THE CULTURE AND CORRELATIVE ELECTRON MICROSCOPY OF POLLEN
MOTHER CELLS IN MEIOSIS : DEVELOPMENT OF TECHNIQUES
AND SOME OBSERVATIONS ON SELECTED TOPICS

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For Lynn

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Abbreviations used in Figures.

- b cross bridges between microtubules.
- c chromosomes.
- er endoplasmic reticulum.
- kMTs kinetochore microtubules.
- MTs microtubules.
- nkMTs non-kinetochore microtubules.
- nmr nuclear membrane remnants.
- np nuclear pores.
- nu nucleolus.
- X points of contact between microtubules and chromatin.

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ABSTRACT

Reliable techniques for the living cell culture and correlative light and electron microscopy (EM) of meiotic pollen mother cells (PMCs) of *Iris spuria*, *Allium triquetrum* and *Tradescantia flumenensis* are described in detail. Living PMCs were successfully cultured in a slide chamber on agar/sucrose medium. Cells were covered with an inert oil to prevent their dehydration, and some cells were cultured from metaphase I to tetrad cell formation over a 20 hour period. Other PMCs were fixed with glutaraldehyde and flat embedded using a modification of the agar sandwich technique of Molè-Bajer and Bajér (1968). This technique was developed to permit the preselection of PMCs at known meiotic stages, for subsequent EM examination. Serial thin sections were cut at known planes of section; and 3-D reconstructions of MT distribution, and MT counts from transverse sections were completed. It was also possible to examine sections of an *Iris* anaphase I PMC which had been previously studied in life.

Anaphase I and II chromosome velocities were analysed in the three species. Mean velocities were approximately 0.5 $\mu\text{m}/\text{min}$ with some variation from cell to cell and between sister half-spindles. In *Allium* anaphase I there was also variation in chromosome velocity within the half-spindle; and this variation was found not to be related to chromosome position on the metaphase I plate. Spindle elongation was zero in *Allium* anaphase I and in *Iris* anaphase II, but was detectable in *Allium* anaphase II (40%) and in *Iris* anaphase I (15%). The extent of spindle elongation in *Tradescantia* could not be determined.

The kinetochore region in the first meiotic division consisted of two closely appressed, but structurally (and functionally) distinct, sister kinetochores. At meiosis II, the two sister kinetochores were separate from each other and faced opposite poles. The kinetochore arrangement probably changes from side-by-side (meiosis I) to back-to-back (meiosis II) during chromosome recondensation at prophase II in these cells.

Bundles of non-kinetochore microtubules (nkMTs) span the interzone between sister chromosome units at metaphase I and II and anaphase II. Bundles of kinetochore MTs (kMTs) do not increase in divergence at any stage of meiosis studied; there was little interaction between nkMTs and kMTs, and MT-MT cross bridges were rare. These observations are not consistent with models of chromosome movement based on MT sliding or zipping. No relationship was found between nkMT distribution and spindle elongation, and the several different nkMT distributions which have been reported for other cell types may be variations on a structural theme.

Spindle endoplasmic reticulum (ER) in meiosis II was found to be derived largely from invaginations and evaginations of the nuclear envelope. Growth of existing spindle ER was proposed to account for the doubling in the amount of ER observed between interphase and prometaphase II. Randomly oriented elements of ER in early prometaphase II spindles may become passively aligned along the interpolar axis and then actively transported polewards at later stages of prometaphase II and metaphase II.

Suggestions for future research are offered.

Chapter 1.

INTRODUCTION

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1.1 LIVING CELL AND ELECTRON MICROSCOPY OF POLLEN MOTHER CELLS.

The most comprehensive living cell and electron microscope (EM) study of any one cell type is undoubtedly the excellent work of Bajer and Molè-Bajer (1971, 1972, reviews) on *Haemanthus katherinae* mitotic endosperm cells. Most other studies have used animal cells; particularly insect spermatocytes in meiosis (Nicklas, Brinkley, Pepper, Kubai and Rickards, 1979) and mammalian cells in mitosis (Roos, 1976). Plant material has been neglected, yet it can provide readily accessible dividing cells, usually in considerable numbers and often with large chromosomes. Some early living cell work was done on plant epidermal and staminal hair cell mitosis (Belar, 1929; Becker, 1938; Wada, 1943) and it is unfortunate that detailed observation has not continued in these materials since they are generally not difficult to culture (personal observations).

