FORCE PRODUCER

A. General Role of Actin

Actin is found in cells throughout the plant and animal kingdoms, in both muscle and non-muscle tissue (reviews in Pollard & Weihing, 1974; Forer, 1974, 1978; Hepler & Palevitz, 1974), and is even found in some prokaryotes (Niemark, 1977; Wang et al., 1976). A generalization which follows from these data is that actin is involved in all cellular motile systems, as argued elsewhere (e.g., Behnke et al., 1971; Forer & Behnke, 1972a, 1972b; Forer, 1974, 1978; Pollard & Weihing, 1974; Pollard, 1977). This is similar to the generalizations that ATP is the universal energy source, and that DNA is the universal genetic system: both of these statements have exceptions, but they remain the general statements that one accepts for any new system until there are data which prove

otherwise. I believe that the same can be said for actin and cell motility; that is, that for any given motile system one expects actin to be involved, unless and until there are data which demonstrate otherwise, such as in cilia and flagella (Gibbons et al., 1976) or vorticellid spasmonemes (Routledge et al., 1976).

In overview, an argument for this generalized role of actin would run as follows. Actin-myosin interactions produce force causing muscle contraction (e.g., Huxley, 1972). Actin-myosin interactions most likely produce force causing amoeboid motions (e.g., Taylor et al., 1973; reviews in Forer, 1974; Pollard & Weihing, 1974; Allen & Taylor, 1975; Taylor, 1976). Actin filaments are involved in protoplasmic streaming in algae (e.g., Palevitz, 1976); in algae the polarity of the actin filaments is directly correlated with the direction of streaming (Kersey et al., 1976). Cell cleavage seems to occur by means of actin filaments in the contractile ring, in the cleavage furrow (Perry et al., 1971; Forer & Behnke, 1972c; Schroeder, 1973, 1975, 1976; Gawadi, 1974), perhaps in consort with myosin filaments (Fujiwara & Pollard, 1976). Actin is involved in acrosome movements (review, Tilney, 1975), in movements of microvilli (Mooseker, 1976), and in movement of animal cells (e.g., Goldman et al., 1976). Actin filaments are found in eukaryote cells ranging from man to amoebae to algae to higher plants (review, Pollard & Weihing, 1974; Forer, 1974, 1978; Hepler & Palevitz, 1974), in places where one expects force production. What more reasonable generalization than to assume that this universal and highly conserved protein is involved in cell motility in all these systems? This is not to say that one knows how actin is involved in any one system (except, perhaps, skeletal muscle), or which systems for sure are exceptions, or what other roles actin might have (e.g., Lazarides & Lindberg, 1974), but rather that one makes this generalization and then uses the large body of data on muscle proteins as a guide to studying these other systems (e.g., Huxley, 1976). What about chromosome movements?

From the 'generalization' just described, and in view of the uncertainties reviewed in Section II, about spindle composition and about which spindle components produce force, one assumes that actin is involved in producing force for chromosome movements. Before I summarize and discuss data which deal with this point I want to state explicitly that the data to date deal primarily with whether or not actin is present in

spindles. If actin is absent from spindles, one can rule out the possibility that actin is involved in chromosome movement. The converse, that actin is present in spindles, only is consistent with the possibility, and does not prove it: only further work can do that. But if actin is present in spindles then one argues from this, and from the generalized role of actin, that actin is probably involved in producing force for chromosome movement. I now discuss the detection of actin in the spindle as determined using various methods, namely, electron microscopy, fluorescently labelled antibodies, and fluorescently labelled HMM, discussing each method in turn.

B. Electron Microscopic Studies of Actin in the Spindle

I first discuss the general problem of detecting actin electron microscopically. Then I discuss the specific data which deal with actin in spindles.

1. Electron Microscopic Detection of Actin

Actin exists as monomeric, globular actin (G-actin), as a non-globular but bound actin (e.g., Tilney, 1975), or as filaments (F-actin) that often contain other components, such as tropomyosin and troponin. Actin-containing filaments from muscle are 6-8 nm in diameter as seen in both negatively stained preparations and in sections, and can be identified as containing actin by means of their reaction with heavy meromyosin or with heavy meromyosin subfragment 1 (review in Pollard & Weihing, 1974; Forer, 1978): actin-containing filaments from muscle have clearly delineated edges and are 6-8 nm wide (Fig. 1), whereas actin-containing filaments that have reacted with heavy meromyosin (HMM) or heavy meromyosin subfragment 1 (S1) have a series of arrowheads down the length of each filament, or at least appear 'decorated', with not clearly delineated edges, and are more than 20 nm wide (Fig. 2). The arrows all point in the same direction on any given filament. Actin-containing filaments from non-muscle cells appear identical to those from muscle cells, and react with HMM (or S1) from rabbit skeletal muscle to form a series of arrowheads, all of which point in the same direction on any given filament. The arrowhead complexes are identical to those formed from rabbit skeletal muscle actin, and are found when rabbit skeletal

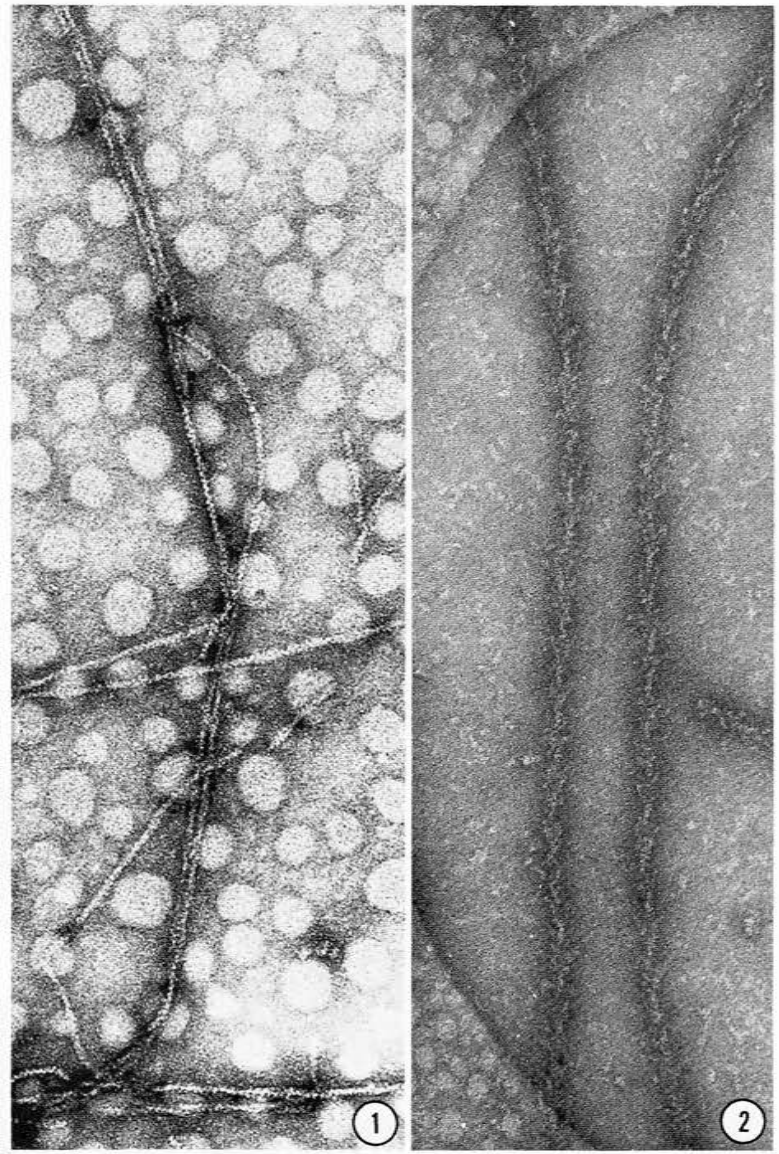


Fig. 1. An electron micrograph of negatively stained rabbit skeletal muscle actin. Fig. 2 is an electron micrograph of negatively stained rabbit skeletal muscle actin that was reacted with S1, to give arrowheads. (Techniques are given in Forer, 1978). In Fig. 2 the arrows all point downwards. Both are at the same magnification (X 190,000).

muscle HMM is mixed with actin from a broad range of non-muscle cells, from man to amoebae to algae to higher plants.

The 'arrowhead' appearance with HMM or S1 is specific for actin-containing filaments, as shown by two kinds of evidence. First, a 'theoretical' argument, namely that the arrowhead appearance is due to the arrangement of the G-actin monomers in the filament: the F-actin filament contains 2 chains of G-actin monomers that twist around each other, and it is this arrangement of monomers that gives rise to the arrowheads (Moore et al., 1970), as follows. There is one HMM binding site per actin monomer, and the binding sites on different monomers twist around the long axis of the filament as the two actin chains twist around. The arrowhead appearance is due to the superposition of the images of HMM (or S1) molecules which are at different angles (with respect to the filament long axis) at different positions along the length of the filament (see Moore et al., 1970, and Huxley, 1973). Thus, since no other known filament has both a similar arrangement of monomers into 2 chains and one HMM binding site per monomer, no other filament will give arrowheads. Second, an 'empirical' argument: HMM has been mixed with various other filamentous cell components, and none of these give a visible arrowhead appearance (or 'decoration') as observed electron microscopically in sections or in negatively stained preparations; a listing of components which do not give visible reaction includes collagen fibrils, 'tonofibrils', intact microtubules, microtubule protofilaments, 10 nm filaments, bacterial flagellae, and fibrin (Forer, 1978). Hence, for both these reasons, the arrowhead formation (or 'decoration') is specific for actin. A further control is to add 1 mM pyrophosphate or 1 mM ATP; both compounds block HMM (or S1) binding to actin (and remove already bound HMM or S1), and thus both cause the disappearance of arrowheads (or of decoration).

It should be emphasized that whilst arrowhead formation is quite specific for identifying actin, it is conceivable that HMM binds to some or to many other cell components besides actin (see, for example, Heywood et al., 1968, or discussion in Forer, 1974, pp. 323-325). Such binding could occur and, for reasons discussed above, would not be seen as arrowhead formation. Hence, even though arrowhead formation is indeed specific for actin, HMM could interact with and bind to other cell components besides actin.

It is relevant to discuss other facets of the electron

microscopic study of actin before considering work dealing with spindle actin. This has recently been reviewed in detail (Forer, 1978); I herein summarize some of the conclusions relevant to the discussion below. The review (Forer, 1978) can be consulted for references and for details on all points discussed from here until the next section (Section B-2, on actin in spindles).

Actin-containing filaments can be seen in myofibrils either after osmium tetroxide fixation or after glutaraldehyde fixation. A certain amount of longitudinal shrinkage of each actin-containing filament is produced by osmium tetroxide fixation; subsequent ethanol dehydration causes further longitudinal shrinkage of actin-containing filaments in the region in which these filaments do not interact with myosin (the I-band), but does not cause shrinkage of such filaments in the region in which they interact with myosin (the A-band). Thus, interaction with myosin seems to prevent the ethanol-dehydration-induced longitudinal shrinkage. The post-osmium longitudinal shrinkage of actin-containing filaments in the I-band is also prevented by holding the ends of the muscle during dehydration. Lateral shrinkage also occurs, such that in sections the spacing between actin-containing filaments is only about 70% of that in vivo.

Single actin-containing filaments from muscle are not straight (as studied using negatively staining), such that, for example, paracrystalline forms need be used to study the structure of actin by means of optical diffraction(from electron microscope pictures). Similarly, in sections single actin filaments outside the A-band are rarely seen to be straight and continuous from A-band to Z-line. Actin-containing filaments are seen more clearly in glycerinated myofibrils than in non-glycerinated myofibrils, and one can recognize a group of filaments more readily than one can follow any single filament.