The culture of living meiotic plant cells, in contrast, is difficult and has been attempted only rarely (Shimakura, 1934, 1937; Yasui, 1952; Fujii and Yasui, 1954; Inoué, 1953, 1964; Garot, Lebrun-Peremans, Moreau and Gillies, 1968; Lambert, 1978). Most of these groups found that a pure sucrose solution, rather than a balanced salt solution, was most effective as a culture medium for pollen mother cells (PMCs). Balanced salt (Ringer) solutions were found by Shimakura (1934) to maintain PMCs of *Tradescantia virginiana* at a particular stage but they did not support the continuation of meiosis. Pure sucrose solutions, more or less exactly isotonic with the PMCs, were usually found in the above studies to permit meiosis to proceed in favourable species. In many (most?) other species, sucrose solutions may not be sufficient to support meiosis.

Shimakura (1934) suspended PMCs in a relatively large hanging drop preparation and obtained a photographic image of PMC chromosomes of good quality, considering he was only able to use a bright field light microscope. The photographic image quality obtained by Fujii and Yasui (1954), in contrast, was quite low. This was because, in order to improve cell viability and to permit meiosis to proceed, it was necessary to explant PMCs still covered with tapetal cells and tapetal fluid. Both studies were completed in the early 1930's, before the development of the phase contrast or the improved polarizing microscopes.

Inoué (1953, 1964) was mainly concerned with spindle fibres and their birefringence (BR) in the polarizing microscope (see section 1.21) and while he reports continuing meiotic division in several plant species, photographic documentation is not given. Inoué's technique (Fuseler, pers. comm. to G.K. Rickards, 1977) was to use a hanging drop preparation, and he is the

only worker to have had success with Ringer solutions as culture media.

The work of Garot *et al.* (1968) and Lambert (in Hufford, 1978; as a text figure), both on *T. paludosa*, are the only reports of high resolution phase contrast light microscopy (LM) and ciné-micrography in meiotic plant cells. Dr Lambert and her students have also had some success with *Paeonia* and *Ginkgo* PMCs, and *Psilotum* spore-mother-cells (pers. comm. April, 1979).

Longer term *in vitro* test tube culture of PMCs, from early stages of prophase I through to the end of meiosis, has been possible for a number of years (Ito and Stern, 1967; see Stern and Hotta, 1977, for a review). These studies utilize a completely defined medium (e.g. modified White's, 1963, medium), but some preliminary studies reported here, using defined media for short term slide culture of PMCs, have shown no improvement in cell health over pure sucrose solution (see Ch. 3). However, defined media may prove to be essential to the culture of PMCs which do not survive in sucrose solution.

Electron microscope investigations of meiotic plant cells have concentrated mainly on prophase I and especially on synaptonemal complexes (e.g. Gillies, 1973; Church and Moens, 1976). Studies on later stages are limited (Sato, 1958, 1969; Sakai, 1968, 1969a and b, 1975; Wilson, 1968; Braselton and Bowen, 1971; Wagenaar and Bray, 1973), and all have involved the sectioning of whole anthers. However, detailed studies of meiosis require prior knowledge of both the meiotic stage of the PMC and the exact plane of section. This information is only available if cells are flat embedded in a thin wafer of plastic before sectioning. The flat embedding technique has been widely used with mitotic plant cells (Molè-Bajer and Bajer, 1968) and mitotic and meiotic animal cells (e.g. Fuge, 1977, 1980, Nicklas *et al.*, 1979) and has contributed greatly to our knowledge of the ultrastructure of dividing cells. It is sometimes desirable to know the behaviour of a particular cell or a particular chromosome immediately prior to fixation, and this also is now possible in many cells. A particular cell can be filmed for some time, then fixed, embedded and sectioned and the same cell can be examined in the EM (Molè-Bajer and Bajer, 1968; Nicklas *et al.*, 1979). Such studies can give considerable insight into particular problems such as orientation and reorientation (Roos, 1976; Nicklas *et al.*, 1979). Correlative L and EM studies, as reported here, have not previously been attempted in meiotic plant cells, although Lambert (pers. comm., April, 1979) reports some unpublished results with *T. paludosa*.

The recent success of the techniques for plant cell protoplast isolation and culture (Cocking, 1977 review) suggests the possibility that protoplasts could be obtained from PMCs. In fact, Ito (1973) and Bajaj (1974) report some success in tube culture of PMC protoplasts isolated at prophase I. In relation to the study of meiotic chromosome movement, it might be possible

to flatten PMC protoplasts, as is done with endosperm cells and spermatocytes, to give a high quality phase contrast image of chromosomes. Unfortunately, viable PMCs at stages with visible chromosomes could not be obtained in this study.

The object of this thesis was to develop reliable techniques for living cell culture, ciné-micrography, and for correlative EM including serial sectioning, of PMCs in meiosis. These aims were achieved and as well some initial observations are presented and discussed.

Chromosome velocities and the duration of meiotic stages were compared with those in other species. Sister kinetochore arrangement in the first and second divisions of meiosis, microtubule (MT) distributions and membrane distributions were also analysed and discussed.

These studies are obviously a technical prerequisite for any subsequent experimental work on chromosome movement. This could involve the behaviour of abnormal chromosomes during prometaphase and anaphase I, which could give useful insights into the forces involved in moving chromosomes. Analyses of abnormal chromosomes in living cells are rare (e.g. Bauer, Dietz and Robbelen, 1961; Wise and Rickards, 1977). Plant material can provide a ready source of such mutant cells since individual plants can be cloned to produce a population of genetically identical plants (see Rickards, 1970, Ph.D. thesis; and Rickards, 1977, 1981, in press; Balog, 1979; for studies on interchange and triploid *Allium triquetrum*).

The following is a review of spindle structure and organization, spindle forces, and force production, which will form a background to the present living cell and electron microscope observations of meiosis in PMCs.

1.2 SPINDLE STRUCTURE AND ORGANIZATION

1.21 Spindle fibres

The chemical composition of the spindle is still largely unknown (Forer, 1978b). The existence of chromosomes has never been seriously doubted, since they are often visible in living cells with the standard light microscope (LM). On the other hand, the reality of spindle fibres has often been examined (see Schrader, 1953), and it was not until 1953 that the "illuminating" polarized LM studies of Inoué "shed new light" on this question. Astral, chromosomal and interpolar spindle fibres are clearly visible when viewed with the rectified polarized light optics developed by Inoué (1953), and their existence in living cells is now no longer questioned. Differential interference contrast microscopy also reveals spindle fibres in living cells (Bajer and Allen, 1966).

Under polarized light, spindle fibres appear as bright zones against a dark background (or vice versa); this condition is known as birefringence (BR) and BR chromosomal and interpolar spindle fibres are present in most eukaryotic spindles. Chromosomal fibres are attached to each chromosome at its centromere and interpolar ($\equiv$ continuous) fibres span all or part of the interpolar distance. Astral fibres are also present in most animal spindles.

1.22 Microtubules

Spindle fibres under the electron microscope consist mainly of bundles of discrete linear molecules called microtubules (MTs) (Harris, 1962; Roth and Daniels, 1962). The replacement of permanganate (Sato, 1958) or osmium (Harris, 1962) fixation with double fixation at room temperature with glutaraldehyde and then osmium (Sabitini, Bensch and Barrnett, 1963; Ledbetter and Porter, 1963) resulted in consistently high quality preservation of cellular organelles, especially MTs.

There are several classes of MT in the spindle: astral MTs; kinetochore or chromosomal MTs; interzonal MTs; and interpolar or continuous MTs (Nicklas, 1971). I will follow the definitions of Jensen and Bajer (1973) and Fuge (1974), which refer to MTs which are attached to a chromosome at the kinetochore, or are in a bundle of MTs which is attached to a kinetochore, as kinetochore MTs (kMTs); and those MTs not attached to a kinetochore, nor in a bundle of kMTs, will be called non-kinetochore MTs (nkMTs). These terms avoid any assumptions of MT length.