Similar conclusions can be drawn from studies of actin-containing filaments in non-muscle cells. For example, actin-containing filaments in non-muscle cells are more readily identified in groups than when there is only one such filament. (One cannot hold the ends of the filament bundles in cells as the cells are being dehydrated, or measure lateral spacing in vivo, as one can with muscle, so there are no equivalent data on these points in non-muscle systems.) Another example is increased visibility after glycerination.

Actin-containing filaments in non-muscle cells can only be identified as such by adding HMM in the absence of ATP, and to do this most workers have utilized glycerol to make the cells permeable to HMM and to allow the endogenous ATP to leave the cells. In non-muscle cells, as in myofibrils, glycerination seems to allow actin-containing filaments to be seen more clearly than in non-glycerinated cells.

Actin is highly conserved, chemically, from species to species, as judged by analysis of amino acid sequences. Actin-containing filaments found in non-muscle cells all can react with rabbit skeletal muscle HMM, under proper conditions, to form arrowheads. Nonetheless, while accumulated experience with muscle proteins can be a 'guide' to planning experiments on non-muscle actin, one can not rely completely on non-muscle actin behaving the same as muscle actin, because in many cases non-muscle actins are chemically different from muscle actins. As one example, actin from Physarum is antigenically different from rabbit skeletal muscle actin; indeed, there are at least three genes that code for different actins in single organisms (review in Forer, 1978). As further examples, actin-containing filaments in non-muscle cells are different from actin-containing filaments in skeletal muscle in that some such filaments are more cold labile and have different salt solubility properties than actin-containing filaments from muscle; also, monomeric actin in non-muscle cells is sometimes associated with inhibitors that prevent filament formation. Further, in at least two cells (Physarum and human blood platelets) there are two components which co-electrophorese with actin, one of which will co-polymerise with the other but will not polymerise on its own. Finally, a large proportion of the actin in chick fibroblasts (and chicken brain) will not polymerise under any of a large number of conditions in which skeletal muscle actin polymerises, even though the chicken fibroblast actin is not associated with an inhibitor. Nor will this actin co-polymerise with skeletal muscle actin. However, this actin can be forced to crystallize by means of vacuum dialysis, and the crystals can be broken into component filaments. These filaments look like actin in negatively stained preparations and bind HMM to form arrowhead complexes, but when the filaments are dissolved they do not polymerise into filaments again under conditions in which skeletal muscle actin polymerises: they can be polymerised only by means of vacuum dialysis (Bray & Thomas, 1976). Thus there are clear

differences between skeletal muscle actin and at least some of the non-muscle actins.

Two additional points need be made concerning non-muscle actin. First, in several non-muscle systems one sees more filaments (in sections) after reaction with HMM than one sees (in sections) without HMM treatment. Different explanations for this seem to apply in different systems. In some systems HMM causes polymerisation of actin into filaments: prior to HMM addition the actin existed in non-filamentous form, and addition of HMM caused polymerisation and formation of arrowhead complexes. In other systems, HMM stabilizes actin-containing filaments against loss during the fixation-dehydration-embedment procedure: actin-containing filaments are present before fixation but varying numbers (depending on the system) do not survive fixation and embedment unless they are complexed with HMM.

Second, several non-filamentous supramolecular forms of actin exist, including "nets" and "bundles". Neither of these two forms looks like skeletal muscle actin and neither would ordinarily be recognized as containing actin. Nor does it seem likely that either would bind HMM to form arrowhead complexes. Thus these forms of actin are likely to escape detection. In other cells, some supramolecular aggregates of actin-containing filaments do not bind HMM in situ even after glycerination and addition of HMM. These filaments will bind HMM to form arrowhead complexes if they are first splayed out on a grid, but they do not bind HMM in situ. Thus, with these known examples of absence of arrowhead complex formation, one needs to be cautious before using negative electron microscopic data by themselves to rule out the presence of actin in any given system.

In summary, actin is a universal protein, and actin-containing filaments are found in many cells. Actin-containing filaments are certainly involved in causing skeletal muscle contraction, amoeboid movement, cell cleavage, and protoplasmic streaming, and seem to be involved in animal cell locomotion, extension of cellular processes, and other motile phenomena. One generalizes from this and suggests that, until proven otherwise, one expects actin to be involved in all cellular motility. Actin-containing filaments are identified electron microscopically by their reaction with HMM to form arrowhead complexes, and actin-containing filaments in non-muscle cells often look like their skeletal muscle counter-

parts. But while one can use the accumulated knowledge of the chemistry of muscle proteins as a guide to studying non-muscle actin, there are distinct differences between skeletal muscle actin and at least some non-muscle actins.

I now consider the question of whether actin is a component of mitotic (and meiotic) spindles.

2. Electron Microscopic Identification of Actin in Spindles: the Evidences

The electron microscopic studies which argue that actin is indeed a spindle component are discussed in this section, in which I concentrate on the original data and interpretations. In the next section I discuss critically these evidences, in light of arguments that have been made against these data and interpretations, and in light of counter-data.

Actin-containing filaments have been found in spindles of various cell types studied electron microscopically: the reaction with HMM to form arrowhead complexes was used as a marker to identify actin-containing filaments. The cells in which spindle actin has been seen in this way are listed in Table III. In Table III, the term 'microfilaments', which denotes 6-8 nm filaments seen in glutaraldehyde-fixed cells, is used as shorthand for actin-containing filaments seen in HMM treated cells. I now discuss these results. (References discussed are those given in Table III unless otherwise specified; the data of Schroeder, 1973, 1976, are discussed separately, in Section B-3, since he argues from his data that actin is not a spindle component.)

All studies reporting actin-containing filaments in spindles identified by HMM have used glycerination to make the cells permeable to HMM (and to allow ATP to leave the cells). The glycerination technique is a source of contention, to some, so I summarize details of the techniques used. In all these studies glycerination was with 50% glycerol in standard salts solution (standard salts solution is .1M KCl or .05M KCl; 5mM $MgCl_2$; 6mM phosphate buffer, pH around 6.8); glycerination was at room temperature and glycerination times (before addition of HMM) varied from 3 days (for crane fly spermatocytes) to 20-30 minutes (mouse neuroblastoma cells, or PtK1 cells), or even to as little as 1-4 minutes (PtK1 cells). HMM (in 6% glycerol, or in standard salts solution) was added to the cells either directly after glycerination, or after the

glycerol was gradually diluted. HMM was sometimes added in the cold (4°C), sometimes at room temperature, and treatment with HMM ranged from 1-2 min (PtK1 cells) to 24-36 hrs (crane fly spermatocytes and locust spermatogonia).

The distribution of actin-containing filaments was studied in various stages of division, or, in neuroblastoma cells, in metaphase only. In anaphase and metaphase, actin-containing filaments between chromosomes and poles were oriented parallel to the microtubules; the one exception was metaphase mouse neuroblastoma cells, in which some filaments were parallel to spindle microtubules whilst most were randomly scattered in amongst the microtubules. Associations between actin-containing filaments and microtubules (or, finding that the 2 elements followed closely parallel, nearby paths) occurred not infrequently in locust spermatogonia, PtK1 cells, and Haemanthus endosperm but did not regularly occur in mouse neuroblastoma cells. Actin-containing filaments were also prominent interzonally, in anaphase, and these were either parallel to the interzonal microtubules (locust spermatagonia), or were both parallel to interzonal microtubules and randomly arranged (crane fly spermatocytes and Haemanthus endosperm cells), or the arrangement was not specified (PtK1 cells). In only PtK1 cells was the orientation and distribution of HMM-binding filaments similar to that of microfilaments in glycerinated cells prior to HMM treatment (see Table III); in PtK1 cells microfilaments were also seen in glutaraldehyde-fixed cells (not-glycerinated), though "less prominently" than in glycerinated cells. In other cells either no microfilaments were seen unless treated with HMM (crane fly spermatocytes), or fewer microfilaments were seen without HMM treatment than with HMM treatment (locust spermatogonia, Haemanthus endosperm), or no comparison was given (mouse neuroblastoma cells).

No clear-cut attachments between actin-containing filaments and chromosomes were illustrated (see Table III); in only locust spermatagonia was such attachment described, but these were not illustrated, nor was serial section analysis described to confirm that such attachments were not artefacts of the large depth of field of the electron microscope. Any such attachment between actin-containing filaments and chromosomes, if they exist, then, would seem to be much less obvious with present methods than those between kinetochore microtubules and kinetochores. More likely, no such attachment

TABLE III

Electron microscopic studies of spindle actin using glycerination and reaction with HMM (and/or S1): positive results.

Reference	Cells studied	Glutaraldehyde fixed cells	Glycerinated cells, no HMM (or HMM + ATP, or HMM + pyrophosphate)		Glycerination followed by HMM			
		MFs?	MFs?	MTs?	MFs?	MTs?	Extra-spindle actin?	MFs attached to chromosomes?
Behnke et al., 1971; Forer & Behnke, 1972b	Crane fly spermatocytes (meiosis)	-	No	No	Yes	Yes or No, depending on the stage of division	Yes	Only possibly, at best
Gawadi, 1971, 1974	Locust spermatocytes (meiosis)	-	Few, only	No	-	-	-	-
	Locust spermatagonia (mitosis)	-	Yes ("in small numbers.")	Yes	Yes (more than without HMM)	Yes	Yes	Some seen directly attached, but not necessarily at kinetochores
Schroeder, 1973, 1976	HeLa cells	-	Yes (few)	Yes	Yes (few; never in bundles)	Yes	Yes	No
Hinkley & Telser, 1974	Mouse neuroblastoma cells (mitosis)	-	Yes	Yes	Yes	Yes	Yes	Filaments are rarely near chromosomes: only possible contact, at best
Schloss et al., 1977	Rat Kangaroo (PtK1) cells (mitosis)	Yes	Yes ("more prominent..." than with no glycerination)	Yes	Yes (with orientation and distribution similar to glycerination only)	Yes	Yes	None reported, so presumably none were seen to
	Rat embryo cells in culture (mitosis)	-	Yes	Yes	-	-	-	-
Forer & Jackson, 1975, 1976	Haemanthus (African blood lily) endosperm cells (mitosis)	-	Some	Yes	Yes (in large numbers, more than without HMM)	Yes	No (very few such filaments were seen)	None seen (serial sections were studied)

MFs = microfilaments

MTs = microtubules

- means that no data were reported on these points

exists. Related to this is the question of the polarities of the spindle actin, i.e., the direction of the arrowheads on the actin-containing filaments. In muscle, the actin-containing filaments have arrowheads which point away from the Z-line and towards the myosin, in the A-band, as in Fig. 3. By analogy, one would expect that if actin was attached to a chromosome at the kinetochore the chromosome would be a Z-line equivalent, and arrowheads would point towards the pole (Fig. 4). Or, conversely, if the actin was not attached to the kinetochore but rather to something else then the arrowheads should point towards the chromosome (Fig. 5), in which case the chromosome or associated microtubules (or other components) are analogous to the myosin. Arrowhead directions are seen only rarely in sectioned material (review in Forer,1978), and with respect to spindle actin only 2 articles have given data on actin polarity: (a) in locust spermatagonia (Gawadi, 1974), actin-containing filaments between chromosomes and poles in metaphase (and oriented parallel to spindle microtubules) had arrows pointing predominantly towards the chromosomes (45 out of 62); (b) in Haemanthus endosperm (Forer & Jackson, 1976) only 11 clear arrowheads were seen in the chromosome-to-pole region, out of "hundreds" of filaments photographed in metaphase and anaphase, and 8 out of 11 arrows pointed towards the chromosomes. A further article has emphasized the rarity of arrowheads: in PtK1 cells (Schloss et al., 1977), very few arrowheads clear enough to get directionality were seen in 60 spindles studied. Thus, though the arrowheads would seem to point towards the chromosomes, in agreement with the lack of attachment of actin-containing filaments to the kinetochore, the difficulty in attaching meaning to the polarities is that only a very small percentage of the filaments have clear polarities in electron micrographs.