The question of the reality of spindle fibres can now be posed with respect to MTs. Microtubules can be demonstrated in the spindle with a wide variety of techniques. Perhaps the most convincing is the demonstration by Moor (1967) of MTs in a parallel array in the spindle of unfixed freeze etched yeast cells. These MTs were similar in all respects to MTs in chemically fixed cells of the same organism (Robinow and Marak, 1966). Recent immunological techniques (Cande, Lazarides and McIntosh, 1977; De May, Hoebeke, De Brabander, Gueuens and Joniau, 1976) reveal MT protein (tubulin) in the same regions as MTs. Also, MTs can be assembled from tubulin subunits *in vitro*, and when these MTs are examined under the EM they are found to have the same structure as spindle MTs (Weisenberg, 1972; Olmsted and Borisy, 1972; Kirschner, 1978 review).

In transverse section, MTs are hollow cylinders approximately 24 nm in diameter, and are made up of 13 discrete subunits. These subunits are stacked in parallel arrays giving rise to 13 longitudinal protofilaments. There are two types of tubulin monomer: α and β , each with a mw of 55,000. The basic subunit is thought to be a hetero-dimer (Kirschner, 1978).

The mechanism of MT self assembly is still, to some extent, the subject of controversy (Kirschner, 1978) and will not be discussed here. MT assembly from existing MTs can occur at both ends of the MTs, although at different rates. Bergen and Borisy (1980), and Bergen, Kuriyama and Borisy (1980), have shown that MT assembly occurs at a rate three times faster at the distal end of a flagellar axoneme "seed" than at the proximal end. Furthermore, disassembly occurs at a faster rate at the distal end. Bergen *et al.* (1980) also found that the rate of assembly (and disassembly) of MTs onto isolated centrosomes and chromosomes was identical to that for the distal end of the flagellar axoneme control. They conclude that proximal end assembly and disassembly is inhibited by structural means and centrosomal and chromosomal MTs grow from their distal ends only. This implies that kMTs and nkMTs would be antiparallel in the half-spindle. These observations on MT polarity are in direct conflict with those of Margolis and Wilson (1978) and Margolis, Wilson and Kiefer (1978), since the latter group propose that assembly of kMTs occurs at the kinetochore (proximal) end, and assembly of nkMTs occurs at the equatorial (distal) end. This gives a parallel arrangement of MTs in each half spindle. Margolis *et al.* (1978) propose that MTs assemble at one end and disassemble at the other, suggesting that there is a flow of material along the MT bundles. A poleward migration of material along chromosome fibres can be seen in living cells of *Haemanthus* endosperm with differential interference contrast optics (Hard and Allen, 1977). Margolis *et al.* (1978) looked only at net MT assembly rather than actual MT assembly (and disassembly) at each end (Bergen *et al.*, 1980), and thus Bergen *et al.*'s interpretation is more appropriate. However, these and earlier studies (McGill and Brinkley, 1975; Snyder and McIntosh, 1975; Telser, Moses and Rosenbaum, 1975; Gould and Borisy, 1978a; Pepper and Brinkley, 1979; Summers and Kirschner, 1979) have all used colcemid-treated chromosomes and it is not known whether these resemble *in vitro* chromosomes (Tippit, Pickett-Heaps and Leslie, 1980). As noted by Tippit *et al.* (1980, p. 415), "the kinetochore may renucleate MTs already bound to the kinetochore, but not removed by colchemid treatment; or the kinetochores may be 'seeded' by binding to small MT fragments collected during chromosome preparation". In view of these criticisms it is clear that further *in vivo* studies are required.

Tippit *et al.* (1980) suggest that the kinetochore does not nucleate MTs at all in diatoms; rather, the kinetochore fibre appears to be primarily composed of MTs from the poles. They further suggest that a situation similar to this may exist in other cell types. If this is so, MT polarity should be parallel in the half spindle. The question of MT polarity in the spindle is clearly unresolved at present.