While perhaps not directly relevant to the discussion at hand, it is clear from the data summarized in Table III that microtubules in meiotic spindles seem to react differently to glycerination than do microtubules in mitotic spindles: in glycerinated meiotic cells (crane fly spermatocytes, locust spermatocytes) one does not see microtubules; in glycerinated mitotic cells (the remainder in Table III) one does. While the data so far are rather limited, this does not seem to be simply a technical difference, because microtubules were seen in glycerinated locust spermatagonia in the same testis lobes as the spermatocytes. That microtubules are seen after HMM

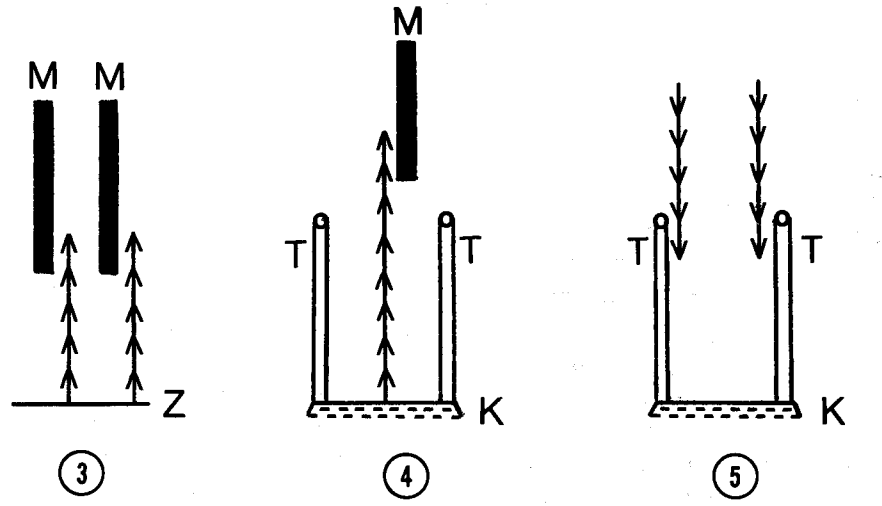


Fig. 3. Illustrates the arrangement in skeletal muscle: the Z-line (Z) is the site of attachment of actin-containing filaments, that have polarities (arrows) pointing towards the thick myosin filaments (M). Figs. 4 and 5 represent possible attachments (and polarities) of actin-containing filaments in spindles. In Fig. 4, actin-containing filaments are attached to the kinetochore (K), as are microtubules (T), with actin polarity (arrows) pointing towards the myosin filament (M) (that may or may not exist in spindles). In Fig. 5 the actin-containing filament is anchored somewhere in the spindle, with polarity (arrows) towards the kinetochore (K) and kinetochore microtubules (T).

treatment of meiotic cells whereas none were seen without HMM treatment suggests strongly that actin interacts with these microtubules, as discussed in detail previously (Forer, 1974).

It is relevant to later discussion to note that, unlike the situation in the animal cells studied, there was very little extra-spindle actin seen in Haemanthus endosperm cells. This finding has been confirmed by serial section reconstruction analysis of other Haemanthus endosperm cells (Forer & Jackson, in preparation), glycerinated in various other ways (e.g., using 25% glycerol with 0.1% Triton-X-100 added).

Summarising these data on HMM-binding filaments in the

spindle, one can say that while actin is found in the spindle, the actin-containing filaments do not seem to be attached to chromosomes, and the qualitative observations made to date do not allow one to decide whether or not actin-containing filaments are associated in a regular way with chromosomes or with kinetochore-associated microtubules. All authors nonetheless argue, cautiously, that actin-containing filaments probably have a role in producing force for chromosome movement. All admit the possibility of various kinds of artefacts, but nonetheless argue for a functional role for spindle actin. Forer & Behnke (1972b) argue that the changes in orientations and arrangements of actin-containing filaments in various stages suggest that actin-containing filaments are genuine spindle components, with a functional role. Gawadi (1974) argues likewise, and suggests that actin-containing filaments interact with microtubules in such a way that, by analogy with muscle, the microtubules are surrogate Z-lines (Fig. 5): actin-containing filaments "terminate" at microtubules (i.e., they interact with microtubules in a fixed positional relationship). As a corollary, she suggests that chromosomes are surrogate myosins. Forer & Jackson (1976) suggest that actin-containing filaments are on the outside of microtubule bundles, and have some functional role. Schloss et al. (1977) suggest that there is an intimate interaction between microfilaments (actin-containing filaments) and microtubules, with the microfilaments being closely applied to the microtubule wall and thereby only rarely being seen in glutaraldehyde-fixed (non-glycerinated) cells.

Before considering objections to these data, and before considering counter-data, it is relevant to consider electron microscopy of glutaraldehyde-fixed (not-glycerinated) cells, because 6-8 nm (diameter) microfilaments have been reported in spindles in various cells. Ryser (1970) described the regular occurrence of large numbers of microfilaments in the intranuclear spindle of Physarum, in all stages of mitosis; these microfilaments looked like cytoplasmic actin filaments, and Ryser (1970) argued that these were spindle actin filaments that were probably involved in generating force for chromosome movements. Lewis et al. (1976) described intranuclear microfilaments in Paramecium micronuclei during mitosis, but the documentation of the presence of microfilaments was not compelling. Müller (1972) described microfilament bundles seen in early-prometaphase and mid-prometaphase spindles in crane fly spermatocytes, and Forer & Brinkley (1977) described single microfilaments in mid-anaphase crane fly spermatocyte spindles, near microtubules. In Haemanthus endosperm cells Bajer & Molè-Bajer (1969) found "frequently in prophase and later stages a continuous transition between MTs [microtubules] and bundles of very thin fibrils..." the size of microfilaments. Goode (1975) found microfilaments in embryonic chick heart muscle cells in mitosis, but only a few were seen and, to quote Goode (1975), there was "no ultrastructural evidence of a specific regular interaction of mitotic apparatus microtubules and any other filament type... at any stage of mitosis." On the other hand, microfilaments were consistently seen in spindles of Xenopus heart cells in primary tissue culture, presumably fibroblasts (Euteneuer et al., 1977). Individual microfilaments were seen rather than bundles, and these were scattered in amongst the spindle microtubules and oriented primarily parallel to the microtubules; these filaments were seen in all stages of division, and the authors argue that in view of the regular orientation and distribution of the filaments they are probably involved in the function of the spindle. Microfilaments were seen in the stem body of human cells in culture (WI-38 and HeLa cells), but these were not seen in metaphase or anaphase spindles (McIntosh & Landis, 1971). In PtK1 cells, McIntosh et al. (1975) identified microfilaments in cross-sectioned spindles (presumably in serial-sections), quite near to microtubules, and Schloss et al. (1977) described microfilaments in longitudinally-sectioned spindles, also quite close to microtubules.

There are difficulties in interpreting the above observations however, in that, for example, the distribution of these filaments is not known, so they may not be regularly associated with chromosomal spindle fibres. Further, all 6-8 nm filaments do not necessarily contain actin. Positive identification of actin requires interaction with HMM, or other marker. One would need to describe the positions of these microfilaments in spindles in glutaraldehyde-fixed (non-treated) cells and show that those filaments which react with HMM have the same distribution as the filaments found in glutaraldehyde-fixed (not-glycerinated) cells, if one wished to use these observations to prove that actin-containing filaments exist in these spindles in vivo. Nonetheless, that microfilaments are seen in glutaraldehyde-fixed (not-treated) Haemanthus endosperm cells, PtK1 cells, and crane fly spermatocytes,

the same cells in which actin-containing filaments (which react with HMM) are found (Table III) is encouraging to those like myself who believe that actin-containing filaments are involved in moving chromosomes. I now consider arguments and data that are counter to the view that actin-containing filaments are functional spindle components.

3. Electron Microscopic Identification of Actin in Spindles: Counter-arguments and Counter-evidences

Porter (1973) has discounted the evidence identifying actin-containing filaments in the spindle; to quote him, this is "because I suspect contamination in experiments which use heavy meromyosin to detect actin". There are two interpretations of this statement. One of these can be ruled out immediately, and that is that the HMM is contaminated with actin-containing filaments. One does not see actin-containing filaments in electron microscopical observation of negatively stained HMM preparations using the same HMM as used to identify spindle actin (e.g. Forer & Behnke, 1972a, 1972b); contamination with actin-containing filaments is readily seen under such conditions, when HMM preparations are contaminated (e.g., Forer, 1978), and hence one concludes that this objection is not valid. It is possible, however, that this statement really refers to "contamination" of the spindle by extra-spindle actin; this argument is discussed below.

Arguments against the presence of actin-containing filaments in crane fly spermatocyte spindles were presented by LaFountain (1974, 1975). Crane fly spermatocytes were not treated with HMM but were directly fixed with glutaraldehyde and then post-fixed and processed in the usual way (LaFountain, 1974), or were fixed with glutaraldehyde-tannic acid and then post-fixed and processed in the usual way (LaFountain, 1975). Microfilaments (5-7 nm in diameter) were seen in cleavage furrows, oriented equatorially, in both glutaraldehyde and glutaraldehyde-tannic acid fixed cells, and none were seen in other regions of the cortex in metaphase and anaphase (LaFountain, 1974). No microfilaments were seen in the spindle, with either fixative. LaFountain (1974, 1975) argued that spindle microfilaments found after HMM treatment do not really exist in spindles in vivo, because, to quote LaFountain (1974), "Since cortical filaments have been preserved by our techniques, one would expect that at least some microfilaments in spindles would have been preserved and detected, provided they are present in the spindle"; hence, he questions "whether spindle microfilaments actually exist in vivo." Where then did the actin-containing filaments come from that were seen in crane fly spermatocyte spindles after glycerination and HMM treatment? LaFountain (1975) speculated that perhaps "glycerinated spindles are "contaminated" with actin...", which originally was outside the spindle. Or, that microtubules bind HMM to give arrowhead complexes as the microtubules are broken down by the glycerination. Or that the actin exists in spindles in the globular (non-polymerised) form, and, presumably, polymerises during the glycerination-HMM treatment.

There are several flaws in the LaFountain (1974, 1975) argument. First, one suggestion can be eliminated immediately: microtubule protofilaments do not bind HMM in any visible manner (review in Forer & Behnke, 1972a), and thus the spindle 'decorated filaments' are not simply microtubule protofilaments, and are actin-containing filaments, as reviewed above. Also, in other cell types microtubules exist in the same spindles as do HMM-binding filaments (Table III). Second, all cortical filaments have not been preserved by LaFountain (1974): actin-containing filaments are seen (after glycerination and HMM treatment) around the entire circumference of crane fly spermatocytes, in large numbers, in regular orientations, and in all stages of meiosis (Forer & Behnke, 1972c). These were not seen by LaFountain (1974, 1975). Similar prominent bundles of actin-containing filaments are seen in cortices of many animal cells (review in Holtzer et al., 1973), so this contradiction is not due to the observations of one set of workers only. LaFountain's original argument was: all cortical microfilaments are preserved and seen, spindle microfilaments are not seen, therefore spindle microfilaments do not exist in vivo. But since the prominent and regularly arranged bundles of microfilaments in the cortex outside the cleavage furrow were not seen, this argument reduces to: cleavage furrow microfilaments are seen, but other cortical microfilaments are not and neither are spindle microfilaments. That is to say, LaFountain missed one prominent class of actin-containing filaments and could have missed as well the less prominent microfilaments in the spindle. Hence this argument is not compelling. Third, microfilaments have been seen in non-treated glutaraldehyde-fixed cells, as described above,

including spermatocytes of the same species of crane fly studied by LaFountain (see Forer & Brinkley, 1977). Indeed, despite LaFountain's claims to the contrary, possible longitudinal-section images of microfilaments can be seen in Fig. 1 of LaFountain (1975) which look like the 'microfilaments' illustrated in Forer & Brinkley (1977) and in Schloss et al.(1977). Likewise, there are cross-sectional images in the spindles (from glutaraldehyde-tannic acid fixed cells) which look like cross-sectioned microfilaments: the profile at 2.8 cm.from the left and 1.1 cm. from the bottom of Fig. 2b of LaFountain (1975) looks exactly like cross-sectioned actin-containing filaments from muscle illustrated in the inset to Fig. 2a of LaFountain (1975). The final point is the argument that spindles are "contaminated" with extra-spindle actin: this is discussed below. In sum, the arguments of LaFountain (1974, 1975) are quite unconvincing, except possibly for the speculation that spindle actin might be "contamination" from without.

Physiological lines of evidence have been used to argue that actin-myosin interactions are not involved in mitosis. One such argument is based on studies of the isolated mitotic apparatus (MA). Sakai et al. (1975) reported that chromosome movement could be induced in MA isolated in glycerol, if the MA were suspended in tubulin, in microtubule polymerising medium plus ATP. Sakai et al. (1976) extended these observations to test the nucleotide specificity of the movement and to test the effects of antibodies against dynein and of antibodies against myosin. They found that chromosome movement required ATP and that other nucleotides could not substitute for the ATP. They found, further, that antibodies against flagellar dynein (the flagellar ATPase) blocked chromosome movement in isolated MA whilst antibodies against egg myosin (from the same species from which MA were isolated) had no effect on chromosome movement. Therefore, as argued by Mohri et al. (1976), Sakai et al. (1976), and Mabuchi & Okuno (1977), chromosome movement is most likely due to dynein ATPase interacting with microtubules and is not likely to be due to myosin interactions with actin.

There are several flaws in this argument, though. First is the question of how well the movement in MA in vitro represents chromosome movement in vivo. Anaphase in sea urchin zygotes is reasonably fast, and usually lasts no more than 10 minutes in vivo; during this time the chromosomes move polewards 10-15 microns, at velocities, therefore, of about 1

micron per minute. In the in vitro movement, on the other hand, in isolated sea urchin zygote MA, the chromosome to pole distances shortened by only 2-3 microns; this shortening took place in 1-2 hours, so the in vitro chromosome velocities measured by Sakai et al. (1976) are about 1.5 microns per hour, or about 60 times slower than in vivo. Also, chromosomes in vitro moved only a fraction of the distance moved in vivo (3 out of 10 microns). Thus, for both these reasons, the chromosome movement studied in vitro is quite different from in vivo movement, and the relevance of the antibody experiments to the in vivo situation is not clear. Second is the question of how accurate the measurements are. In general one needs to consider resolution limits of the microscope and consequent accuracy of measurements, but not enough experimental information is given in the paper by Sakai et al. (1976) to judge these points. Nonetheless, inspection of the published curves leads one to question the accuracy of the measurements, for the following reason. In the published curves, Sakai et al. (1976) give measurements of pole-to-pole distances (spindle lengths), chromosome-to-pole distances, and interzonal distances (distances between the separating chromosomes). Clearly, the spindle lengths should equal the interzonal distances plus two times the chromosome-to-pole distances, within the accuracy of the measurements. The correspondence between these numbers, then, is a measure of the accuracy of the measurements. If one inspects the curves, one finds many instances of 2 to 4 micron differences between spindle lengths and the sum of interzonal distances plus two times the chromosome-to-pole distances: examples include Figs. 1b, 2b, 3a, 3b, 3c, and 5b of Sakai et al (1976). Therefore, since individual curves are inaccurate to within 2-4 microns, and since the total movement (2 times the shortening of the chromosome-to-pole distances) is only 4-6 microns, one questions how much movement indeed has occurred. The final points are missing controls: would the antibody against myosin block the contractile function of myosin in muscle (or other myosin system) under the conditions of the experiment? No such experiments were reported, but are necessary, because McIntosh et al. (1975) were unable to block the contraction of glycerinated muscle by application of antibodies against myosin or of antibodies against actin. Also, one needs a control to ensure that the antibody against dynein does not affect chromosome movement indirectly via an effect on the tubulin polymerisa-

tion medium in which the MA need be suspended. Thus, in sum, the observations are of inadequate accuracy and have questionable relevance to postulated roles of myosin involvement in chromosome movement.

Another physiological experiment has been used to argue against actin-myosin interactions being functional in mitosis; in this experiment antibody against myosin was injected into blastomeres (at the 2 cell stage) at varying times before cell cleavage (Mabuchi & Okuno, 1977). Sakai et al. (1976) have argued from these experiments in the following way. Injection of antibody blocked cleavage, but did not block chromosome motion. The antibody does function after being injected into the cell, since cleavage is blocked; therefore, they argue, "the fact that no inhibition of chromosome motion was caused by the anti-egg myosin γ-globulin provides evidence that the chromosome motion ... is not dependent upon a myosin-actin system ..." (Sakai et al., 1976). (Mabuchi & Okuno, 1977, who presented the relevant data, do not make so explicit an argument.) One must examine the experiments in detail in order to critically assess this argument, so I first summarize the relevant experimental details.

Mabuchi & Okuno (1977) prepared antibodies against myosin isolated from starfish eggs. The antibodies had no effect on myosin Ca-ATPase activity, nor on myosin Mg-ATPase activity, but did block the actin-activated Mg-ATPase activity of myosin. Mabuchi and Okuno injected antibodies into single blastomeres of starfish embryos at the 2-cell stage: they injected different doses of antibody and injected at various times in the cell cycle, namely in interphase (before nuclear membrane breakdown), after nuclear membrane breakdown, and just at the start of cleavage. The cells took 15 minutes to progress from nuclear membrane breakdown to cleavage, and the injected antibody was spread throughout the cell in less than one minute (as measured using fluorescently labelled non-immune serum); thus, they argued, diffusion was not a limiting factor in the experiments. The results were as follows. In injections prior to nuclear membrane breakdown, 0.3 ng of antibody blocked cleavage in 100% of the cells (0.2 ng blocked cleavage in only 50% of the cells). In injections after nuclear membrane breakdown, however, 0.5 ng of antibody, almost twice the previous concentration, inhibited only 50% of the cell cleavages. Injections at the onset of cleavage did not block cleavage at all: after injection of 0.5 ng of antibody there was furrow

regression in 2 out of 9 cells, whereas after injection of 0.5 ng of pre-immune gamma-globulin, in the controls, there was blockage of cleavage in 1 out of 8 cells. If one accepts that cell cleavage is a result of actin-myosin interactions in the cleavage furrow ('contractile ring'), as Mabuchi & Okuno (1977) do, then one concludes from this aspect of the experiment that the antibody against myosin blocks the setting up of a functional contractile ring, and hence blocks cleavage, but that the antibody does not block the actin-myosin interactions once the contractile ring is already set up.

How about the supposed lack of effect on chromosome movement? This was studied in 17 cases in which antiserum was injected prior to nuclear membrane breakdown, in all of which cleavage was blocked. (No doses were reported.) In 5 cases no MA were formed; in the remaining 12 cases the MA that were formed were, to quote Mabuchi & Okuno (1977), "small and obscure". (There was no documentation of presence or absence of MA, or of altered MA, or even mention of which microscope system was used for these observations, however.) Did the "small and obscure" MA function normally? As far as I can determine from the published work (Mabuchi & Okuno, 1977), chromosome movement was neither seen nor measured; the only relevant data presented were that "formation of daughter nuclei was observed in 9 out of the 12 cells..." (Mabuchi & Okuno, 1977). Presumably the argument regarding lack of effect on chromosome movement derives from the observation that 2 daughter nuclei were formed in each of these 9 cells. I now consider these experiments with regard to the conclusion of Sakai et al. (1976) that chromosome movements in mitosis are "not dependent upon a myosin-actin system ...".

Several assumptions inherent in the experimental approach should be considered before considering details of the results. The first point is the validity of experiments in which non-immune serum is used to estimate the diffusion of specific antiserum (in this case, antiserum containing antibodies against myosin). Experimentally, Mabuchi & Okuno (1977) observed that non-immune serum freely diffused throughout the cell, in less than one minute, and they assumed, therefore, that antibodies against myosin would do likewise. But this assumption is not necessarily valid. For example, there is a large amount of non-diffusible myosin in the cell cortex (Mabuchi, 1973, 1974, 1976), and if there are fewer antibody molecules injected into the cell than there are

myosin molecules, high affinity antibodies would be sequestered at the positions of the myosin and no antibody molecules would be free to diffuse elsewhere in the cell. [It is relevant to note that antibody against dynein reacts in exactly this way: antibodies against dynein blocked the beating of sperm, and after washing away excess antibody and immersing the sperm in solution not containing antibodies, the antibodies against dynein remained bound to the sperm, and the sperm were not reactivated (B. Gibbons et al., 1976).] One therefore needs to do a different control. One such control would be to add fluorescently-labelled antibodies against myosin, and to see whether such antibodies are indeed sequestered and to see if antibodies are present in the spindle (or other region of interest). Another possibility is to demonstrate chemically that the antibodies are in large excess over intracellular myosin, and that not enough additional myosin is synthesized after the antibodies are added to deplete the antibody pool.

A second point is the assumption that antibodies directed against myosin ATPase will block contractile processes involving myosin. That is to say, it is assumed that if cell cleavage involves myosin interactions with actin and if chromosome movement (or anything else) involves myosin interactions with actin, then addition of antibodies that are against myosin ATPase will block (stop) cell cleavage, chromosome movement, or anything else that requires myosin interactions with actin. But this assumption is not valid: several experiments demonstrate that antibody directed against myosin might completely block actin-myosin interactions if the antibodies are added with myosin before actin and myosin are able to interact, but that the antibodies do not completely block actin-myosin interactions if the antibodies are added after actin and myosin are able to interact. For example, antibodies against muscle myosin blocked a large proportion of the myosin Ca^{++}-ATPase and Mg^{++}-ATPase activity, they blocked a large proportion of the actin-activated Mg^{++}-ATPase, and they blocked actomyosin superprecipitation, but they did so only if the myosin reacted with the antibodies prior to interaction with actin: there was no effect when antibodies were added directly to actomyosin (Puszkin et al.,1975). In another example, McIntosh et al. (1975) were unable to block the contraction of glycerinated muscle by addition of antibodies against myosin or against actin. In a further example, antibodies against Physarum

myosin (Kessler et al., 1976) greatly inhibited the myosin Ca^{++}-ATPase, but antibodies that were added to actomyosin did not block superprecipitation: superprecipitation was delayed (the amount of delay depended on the amount of antibody), but the superprecipitation itself was not blocked, nor was there much effect on the extent of superprecipitation, as judged by change in absorbance (Nachmias & Kessler, 1976). Thus, in any experiments on the effects of antibodies on chromosome movement it is necessary to measure chromosome velocities, and to determine if the start of anaphase is delayed, for, by analogy with these experiments, antibodies might cause chromosome movement to be delayed, or slowed, without causing movement to be completely blocked. In yet another example, antibodies against myosin blocked cleavage only when they were injected into cells much prior to the time the contractile ring was set up (Mabuchi & Okuno, 1976), as discussed previously. As a final example it is relevant to consider the effect of antibodies against dynein-ATPase on the movement of sperm (B. Gibbons et al., 1976; I. Gibbons et al., 1976). Addition of antibodies to reactivated (beating) sperm resulted in gradual decrease of the beat frequency; the beat frequency declined rapidly in the first 5 - 15 minutes, and then more slowly up to 60 minutes, but even after 60 minutes the sperm continued to beat, albeit with a greatly reduced frequency. The amount of reduction in beat frequency depended on the amount of antibody added. These results emphasize the point made above, that chromosome velocities need be measured, for, by analogy with the results on sperm, one might even expect slowing down of movement rather than cessation. Also relevant is the comparison between adding high concentrations of antiserum to "rigor" sperm vs. to reactivated, motile sperm. When high concentrations of antibodies against dynein-ATPase are added to reactivated sperm the beat frequency is greatly reduced, but sperm continue to beat. But when antibodies are incubated with rigor sperm for 30 sec. prior to addition of reactivation medium, then the sperm do not beat (B. Gibbons et al., 1976). Here, too, then, function which is blocked completely by incubation of antibodies with the separate (non-functional) component is not blocked when added to the same component which is in a functional state.