Microtubules can be revealed in the LM with the immunofluorescent technique (Brinkley, Fistel, Marcum and Pardue, 1980, review). The procedures are described in detail in Brinkley *et al.* (1980) and Peterson and Berns (1980); and two variations are available: direct, and indirect immunofluorescence using tubulin antibody. In interphase cells beautiful 3-D images of MT networks are seen (Fuller, Brinkley and Boughter, 1975; Brinkley, Fuller and Highfield, 1975, 1976; Weber Bibring and Osborn, 1975a; Weber, Pollack and Bibring, 1975b; Weber, 1976. For a review of the recent literature see Brinkley *et al.*, 1980). In mitotic spindles of animal cells the technique reveals distinct spindle fibres (Nagayama and Dales, 1970; Dales, 1972; Fuller *et al.*, 1975; Brinkley *et al.*, 1975, 1976; Weber *et al.*, 1975a; Weber, 1976; Cande *et al.*, 1977; Connolly, Kalnins, Cleveland and Kirschner, 1978; Welsh, Dedman, Brinkley and Means, 1979; Brinkley *et al.*, 1980). Immunofluorescence of plant cells, and of mouse meiotic spindles, has also revealed distinct spindle fibres (Franke, Seib, Osborn, Weber, Herth, and Falk, 1977; and Weber, Osborn, Franke, Seib, Scheer and Herth, 1977; for plant cells, and Wasserman and Fujiwara, 1978 for meiotic cells). Fluorescence is dispersed by agents known to disrupt MTs in the EM (Weber, 1976) and correlative LM and EM studies show that MTs are indeed labelled with antibody (De May *et al.*, 1976; Pepper and Brinkley, 1977; Osborn, Webster and Weber, 1978).

Formalin fixed cells have been used routinely in these studies, but formalin is known to depolymerize MTs (Forer, Kalnins and Zimmerman, 1976; Pepper and Brinkley, 1977). The antigenicity of tubulin is apparently not significantly altered during formalin fixation; thus conjugation with anti-tubulin still occurs. The intact, but chemically altered, MTs of glutaraldehyde fixed cells do not bind to antibody against native tubulin. Antibody against glutaraldehyde fixed tubulin has recently been used to give a much clearer image of MT distribution (Eckert, 1979; Eckert and Snyder, 1977, 1978; Weber, Rathke and Osborn, 1978), and it is now possible to identify individual MTs with both immunofluorescence and electron microscopy (Osborne *et al.*, 1978).

The peroxidase-antiperoxidase (PAP) technique (De May *et al.* 1976; De Brabander, De May, Joniau and Guenens, 1977; Pepper and Brinkley, 1977) uses enzyme rather than fluorescent labelling, and glutaraldehyde fixation of cells is preferred. Microtubules are visible in the LM, and under the EM they are coated with layers of enzyme. The staining distribution in dividing cells is the same as with fluorescent dyes and is sensitive to mitotic poisons. There are variable amounts of diffuse staining in the cytoplasm and on membranes of several organelles and these might localize MT subunits (De Brabander *et al.*, 1977).

1.23 Microtubules and birefringence

Microtubules are the most likely major contributors to the BR observed in living (and fixed) cells since they are found in positions identical to the BR fibres. They are the type of molecule capable of retarding polarized light (Sato, Ellis and Inoué, 1975), and both MTs and BR are sensitive to the same external stimuli (Fuseler, 1975a; Salmon, 1975a, b, c, 1976; Inoué and Ritter, 1975 review). Cassim, Tobias and Taylor (1968), Sato (1969), Sato, Inoué and Ellis (1971), and Sato *et al.*, (1975) calculated expected BR from observed numbers of MTs and all found a good fit of expected BR with observed BR. Forer (1976) and Marek (1978) have criticised these results on the grounds that measurements of retardation do not take into account the thickness (and therefore volume) of oriented material that the polarized light has passed through. Birefringence should be expressed as retardation per unit volume, or the coefficient of BR. Marek (1978) further derives "volume BR" as a measure of the total oriented material in the spindle. He calculated the expected number of kMTs in *Melanoplus* spermatocytes from the observed volume BR and found that the observed number of kMTs was half the expected value. He notes that *Melanoplus* spermatocytes commonly lose 50% of their BR during fixation. Thus MTs could be lost, although a contribution to BR from other material is also possible.