A third point to consider is whether myosin in the spindle would be the same as myosin in the cortex, and whether antibodies against the active site of one myosin would necess-

arily block the active site of the other myosin. Myosins from different tissues do not cross-react with antibodies against myosins of the other tissues (e.g., Bruggmann & Jenny, 1975; Puszkin et al.,1975; Fujiwara & Pollard, 1977; Pollard et al., 1976, 1977), and indeed, antibodies against the ATPase of dynein-1 from sperm do not inhibit the ATPase of dynein-2 (B. Gibbons et al., 1976), so it is not unreasonable to expect that different myosins in the same cell would not be blocked by antibodies to only one of the types of myosin. Nor is it unreasonable to expect that there are different myosins in the same cell, for indeed 3 different actins occur in single cell types (review in Forer, 1978), and recent evidence indicates that there may be as many as 29 different genes coding for actins (Tobin & Laird, 1977; and S.L. Tobin, personal communication). Hence the possibility that the injected antibody would not even react with spindle myosin (if it exists).

The final point is that even if one could prove definitively that there was no myosin in spindles, this does not rule out a functional role for actin, for, as Tilney has emphasized (e.g., Tilney, 1975), some motile systems utilize actin, but without myosin.

I now consider details of the experiments of Mabuchi & Okuno (1977). First, as discussed above, the antibodies blocked cleavage only if injected much earlier than cleavage, so, in agreement with other work discussed above, one assumes that the antibodies do not block a functioning system once it is set up but that the antibodies will block the setting up of a functional system. [Mabuchi & Okuno (1977) give data that their antibodies inhibit the actin-activated Mg^{++}-ATPase of myosin, which might be considered as a counter-argument; but Mabuchi & Okuno (1977) do not state whether this was done by pre-incubating the myosin with antibody, as is common (e.g., Trenchev & Holborow, 1976; Gröschel-Stewart et al., 1977), or whether the antibody was added after the actin and myosin were already mixed.] One could argue from the Mabuchi & Okuno (1977) data, therefore, that myosin is a spindle component: since the antibodies block formation of a functional myosin structure, and since either no MA or "small and obscure" MA were formed after injection of antibodies, one could conclude that there is spindle myosin, and this myosin functions in setting up normal-sized MA. As a second point, even if one uses the mere presence of daughter nuclei as criteria for the occurrence of chromosome movement, Mabuchi & Okuno (1977) show

that chromosome movements never occurred in almost half the cells (in 5 cells no MA were formed, and in 3 cells no daughter nuclei were found, for a total of 8 out of 17); this is quite different from the interpretation "no inhibition of chromosome motion". Further, in the other cells chromosome movements were never measured, and thus they could have been slower, or anaphase could have been delayed.

I conclude that the data of Mabuchi & Okuno (1977) are not really relevant to the question of myosin involvement in chromosome movement, because of lack of data dealing with the basic assumptions discussed above (namely, diffusion of antiserum vs non-immune serum, cessation of movement vs slowing or delaying of movement), and that even accepting their interpretation spindle myosin may not be inhibited by their antibodies, or spindle actin may function without myosin. Further, their data could even be used to argue for the presence and function of spindle myosin.

Injection of antibodies against myosin were also reported to block cleavage but not chromosome movements by Kiehart et al. (1977). These experiments seem to rule out some objections I raised against those of Mabuchi & Okuno (1977), but only when detailed descriptions of the experiments are available will one be able to critically evaluate the results.

Schroeder (1973, 1976) has argued on several grounds that actin has no role in chromosome movement. For one, Schroeder (1973, 1976) studied HeLa cells after glycerination and HMM treatment; he found some actin-containing filaments in these spindles, but not "in functionally significant numbers..." (Schroeder, 1973). In metaphase spindles, between chromosomes and poles, "A few - but only a few - solitary filaments can be seen ...", and Schroeder has "never seen bundles of filaments in this region..." (Schroeder, 1976). In anaphase, actin-containing filaments are seen interzonally, and in the cell cortex, but "are practically never seen in chromosome-to-pole regions" (Schroeder, 1976). Second, not only are there only "a few" actin-containing filaments, the distribution of these filaments is wrong: the filaments "either remain fixed in place during chromosome motion or are preferentially located behind the moving chromosomes..." (Schroeder, 1976), both of which seem "inconsistent" with actin associations with a traction fibre. Third, Schroeder (1976) studied mitoses in 7-day chicken embryo gizzard cells, in which the smooth muscle contractile filaments were already present. Schroeder saw

solitary filaments in the spindles, that looked like the muscle filaments, but "there is no preferential concentration, alignment or association of these putative actin microfilaments in any portion of the MA at any stage of division..." (Schroeder, 1976). This is "additional evidence against a significant role for actin in chromosome motion..." (Schroeder, 1976). The final argument is that there are "sharp contradictions" between the results of electron microscopic detection of actin in spindles and the results of fluorescent microscopic detection of actin in spindles, and therefore "the data are seriously contradictory and tend to nullify each other..." (Schroeder, 1976). I discuss this last point in Section IV-D, after considering in detail the methods and results of fluorescence microscope detection of actin; I now consider the first three points raised by Schroeder.

First, in considering the absence of "functionally significant numbers of filaments", the wrong distribution of filaments, and the absence of filaments in spindles of glutaraldehyde-fixed (non-treated) cells, one should point out that, as summarized above, others have studied similar cells, both before and after glycerination and HMM treatment, and have given different descriptions of actin-containing filaments in spindles (e.g., Euteneuer et al., 1977; Schloss et al., 1977). Part of the difficulty in resolving this apparent contradiction in results is in the question of what one expects to find, and is in the lack of quantitative data from either Schroeder or those like myself who claim that actin in the spindle is present in proper amounts, orientations, and distributions to be functional (Table III). To consider one point, Schroeder argues that in HeLa cells there are only a "few" actin-containing filaments, in numbers that are not "functionally significant". The data summarized in Section II of this chapter show that only 1 actin filament may be required per chromosomal spindle fibre, so that even "a few" may be functionally significant. For example, HeLa cell spindles have 1000 - 1500 microtubules (e.g., McIntosh & Landis, 1971), so that 100 actin-containing filaments would be "a few" by comparison, yet might be more than enough to do the job, if they were distributed properly. Nor is there any a priori reason to expect bundles of filaments. I can only conclude that this disagreement on whether actin-containing filaments in the spindle may be functional can not be resolved without quantitative data on the distribution of actin in the spindle, primarily to see if

there are actin-containing filaments associated with each and every chromosome or chromosomal spindle fibre; this is the requirement of any force-producer, and if actin-containing filaments are not so distributed, then the actin-containing filaments can not be functionally involved in causing chromosome movement. Until we have these data, the disagreement is essentially unresolvable.

Second, it is relevant to consider another aspect of this disagreement, and that is the difficulty in seeing microfilaments in non-treated cells. Schroeder has made his expectations clear: to quote him (Schroeder, 1976), "I would expect that if actin were involved in force production (in the spindle) that its location would be easily and reproducibly visualized". This expectation has indeed not been fulfilled; but this expectation need not fit reality. McIntosh et al.(1976), Sanger & Sanger (1976), Cande et al. (1977), and McDonald et al. (1977), for example, have emphasized the problem of fixation of actin filaments and suggest that, perhaps like spindle microtubules that were rarely seen prior to the advent of glutaraldehyde fixation, spindle microfilaments are just not well preserved with present methods of fixation. The literature on fixation of actin-containing filaments in muscle, discussed above, emphasizes this point (review in Forer, 1978). On the other hand, Schloss et al. (1977) suggest another possibility: they suggest that microfilaments are not seen in PtK1 cell spindles because they are obscured by closely apposed microtubules. Schloss et al. (1977) document this in PtK1 cell spindles, and recent work by Jackson and me (in preparation) on serially-sectioned spindles in Haemanthus endosperm cells confirms such closeness between microfilaments and microtubules. So, for these and other reasons, that Schroeder's expectation is not fulfilled does not mean that actin does not function in spindles.

Two other counter-arguments remain to be discussed. One is that though actin is seen in the spindle, "the possibility that the actin was, in life, located elsewhere in the cell has certainly not been excluded..." (Nicklas, 1975); this argument, that actin "relocates" during glycerination and HMM-treatment, has also been presented by Porter (1973) and LaFountain (1975), as discussed previously. The second is that actin is "trapped" in the spindle, and has no real role: to quote Sato et al. (1976), "The spindle is ... formed at each mitosis in a cytoplasmic environment that contains a

quantity of actin and some actin filaments. Thus it is not surprising to find some actin-like filaments both inside and outside the spindle...".

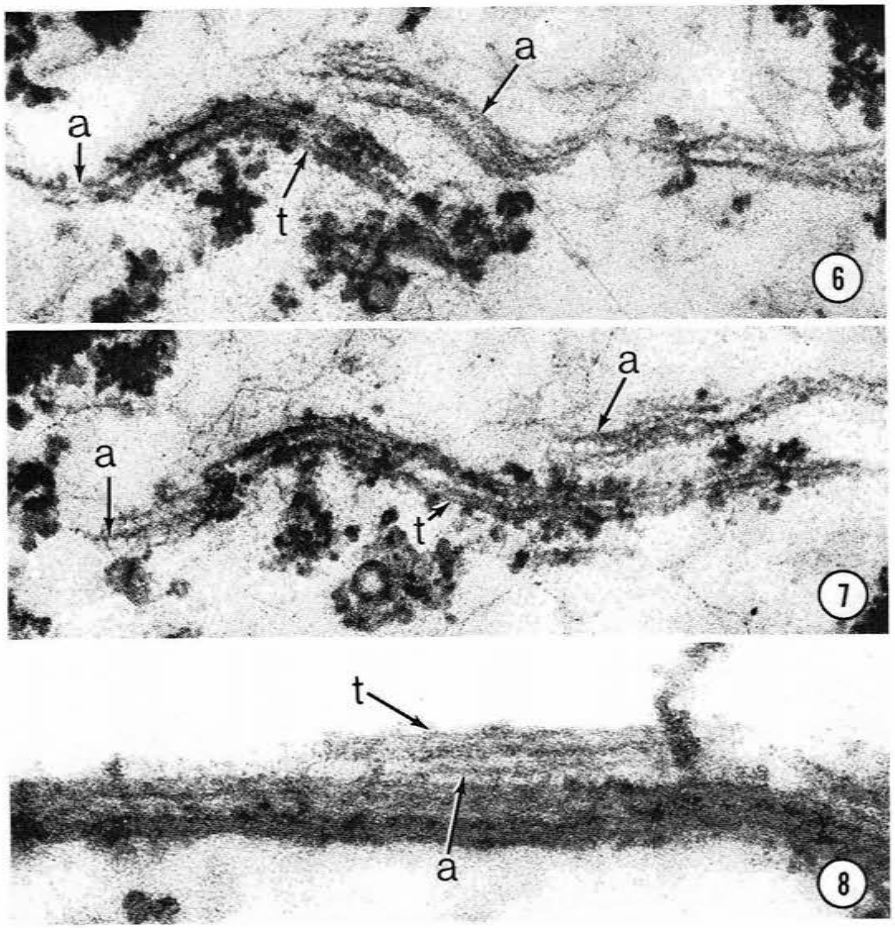
Forer & Jackson (1976) have tried to deal with the relocation problem by studying spindles in Haemanthus endosperm cells: after glycerination and heavy meromyosin treatment there were very few actin-containing filaments outside the spindle and relatively large numbers inside the spindle. Thus there was no large extra-spindle source of actin-containing filaments which could "contaminate" the spindle. Jackson and I have subsequently studied (and photographed) Haemanthus endosperm cells undergoing glycerination, and we have found that cells glycerinated as in Forer & Jackson (1976) shrink considerably when they first are placed in 50% glycerol, but that after 3 minutes or so the cells suddenly expand, to near original size, whilst the chromosomes maintain positions near those seen originally. Thus actin could "relocate" during the shrinkage period. However, cells prepared in different ways had different degrees of shrinkage and swelling, yet all had the same distribution of actin. This argues against "relocation", but some change in shape has been observed during all treatments studied to date so we can not definitively rule out "relocation" of actin-containing filaments during the procedures.