Forer (1976) compared the BR distribution of one chromosomal fibre with that in the interzone, by microdensitometry of photographic negatives. He found that the considerable differences in BR in the two regions were not reflected in actual MT numbers. The reverse experiment of calculating expected BRs in the two regions based on observed MT densities, showed that observed BR was three to six times lower than expected BR. Forer (1976) concludes that some other component that masks the BR of the MTs must be present in the interzone.

Forer (1976) and Forer *et al.* (1976) report a series of experiments on the isolated mitotic apparatus (MA) which suggests a second component to spindle BR. The isolated MA fixed in glutaraldehyde contains normal numbers of MTs, but when fixed in formaldehyde it has none, although the MA stains beautifully with antitubulin immunofluorescence, suggesting that the MTs have been depolymerized and the tubulin subunits have stayed *in situ*. If the MA is treated with KCl only 50% of the normal BR is seen, yet on fixation with glutaraldehyde normal numbers of MTs are found, indicating that a non-MT component of the BR has been extracted. But if the extracted MA is fixed with formaldehyde, no MTs are found and, also, very little tubulin staining is observed, suggesting that the tubulin subunits have been washed out during formaldehyde fixation. Forer suggests binding between the two components

accounts for these observations. The tubulin is not washed out in the formaldehyde fixed untreated MA because it binds to "substance Y" (Forer certainly means "actin" here!). Substance Y is extracted with KCl, there is no binding to MTs, and thus in formaldehyde fixation the tubulin is washed out. It is possible, though Forer *et al.*, (1976) consider it unlikely, that the KCl treatment alters the antigenicity of the tubulin and thus destroys the binding of antitubulin and tubulin, negating the necessity for the subsequent extraction of tubulin. But if substance Y is bound to tubulin, and if Y is indeed extracted, the extraction of Y might (itself) alter the binding site on the tubulin for antitubulin. The point could be resolved in two ways. Firstly, the BR of KCl extracted, formaldehyde fixed MAs should be zero, since both components have been extracted. Secondly, tubulin should be present in the fixative solution after extraction and could be demonstrated by gel electrophoresis. Neither of these experiments have been conducted, nor mentioned. Although the explanation may be somewhat open to question, the evidence for a non-MT contribution to BR is still convincing.

Other evidence shows that up to 50% of the BR can remain in isolated MAs despite the absence of all MTs (Goldman and Rebhun, 1969; Rebhun, Rosenbaum, Lefebvre and Smith, 1974); and isolated MAs treated with pressure can lose up to 70% of their BR without any change in MTs, but with some changes in non-MT material (Forer and Zimmerman, 1976). La Fountain (1974, 1976), in his analyses of BR changes following glutaraldehyde fixation, found that the measured BR in living spermatocytes does not change upon fixation of anaphase cells, but drops by one half in metaphase cells; similarly McIntosh, Cande, Snyder and Vanderslice (1975b) found a significant drop in BR in some cells after fixation. Both groups of workers present data that suggest that the loss of BR is not caused by a reduction in the number of spindle MTs. These results also suggest a non-MT contribution to BR. Forer (1974, 1976, 1978) has proposed that this component is actin.

Recently, Nicklas, Brinkley, Pepper, Kubai and Rickards (1979) found that BR can drop by up to 60% on fixation and, most importantly, the amount of the drop is quite sensitive to minor changes in fixation technique, especially glutaraldehyde concentration, buffer type, and duration of fixation. By adjusting these parameters they were often able to keep the percentage BR drop to about 20-30%. While this drop might not be due to loss of MTs (McIntosh *et al.*, 1975b), the relative ease with which high BR loss (60%) can be achieved is disquieting and may reflect real loss of MTs during fixation in some cases.