Could actin be "trapped" in the spindle? Actin-containing filaments found in intranuclear spindles (Ryser, 1970; Lewis et al., 1976) would rule out this objection (and would rule out the "relocation from the cytoplasm" objection), except that these filaments have not been demonstrated (by HMM-binding) to contain actin. And even if so, this does not necessarily mean that such filaments would function in chromosome movement. A key to deciding whether actin might function in causing chromosome movement is to see if actin is associated with each and every chromosomal spindle fibre and/or chromosome. Such a consistent finding would also argue against "relocation" and "trapping", since these would predict random arrangements of actin.

To see if there is such a consistent arrangement of actin-containing filaments in spindles, Bill Jackson and I have begun serial-section reconstruction of actin-containing filaments in Haemanthus endosperm cells, and I summarize briefly some initial findings. First, as noted above, we confirm that there is little extra-spindle actin. Second, we confirm

exactly the description by Schloss et al. (1977) regarding intimate associations between actin-containing filaments and microtubules. Throughout the spindle, actin-containing filaments and microtubules are in very close association; the two are often so close together that microtubules obscure the actin-containing filaments, such that, as in Schloss et al. (1977), actin-containing filaments are seen to enter the sections just as microtubules leave the sections. The same situation applies with the same microtubules and actin-containing filaments through several serial sections, and over distances of microns. (Some such associations between actin-containing filaments and microtubules are illustrated in Figs. 6 - 8.) Third, we have so far analyzed longitudinal serial sections through 4 kinetochores (and associated microtubules), in one metaphase cell. We have found several actin-containing filaments associated with each group of kinetochore microtubules; the filaments were in amongst the microtubules as well as on the outside of the bundle, and were found from near the kinetochore to at least several microns from the kinetochores towards the poles (Forer & Jackson, in preparation). At least preliminarily, then, these findings support the view that actin-containing filaments in the spindle function in causing chromosome movement.

In conclusion, I have summarized various arguments raised against the possibility that actin-containing filaments function in mitosis. Many of these arguments were dismissed, but one is left with the 3 counter-arguments that actin "relocates" during glycerination, or is "trapped" in the spindle, or just does not function in causing chromosome movement. There are arguments against "relocation" from the cytoplasm, since very little extra-spindle actin exists in some cells in which spindle actin is prominent. Nonetheless, final resolution of these arguments will only come about when one has quantitative data describing the distribution of actin-containing filaments in spindles or when one can otherwise decide whether there are actin-containing filaments associated with each and every chromosomal spindle fibre (and/or chromosome). Serial-section reconstruction of 4 kinetochores in one Haemanthus endosperm cell at metaphase confirms such an association, but more cells need to be studied in this regard. Fluorescence microscope analysis of spindle actin purports to show such a regular arrangement of actin (a very important statement to be able to make in stating a case for actin functioning in mitosis), and



Figs. 6 and 7. Electron micrographs from consecutive sections of a glycerinated Haemanthus endosperm metaphase cell that was reacted with rabbit skeletal muscle HMM; these illustrate the closeness of spindle microtubules and actin-containing filaments, as described in the text. Some actin-containing filaments are labelled a, and some microtubules are labelled t. Both are X 70,000. Fig. 8 is an electron micrograph of a section of a glycerinated Haemanthus endosperm cell that was treated with HMM, illustrating the closeness of an actin-containing filament (labelled a) and microtubules (one of which is labelled t). X 115,000.

I discuss these data next. Unfortunately for proponents of actin function in mitosis, such as me, these data are not compelling. I discuss first the data using fluorescent labelling via antibodies, and then the data using fluorescently labelled HMM.

C. Studies of Actin (and Myosin) in Spindles using Antibodies and Fluorescence Microscopy

I first outline the general approach, then describe the individual experiments, and then discuss critically the interpretation of these experiments.

The general approach in localising intracellular antigens using fluorescence and antibodies is as follows (see Goldstein, 1976, for example, for references). One purifies an antigen, and then obtains antibodies to the antigen by injecting the antigen into an animal (e.g., a rabbit). Serum is taken from the animal prior to injections (this is the control 'pre-immune serum') and serum is taken from the animal after injections (this contains the antibodies). One determines that the antibodies are specific for the antigen injected (and not to minor components in the injected solution) and one determines that the pre-immune serum does not contain antibodies which react with the cells (e.g., Trenchev & Holborow, 1976). Then one adds the specific antibodies to a cell which contains the antigen (the cell must first be made permeable to the antibodies), and, after rinsing away antibodies which are not tightly bound, one identifies the position of the antibodies (and hence the specific antigen) by means of a fluorescent marker. In the 'direct fluorescence' technique the fluorescence is inherent in the antibodies themselves. In the 'indirect fluorescence' technique one adds fluorescently-labelled antibodies from a second animal which are directed against the antibodies from the first animal: one injects antigens into rabbits, for example, reacts rabbit antibodies with the cell, and then determines the localisation of the rabbit antibodies by using commercially available fluorescently-labelled goat antibodies directed against rabbit immunoglobulins.

Experiments using antibodies against actin and indirect immunofluorescence of spindles in PtK1 cells in culture were described by Cande et al. (1977). Antibodies were prepared against sodium-dodecyl-sulphate (SDS) - treated calf thymus or

chick fibroblast actin by injection into rabbits. Antibodies against tubulin were made in a similar way using SDS-treated porcine brain tubulin. The specificity of the actin antibodies was confirmed by 3 lines of evidence (Lazarides, 1975): (1) there was a single precipitin line against partially purified actin in immunoelectrophoresis and double immunodiffusion tests; (2) pre-absorption of the antibody with heat-treated actin destroyed the fluorescence staining of cells and of myofibrillar I-bands; pre-absorption with native tubulin had no effect on these staining reactions; and (3) there were different staining patterns when cells were stained with different antibodies (viz., against actin, tropomyosin, and α - actinin). Similar studies demonstrated the specificity of the antibodies against tubulin.

The techniques used to stain the PtK1 cells were as follows. Mitotic cells were gently lysed in a medium containing 0.04% Triton-X 100 and tubulin, under conditions in which anaphase movements continue for 20 min. or so (Cande et al., 1974). Then the cells were fixed (in 3.7% formaldehyde, pH 6.4, in a salt solution containing 2% Carbowax), then rinsed with salt solution, and then incubated in non-immune goat serum; this last step is done in order to absorb goat serum to all non-specific absorption sites, so that later staining with fluorescently-labelled goat serum antibodies against rabbit immunoglobulin will be specific. Then, after rinsing, cells were reacted with the rabbit antibodies, then rinsed, then reacted with fluorescently-labelled goat antiserum against rabbit immunoglobulins (antibodies), and then rinsed, mounted in glycerol, and observed. Some cells were not lysed, but instead were fixed directly in formaldehyde; then they were treated with 50% acetone, then 100% acetone, and then 50% acetone (to make the cells permeable to antisera), and then treated with non-immune goat serum and processed as were the others.

The results were as follows, considering first the results for antibodies against actin. Staining with pre-immune serum gave no fluorescence. Staining non-lysed cells resulted in spindle staining, but background cytoplasmic fluorescence tended to mask details. Staining lysed metaphase cells demonstrated an amorphous staining of the spindle pole (a "halo") and "fibers running from chromosome to pole..." (Cande et al., 1977). (These cells have 11 chromosomes; the exact number of chromosome-to-pole fibres could not be counted, for technical

reasons, but 6 such fibres were seen in one cell.) No such fibres were seen to cross the metaphase plate and, consistent with the idea that these correspond to chromosomal spindle fibres, the fibres were of unequal length in prometaphase. In anaphase the fluorescent fibres were shorter than in metaphase (as chromosomes moved closer to the poles), there were still amorphous "halos" at the poles, and no fluorescent fibres were seen interzonally (though later in anaphase there was diffuse interzonal fluorescence). Staining with antibodies against tubulin, using lysed cells, gave different results than did staining with antibodies against actin: fluorescent fibres were seen between chromosomes and poles, with no fibres extending across the metaphase plate, but rather than a polar "halo" there were distinct astral fibres radiating into the cytoplasm. Also, there were distinct interzonal fibres in anaphase when antibodies against tubulin were used. When non-lysed cells were used, the tubulin antibody was localised more-or-less the same way as in lysed cells, with the differences that there were fluorescent fibres which crossed the equator, in metaphase, and that the chromosome-to-pole fibres were somewhat less distinct. From electron microscopic observation of cells treated with antibodies against actin, Cande et al. (1977) concluded that the spindles were severely extracted, that the numbers of spindle microtubules were "substantially less..." than in non-treated cells, and that there was "fuzzy amorphous..." material near microtubules which "may represent antibody binding to poorly preserved actin filaments..." (Cande et al., 1977). Cande et al. (1977) argue from these results that, though they can not rule out the possibility that actin is trapped in the spindle, they have ruled out other possible artifacts; since the results from localising actin differ from those from localising tubulin, they argue, this "rules out the possibility that the actin localization is just a visualization of the microtubule distribution in the formalin-fixed spindle..." (Cande et al., 1977). They conclude that "actin antigenicity is associated with the mammalian mitotic spindle, and that the spatial distribution of this antigenicity changes during spindle formation and function...", and, further, that "actin in a fixable form is a component of the chromosomal spindle fibers..." (Cande et al., 1977).

My criticisms of these interpretations deal with the entire immunofluorescence technique as it has been applied to

date in studying cellular actin, myosin, and tubulin (e.g., Weber, 1976; Brinkley et al., 1976; Forer et al., 1976; Franke et al., 1977; and Pepper & Brinkley, 1977, to cite some recent examples which used immunofluorescence and antibodies against tubulin). Thus I will describe the experiments using antibodies against myosin before I discuss those just summarised dealing with antibodies against actin.

Goode (1975), Mohri et al. (1976), and Fujiwara & Pollard (1976) have described immunofluorescence of mitotic cells after reaction with antibodies against myosin. Goode (1975) used antibodies against chicken myosin and, using the direct fluorescence technique, added the antibody to glycerinated chicken embryonic cells in mitosis. He found fluorescent material "occasionally present in or around the mitotic spindle of myocardial cells..." (Goode, 1975); the fluorescent images (e.g. Figs. 5, 9, and 10 in Goode, 1975) seem to show fibres extending between chromosomes and poles. Mohri et al. (1976) used rabbit antibodies directed against Asterias egg myosin, in the indirect fluorescence technique, and they stained MA which were isolated from sea urchins using 1M glycerol (the method of Sakai et al., 1977). The isolated MA were fixed in 10% formalin, were air dried on a slide, were rinsed, were incubated in rabbit antibodies, were rinsed, were incubated in fluorescent goat antibodies (against rabbit immunoglobulins), were rinsed, were placed in glycerol, and were studied. "Non-immune..." rabbit serum gave no reaction. Antibodies against myosin stained the MA slightly to give diffuse staining throughout, except for chromosomes, which did not stain, and except for discrete and brighter staining of spindle poles, which appeared as amorphous "halos".