Microtubules can be lost during fixation. A variation in total MT number of 40-50% was common between transverse sections of cells at the same stage but fixed at different times from different cultures (Brinkley and Cartwright, 1971). Variation was less (10%) if the cells were from the same batch. Brinkley and Cartwright (1971) note "... it is likely that culturing conditions and/or fixation affect microtubule number". While actual numbers varied the MT distribution profile from pole-to-pole was always the same shape and they argue that no particular class of MTs was preferentially lost. Similar conclusions from similar results are drawn by McIntosh and Landis (1971). However, nkMTs are more susceptible than kMTs to a number of environmental effects (Brinkley and Cartwright, 1975; Inoué and Ritter, 1975) and could respond in different ways to fixation. A more disturbing example can be seen in a comparison of kMT number in crane fly spermatocytes. La Fountain (1976) found 70-90 kMTs per kinetochore at anaphase, while Forer and Brinkley (1977) found only 20-30 in the same species. Clearly, fixation of MTs can be variable even in apparently well fixed cells using similar techniques. Thus, studies relating MT numbers directly to BR are of limited value.

1.24 Calcium and the control of microtubule assembly

Divalent cations, especially calcium ions, are involved in MT assembly (Weisenberg, 1972; see Olmsted, 1976; Kirschner, 1978 reviews) and Ca^{++} has been postulated to be involved in the regulation of MT assembly in axopodia (Schliwa, 1976a and b), in the cytoplasmic MT complex at interphase (e.g. Fuller, Artus and Ellison, 1976), during ciliary beating (Schmidt and Eckert, 1976; Zanetti and Walker, 1978) and in the mitotic spindle (Harris, 1975; Kiehart and Inoué, 1976; Wick and Hepler, 1976; Hepler, 1977, 1980; Sissen and Ved Brat, 1977).

In general, an increase in Ca^{++} concentration in a cell will cause MTs to depolymerize (Schliwa, 1976a), and in dividing cells this usually causes retardation of mitosis and stimulation of cytokinesis (Sissen and Ved Brat, 1977; Kiehart and Inoué, 1976). Recent studies suggest that calcium induced depolymerization of MTs *in vitro* proceeds by an all or nothing mechanism: individual MTs are either completely depolymerized by Ca^{++} or are unaffected (Weisenberg and Piazza, 1978). However, results consistent with simple end disassembly were obtained with dilution induced depolymerization. These observations are relevant to *in vivo* conditions and in particular, to theories of chromosome movement, which will be discussed later.

Calcium has been located in the spindle with an electron probe microanalyser (Timourian, Jotz and Clothier, 1974; Forer, Gupta and Hall, 1980). Forer *et al.* (1980) note that the quantity of calcium in the spindle is too

high (6 mM/kg wet weight) to be compatible with the presence of MTs (see e.g. Salmon and Segal, 1980) and they suggest therefore, that Ca^{++} is probably sequestered. Vesicles capable of taking up Ca^{++} from the medium have been isolated from the mitotic apparatus of sea urchin oocytes by Silver, Cole and Cande (1980). Wick and Hepler (1980) found calcium specific antimonate staining on tubules and cisternae of endoplasmic reticulum (ER) in the spindle and they suggest the ER sequesters Ca^{++} from the spindle matrix. A Ca^{++} sequestering protein (calmodulin), which might mediate Ca^{++} effects on MT assembly, has been localized in mitotic spindles by immunofluorescence (Marcum, Dedman, Brinkley and Means, 1978; Welsh, Dedman, Brinkley and Means, 1979).

Microtubule associated proteins (MAPs) are important in *in vitro* MT assembly (Kirschner, 1978) and several MAPs have been found in spindles by immunofluorescence techniques (Connolly, Kalnins, Cleveland and Kirschner, 1977, 1978; and Sherline and Schiavone, 1978). The fluorescence pattern is the same as for antitubulin. Sherline and Schiavone (1978) suggest that MAPs produce periodic side arms on MTs and thus contribute to MT sliding.

Nucleotides (Olmsted, 1977; Weisenberg and Deery, 1976), sulph-hydryl groups (Rebhun, 1976) and polycations (Erickson and Voter, 1976) have also been implicated in the control of MT assembly.