Fujiwara & Pollard (1976) used direct immunofluorescence to stain mitotic spindles in HeLa cells in culture, using antibodies against myosin, and myosin prepared from human blood platelets. The specificity of the antibodies was tested carefully using various methods. For example, using immunoelectrophoresis, the antibodies did not react with actin, or with the head (ATPase) region of myosin, but did form single precipitin lines with purified platelet myosin or with purified rod portion of platelet myosin (the non-ATPase portion), and they did form a single precipitin line when reacted with crude platelet extract. (Crude platelet extract and crude HeLa cell extract consisted of the material which was soluble when whole human platelets, or HeLa cells, were broken open into 40 mM sodium pyrophosphate solution.) Further, using double immunodiffusion, single (confluent) precipitin lines were formed with purified platelet myosin, rod portion of platelet myosin, crude HeLa cell extracts, and crude platelet extracts, whilst there was no reaction with myosin from human skeletal or cardiac muscle. Finally, using tandem crossed immunoelectrophoresis, there was one reacting component in crude platelet extract which was identical in position with (i.e., the line fused with that of) purified myosin. (It might be relevant to note that these tests were done with antibodies which were not fluorescently labelled.) Further care was taken to ensure that fluorescently-labelled antibodies did not react non-specifically: only those with 2-5 dye molecules per protein molecule were used for cytochemical localisations. In addition, specific antibodies against myosin were purified (by affinity column chromatography) from the fluorescently-labelled immunoglobulins.

The staining procedures were as follows. HeLa cells were treated with acetone at -20°C, for 5 min., and then air-dried. Air-dried cells were treated with antibodies, rinsed, and then placed in 90% glycerol and studied. Equivalent results were obtained when acetone-fixed cells were post-fixed with 1 to 1.5% formalin. Various controls were run, to assure that staining was due to specific antibodies, and these included staining with pre-immune serum and staining with antibodies pre-absorbed with myosin, neither of which gave staining of spindles. A further control was the absence of staining of spindles when non-human cell lines were studied, namely in rat-kangaroo (PtK2) cells or in salamander cells.

Fujiwara & Pollard (1976) found staining (fluorescence) throughout the HeLa cell cytoplasm, but there were higher intensities of staining in the spindle and in the cleavage furrow than elsewhere. Spindle staining was between the chromosomes and poles, in both metaphase and anaphase, but this was diffuse, and was not seen as discrete fibres. In anaphase the interzone also stained, without visible fibres, "but much of this intensity is attributable to the cleavage furrow surrounding this part of the cell..." (Fujiwara & Pollard, 1976). Purified antibody against myosin stained the spindle less intensely than did whole immunoglobulins; Fujiwara and Pollard discussed possible explanations for this, but because of this result they conclude, cautiously, that they "believe that myosin is concentrated in the mitotic spindle, but feel that this

point needs further investigation..." (Fujiwara & Pollard, 1976).

In evaluating the immunofluorescence results, both for actin and myosin distributions, I think three important criteria need be kept in mind: (1) are the antibodies specific for the antigen in question? (2) If the antibodies are specific for that antigen (i.e. if they do not react with minor components which may have been injected), do any other proteins in the cell share antigenic determinants with that antigen? (In this case antibodies would react both with the antigen and with the other components.) (3) Is there movement of antigen during the procedures of fixation and antibody treatment? I will look at these criteria in turn.

The various experiments using fluorescently marked antibodies generally have included sufficient controls to demonstrate that the antibodies are specific for the particular antigen in question, though some caution should be exerted even here because Fujiwara & Pollard (1976) demonstrate that non-specific antibodies (which react with more than one component in the antigen preparations) can pass some of the usual tests for specificity.

The second criterion is one of the main reasons why the results can not be accepted as demonstrating spindle actin and spindle myosin (or, for that matter, as demonstrating spindle tubulin by means of fluorescence microscopy): no-one has demonstrated that there are no other components in the cell which share antigenic determinants with the antigen in question. To illustrate what I mean by this, one can look at the experiments done by Fujiwara & Pollard (1976), who demonstrated that when antibodies against myosin reacted with crude cell extract, only one component in the extract reacted with the antibodies. (The antibodies against tubulin and against actin have not been tested even in this way, as far as I am aware.) This commendable experiment is closer than most to a rigorous control, but two things are missing. First, while the antibody against myosin reacts with only one soluble cell component, there may be several components in the insoluble (discarded) fraction with which the antibody reacts, and which would show up as fluorescence in the cytochemical studies. Second, and perhaps more importantly, this test, that the antibody reacts only with one component in the cell extract, was done under conditions that were quite different from the cytochemistry: the immunoelectrophoresis was done with more-

or-less native proteins while the cytochemistry was done on proteins that were treated with cold, 100% acetone and then air dried. (An additional difference in this particular case is that the immunoelectrophoresis was done using not-fluorescently labelled antibodies, while the cytochemistry was done using fluorescently-labelled antibodies.) Thus, while no native proteins might share antigenic determinants with the antigen in question, denatured proteins might do so. Hence, testing for other reacting components in the cell needs to be done under the same conditions that the cells are treated for immunofluorescence.

This point is important, and not just nitpicking, for there are data which show clearly that denaturation can expose antigenic determinants that otherwise would not react with the antibodies. As one example, one could cite the data of Mabuchi & Okuno (1977): antibody against Asterias egg myosin did not react with native (not-denatured) platelet myosin, or with native rabbit skeletal muscle myosin, but did react with these other proteins after they were treated with 0.05% SDS. Hence the treatment with SDS (denaturation) exposed antigenic determinants that were recognized by the antibodies, but that were otherwise hidden in native proteins. As another example, perhaps even more relevant to the cases under discussion, one could cite the data of Nishino & Watanabe (1977a,b): antiserum directed against 14S dynein from Tetrahymena does not react with native tubulin or native actin, but does react with both denatured tubulin and denatured actin (though not with denatured myosin, tropomyosin, or troponin). Hence there are antigenic determinants in common between actin, tubulin, and dynein when these proteins are denatured. Thus it is quite possible that antibodies thought to be specific for actin, for example, would react with denatured dynein and denatured tubulin, and that the intracellular localisations observed would be combinations of the positions of all 3 proteins, and perhaps others. As a final example, one can consider the γ - subunit of glycogen phosphorylase kinase in the Pacific dogfish: this subunit has an amino acid composition identical to that of actin, it reacts with HMM to polymerise into arrowhead-decorated filaments, and it stimulates the Mg^{++}-ATPase of HMM (Fischer et al., 1975, and E.H. Fischer, personal communication; I am indebted to Sally Tobin for pointing this work out to me). The γ - subunit is ordinarily bound tightly into the glycogen phosphorylase kinase enzyme, under which condit-

tion it does not react with HMM (E.H. Fischer, personal communication), but under denaturing conditions (such as those used for the cytochemistry) the γ - subunit might be freed, and might be able to react with antibodies against actin. Hence if one is to accept the cytochemical localisations, one must rigorously test whether the antibodies in question will react with more than one protein in the cell, and this test must be done under the same conditions as the cytochemistry is done. Otherwise one can question the localisations determined cytochemically as being due to several proteins, and not just to the antigen.

It might be relevant to point out a straightforward way in which this control can be run. Various people have demonstrated that antibodies still react with SDS-treated antigens (e.g., Stumph et al., 1974; Mabuchi & Okuno, 1977; and Olden & Yamada, 1977). Thus one can perform electrophoresis of whole cells in weak SDS: some components will remain at the origin, and others will migrate. Then one gel can be stained, to see where the bands are, whilst a parallel gel can be fixed and treated as if it were the cell being studied (e.g., with acetone, and air-dried; or with formaldehyde, followed by acetone, etc.); then the treated gel can be reacted with fluorescently labelled antibody, to see whether the antibody will bind solely to the antigen or will bind to other bands as well. This, then, performs the required test of specificity under the exact conditions of the cytochemistry. (A somewhat different way has been used: Olden & Yamada (1977) reacted such gels with non-labelled antibodies and then by staining and microdensitometry measured increased protein concentrations at the bands in question.) Only by doing some experiment of this kind will one be able to have confidence in the fluorescence microscopy results, and to believe that the antibodies bind solely to the antigen in question.

The third criterion, adequacy of fixation in immobilising the antigen, should also be considered: 'inadequate fixation' is another serious criticism which can be levelled against the fluorescence microscopy results. Two lines of evidence demonstrate that the fixation using formalin and acetone (or variants of this) is inadequate, in that it permits relocation of antigen. First is electron microscopic study of cells treated in this way. There is agreement amongst several groups of workers that, as seen in sectioned material, the fixation procedures commonly used for fluorescence microscopy studies

preserve only some microtubules (e.g. Cande et al., 1977; Sato et al., 1976), or no microtubules (Forer et al., 1976; or as illustrated in Figs. 1-3, and 6-11 in Pepper & Brinkley,1977). Hence, since at least some of the tubulin has moved during this fixation, other antigens also might move. Indeed, Forer et al. (1976) argue that using fluorescence microscopy one observes only tubulin which binds to another component when the microtubules break down, and hence one sees in fluorescence microscopy the position of the other component and not necessarily the original position of the tubulin. The second kind of evidence indicating translocation of antigen is the appearance after fixation: cells fixed for immunofluorescence look quite different from living cells as viewed in phase-contrast microscopy. The differences are striking: in living cells, chromosomes are phase dark and spindles are light against the darker cytoplasmic background and are clearly separated from the cytoplasm, whereas in cells fixed for fluorescent antibody studies chromosomes may be phase bright, or not seen, and spindles are not seen or are only weakly set-off from the cytoplasm. [In making this evaluation, one needs to use spindles treated with pre-immune serum or the like, in which there is no specific labelling, because addition of antibody to only one part of the cell will change the concentrations of protein in that part of the cell. Hence, I considered Fig. 19 and 20 of Fujiwara & Pollard (1976), and compared it with living cells illustrated in Robbins (1961) and Robbins & Gonatas (1964). Or I considered Figs. 3-5 in Cande et al., 1977 and compared these with living cells illustrated in Roos (1976) and Brenner et al. (1977). Or I considered Figs. 12 and 13 in Franke et al. (1977) compared with living cells illustrated in Figs. 1 and 2 of Franke et al. (1977).] Since contrast in phase-contrast microscopy is a measure of relative path difference, which in turn is a measure of path length and concentration of dry matter, these alterations mean that relative concentrations have changed and that some intracellular materials have relocated; hence there is the clear possibility that antigens have moved from their original positions. This seems not to be due to the glycerol in which the final products are mounted, because cells fixed and prepared for electron microscopy and looked at after they are impregnated (and mounted) in Epon or Araldite still look more-or-less like cells in vivo (see, e.g., pictures of such cells in Bajer and Molé-Bajer, 1969), though, of course, one has here the complication that a

colouring agent (osmium) has been added.

It might be relevant to consider controls that might help decide if relocation does indeed occur during fixation. One way might be to use fluorescently labelled antigen that is added with the fixative (and/or later steps) to see if the antigen selectively binds to some structure, and/or if the antigen relocates during subsequent procedures.

Experiments by Goldman et al. (1977) are relevant in discussing the reliability of fluorescence microscopy using antibodies; these experiments compared electron microscopic identification of bundles of actin-containing filaments ("stress fibres") with immunofluorescence localisations using antibodies against actin. Goldman et al. (1977) found clear discrepancies, in that "stress fibres" were present in some cells, in normal numbers, as identified electron microscopically, yet were not seen using fluorescence microscopy. Hence, for whatever reason, one can not necessarily rely on fluorescence microscopy alone (see Goldman et al., 1977, for discussion of this).

I have raised objection to the cytochemical localisations of actin and myosin, because there may be other cellular proteins with similar antigenic determinants. A counter-argument is implicit in the discussion by Cande et al. (1977) that, as determined by immunofluorescence, the actin localisation is different from the tubulin localisation, and this "rules out the possibility that the actin localization is just a visualization of the microtubule distribution..." (Cande et al., 1977). This argument is not compelling. For one, we know that kinetochore-associated microtubules respond differently from other spindle microtubules when cells are treated in various ways (e.g., Lambert & Bajer, 1977), and that astral fibres respond yet differently (e.g., Forer et al., 1976). Whatever the reason is, there are some differences between the different kinds of spindle fibres, and these differences could give rise to the different staining and yet still not be related to differences in actin or tubulin distribution. Until one can rule out that the antibodies react with several proteins under the conditions of the cytochemistry, one can not rule out alternative interpretations of the data.

It is also relevant to point out that even were all my objections overruled, one still needs definitive proof that each and every chromosomal spindle fibre has actin. This was suggested but not definitively proven in the cases cited above, because there were too many chromosomes per cell. Such proof can probably only be done by studying some cell with only a few spindle fibres, such as crane fly spermatocytes, that have only 5 chromosomal spindle fibres per spindle (e.g., Forer, 1976).

In summary, I have concluded that the immunofluorescence localisation of actin and myosin in spindles is not compelling, because there is the possibility (or even likelihood) that the antibodies reacted with several proteins under conditions of the cytochemical treatment, and because there is reason to believe that antigens are not immobilised by the fixation procedure.

I now consider experiments using fluorescently labelled heavy meromyosin (HMM).

D. Studies of Actin in Spindles Using Fluorescently Labelled HMM

I first outline the general approach, then describe the individual experiments, and then discuss the interpretation of these experiments.

The general approach is as follows. HMM binds specifically to actin to form arrowheads, and this binding is blocked by ATP or pyrophosphate. The general idea, then, is to make the HMM fluorescent (by labelling it with a fluorescent marker), and then to visualize, using fluorescence microscopy, the localisation of the HMM after the HMM has reacted with the actin in the cell (Aronson, 1965).

Spindle actin has been studied, using fluorescent-HMM, by Aronson (1965), Sanger (1975a,b), Sanger & Sanger (1976), and Schloss et al. (1977). Aronson (1965) labelled HMM with fluorescein; this destroyed 90% of the HMM ATPase, and a large fraction of the HMM did not bind to myofibrillar actin-containing filaments. When fluorescent-HMM was reacted with myofibrils the fluorescent staining pattern was nonetheless specific for actin-containing filaments, as demonstrated by the following controls. The myofibrils stained heaviest in the I-band, weaker in the A-band (overlap region), and not at all in the H-gap. Pre-treatment with unlabelled HMM abolished "all but a very faint trace..." of fluorescent staining, and addition of pyrophosphate caused loss of most of the staining. Addition of 0.6M KI (which dissolves out the actin-con-

taining filaments) abolished the staining. Finally addition of fluorescein itself to untreated, glycerinated myofibrils resulted in a staining pattern quite different from that using fluorescent-HMM. Aronson (1965) added fluorescent-HMM to isolated sea urchin zygote spindles, and found that both the spindles and the chromosomes bound the HMM, "although in this instance no attempt was made to establish the specificity of the binding..." (Aronson, 1965), by means of pyrophosphate addition or the like.

Sanger (1975a,b) and Sanger & Sanger (1976) studied fluorescent-HMM labelling of PtK2 spindles, and established the specificity of their fluorescent-HMM by reacting the HMM with myofibrils, and by using pyrophosphate or pre-treatment with unlabelled HMM to block the reaction. Sanger (1975b) glycerinated rat kangaroo (PtK2) cells in the cold, using 50% glycerol; then he reacted them with fluorescent-HMM (in 25% glycerol), rinsed them, and placed them in 50% glycerol for viewing. In metaphase cells he saw discrete fluorescent fibres, 6-9 per cell (though it was difficult to determine the exact number), extending between chromosomes and poles, and the chromosomes were unstained except for a small "dot" where the fibres ended (the kinetochores). There were no continuous fibres, and there were no astral fibres, though there were round spots at the poles. In anaphase the chromosome-to-pole fibres were shorter, and there were no interzonal fibres, though diffuse fluorescence appeared interzonally at late anaphase. Absence of the fluorescence after treatment of mitotic cells with ATP or pyrophosphate was not reported in Sanger (1975b); Sanger & Sanger (1976) did report this control, though, namely that 10mM ATP blocked the staining of the chromosome-to-pole fibres, though some fluorescence remained near the poles. To quote Sanger (1975b), "we conclude...that actin is present in...chromosomal spindle fibers of rat kangaroo cells."

Schloss et al. (1977) labelled S1 with fluorescein, and then separated the fluorescent-S1 (using DEAE cellulose columns) into portions with varying amounts of fluorescein per protein, namely, 1,2,2.5, or 4-5 fluorescein molecules per protein molecule. This was necessary in order to avoid non-specific staining (which was not reversible using pyrophosphate) which occurred when there were 4-5 fluorescein molecules per S1 molecule. The specificity of the fluorescent-S1 was tested in a number of ways. The S1 gave arrowheads with

muscle actin, and the arrowheads were removed by pyrophosphate. The S1 maintained 68% of its actin-activated ATPase after labelling with fluorescein. Stress fibres were stained with fluorescent-S1, and this staining was blocked completely with 4mM pyrophosphate or by pre-treatment with unlabelled S1, whereas pre-treatment with bovine serum albumen had no effect. In labelling spindles, PtK1 cells were glycerinated for as little as 2-4 minutes prior to treatment with fluorescent-S1. In metaphase cells, chromosome-to-pole fluorescence was seen, as discrete fibres. In anaphase these fibres shortened, and there was no interzonal fluorescence. In both stages, the cell cortex also was fluorescent. Both 4mM pyrophosphate and pre-treatment with unlabelled S1 blocked the fluorescence staining, except for staining of the poles. Electron microscopic observations were made on these cells, as described above, and actin-containing filaments were seen. The authors conclude that actin is in the spindle and that "an actomyosin like contractile system may play a role in chromosome movement during mitosis and meiosis..." (Schloss et al., 1977). I now discuss the results of fluorescent-HMM labelling of spindles.

Several points need be made in assessing these results. First is the possibility that when pyrophosphate (or ATP) blocks the staining, this may be due to dissolving out a non-specific binding component rather than blocking the reaction; 1M ATP, for example, solubilizes several muscle proteins (e.g., Cooke, 1972). This criticism is especially relevant to the results of Sanger (1975b) and Sanger & Sanger (1976), since treatment with 10mM ATP is the only control they report for staining spindles with fluorescent HMM. A control for this is first to block (or remove) the staining by treatment with ATP or with pyrophosphate, and then to show that the staining is regained after rinsing and subsequent treatment with fluorescent-HMM (or fluorescent-S1). Schloss et al. 1977) did exactly this control for staining stress fibres with fluorescent-S1, but it is unclear from their statement of the controls for staining spindles (p.800 of Schloss et al., 1977) whether they did this control for staining spindles. Hence it is not clear whether the controls which have been run to date satisfactorily demonstrate specificity. Second is a major problem with the technique as used by all groups: HMM binds to actin, specifically, to give arrowheads, but HMM might bind to other cell components, to not give arrowheads, and to not give binding visible electron microscopically, yet

such binding would be visible using fluorescence microscopy. Examples of components HMM might bind to include ribosomes and polyribosomes: ribosomes are present in spindles (e.g., Hartman & Zimmerman, 1968; Goldman & Rebhun, 1969), as are polyribosomes (e.g., Forer & Jackson, in preparation), and both ribosomes and polyribosomes will bind to myosin and coprecipitate with myosin (e.g., Heywood et al., 1968). Thus, the fluorescent-HMM might bind to spindle ribosomes which are oriented between chromosomes and poles. I do not know if this binding would be sensitive to pyrophosphate, but it might be, since it is sensitive to KCl in exactly the same manner as actin and myosin (Heywood et al., 1968). Another example might be glycogen phosphorylase kinase (Fischer et al., 1975), as discussed previously when considering antibodies against actin. Thus other controls are needed to verify that the binding is indeed to actin and only to actin. One such control might be to block the binding by means of DNAase treatment, or thrombin treatment, which would break down actin-containing filaments (review in Forer, 1978) but which would not be expected to alter ribosomes or polyribosomes. Or one might run electrophoretic gels of the entire cell and then, as in the control proposed for staining by actin antibodies, one could determine if the HMM (or S1) bound to only the actin band or to other bands as well.

A third point is that, until one studies cells in which there are only a few spindle fibres, one does not have compelling evidence to prove that each and every spindle fibre has actin, as discussed previously. The final point is one that has been discussed, and that is the problem of relocation during glycerination. Schloss et al. (1977) shortened the glycerination time to 2-4 minutes to minimize this problem, and they also reported that formalin-fixed cells (not glycerinated) have staining patterns identical to those of glycerinated cells. However, relocation can occur during formalin fixation too, as argued above. Perhaps the best argument against this would be to use intranuclear spindles, or to demonstrate unequivocally that each and every spindle fibre always contains actin.

In summary, there are qualifications to the fluorescent-HMM evidences that actin is in spindles, in that proper ATP or pyrophosphate reversibility controls have not been run, in that non-actin components in the spindle might bind HMM (reference was made to myosin-ribosome binding), and in that it

still has not been demonstrated that each and every chromosomal spindle fibre contains actin (or HMM-binding material); demonstration that each spindle fibre always contains actin would argue forcibly against the "relocation" problem.

Two additional points need to be considered. First is the "contradiction" between the electron microscopic localisation of actin and the fluorescence microscopic localisation of actin. This 'discrepancy', discussed by Schroeder (1976), Cande et al. (1977) and Schloss et al. (1977) is essentially that actin-containing filaments are prominent interzonally, as studied electron microscopically, yet little fluorescence staining is seen interzonally, using either antibodies against actin or fluorescent-HMM. To me this 'discrepancy' is difficult to discuss because of the severe criticisms I have of the fluorescence studies, that, because of missing controls, it is possible that much of the localisation is due to binding with components other than actin. Even disregarding my objections to the fluorescence data, it seems clear to me that one does not really even know if a 'discrepancy' exists, for neither the electron microscopic distribution of filaments nor the fluorescence distribution has been presented in a quantitative manner. Until one knows even roughly how many microfilaments there are in the various regions, and compares these data with even rough amounts of fluorescence, one does not know if a 'discrepancy' exists, let alone what to make of it.

The second point is the striking similarity in results using antibodies against actin and using fluorescent-HMM: both stain discrete fibres between chromosomes and poles, both stain weakly interzonally, both do not stain astral fibres, and both stain the spindle poles as diffuse circular areas. And this pattern of staining is quite different from that seen after staining with antibodies against tubulin. Even though there are severe criticisms of each technique, separately, one nonetheless could argue that the staining pattern indeed reflects spindle actin, because the two different techniques give the same result, and this result is different from results of staining using other fluorescent antibodies. This argument has some force, but because of the uncertainties discussed above one can still think of simple ways to explain the congruence. For example, that actin antibodies bind to non-actin components would mean that these non-actin components had antigenic determinants in common with (or similar to) actin. If these antigenic determinants are chemically similar

to the HMM-binding site of actin, then both the antibodies and the HMM would be expected to bind to the same components. That is to say, if there is any binding to non-actin spindle components (and I have argued that the controls have not at all ruled this out), then one might expect both antibodies and HMM to bind to the same components, since these non-actin components would be similar to actin. Thus while it is tempting to consider that the 2 sets of data together indicate that spindle fibres indeed contain actin, until more controls are done these results are not unequivocal.

In conclusion, the chemical nature of spindles is largely unknown, but microtubules are universally associated with chromosomal spindle fibres; these fibres are the most likely sites of force production. The microtubules occupy only a small fraction of the spindle, and it is not known whether it is the microtubules or other spindle fibre components that produce the force for chromosome movement. To me, the most likely hypothesis is that actin is involved in producing the force. I have argued that actin is indeed a bona fide spindle component, and I have summarised the various evidences on this point. I have summarised the counter-arguments, and dismissed many of them. But some counter-arguments have not been eliminated. One question to be answered in determining whether actin functions in chromosome movement is to see if actin is associated with each and every chromosomal spindle fibre. Quantitative electron microscopy can be done to demonstrate this (or deny this), but so far has not been done in sufficient detail. Fluorescent-HMM data (and fluorescence microscope data localising actin antibodies) suggest that chromosomal spindle fibres contain actin, but these experiments are not definitive because further controls need be done.

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