1.25 Actin, actin-like protein, microfilaments.

Direct evidence both for and against actin microfilaments in the spindle comes from several sources.

Microfilaments have occasionally been reported in glutaraldehyde fixed spindles (Bajer and Molè-Bajer, 1969; Müller, 1972; Goode, 1975; McIntosh, Cande and Snyder, 1975b; Euteneuer, Bereiter-Hahn and Schliwa, 1977; Forer and Brinkley, 1977; Schloss, Milsted and Goldman, 1977) in intranuclear spindles of *Physarum* (Ryser, 1970) and *Paramecium* (Lewis, Witkus and Vernon, 1976) and commonly in the contractile ring (Schroeder, 1976). In general, however, thin filaments of actin dimensions are absent in most standard glutaraldehyde preparations of dividing cells. Schroeder (1976) states that "I would expect that if actin were involved in force production that its location would be easily and reproducibly visualized". But it is not known just how much actin would need to be present in order to move chromosomes. If only a small amount of actin is required then thin actin filaments would be difficult to detect amongst other spindle components, e.g. MTs.

Fixation involving tannic acid plus glutaraldehyde has been used to reveal actin in the cleavage furrow at telophase (La Fountain, 1974, 1975; La Fountain, Zobel, Thomas and Galbreath, 1977). No actin filaments were observed in the spindle with this technique, although microfilaments have

been observed in glutaraldehyde fixed spindles of the same species (Forer and Brinkley, 1977). Forer (1978a) argues that La Fountain does not describe the prominent cortical bundles of actin commonly seen in many animal cells, and he argues that if the tannic acid treatment fails to reveal these filaments, it might also miss spindle actin.

The reason that actin filaments are not routinely seen in the spindle may be that osmium tetroxide degrades actin during fixation (La Fountain, *et al.* 1977; Szamier, Pollard and Fujiwara, 1975; Maupin-Szamier and Pollard, 1978). The evidence of Maupin-Szamier and Pollard (1978) is based on viscometric and EM studies of polymerized actin, and under the most damaging procedure (e.g. 2% osmium), the actin actually dissolves! By varying the conditions for osmium fixation, such as osmium concentration, type of buffer, temperature etc., optimum conditions for actin fixation may be obtained. The presence of tropomyosin bound to actin appears to assist in actin preservation. In all actin filament systems known to contain tropomyosin (e.g. muscle, brush border, stress fibres, acrosome process) actin filaments are easily preserved; but where tropomyosin is absent (e.g. spindles), actin is degraded by osmium (Maupin-Szamier and Pollard, 1978). The addition of tropomyosin to fixatives may assist the fixation of spindle actin (Szamier *et al.*, 1975).

Since direct visualization of actin in spindles has been relatively unsuccessful, indirect methods have been tried. Evidence for two components of spindle BR were discussed in the section on MTs and BR (sect. 1.13).

The first indirect evidence for two spindle fibre components involved in force production came from the ultraviolet light (UV) microbeam experiments of Forer (1965, 1966). He showed that chromosome velocity can be greatly reduced by UV irradiation of the spindle fibres, without loss of spindle BR and, conversely, spindle BR can sometimes be locally reduced without slowing the chromosomes. Forer (1966, 1978a) suggests that the two components are MTs and actin.

Prophase chromosome movements in cricket (*Acheta domesticus*) spermatocytes occur one hour or more before the breakdown of the nuclear membrane (nm) (Rickards, 1975, 1980 in press). The movements are saltatory and occur at relatively high speed over moderate distances. They are usually based at the end of the chromosome, but occasionally also at the kinetochore. In either case the movements are intimately associated with the nm during the entire movement. The movements are separate from, but apparently polarized by, the extranuclear asters and, like saltatory granule movements, are sensitive to colchicine. Rickards proposes that there is a separate force producer inside the nm and this force producer is somehow polarized by MTs outside the nm. He suggests that a similar two component system could therefore also be necessary for later prometaphase and anaphase chromosome movement.

Antibody injection experiments (Kiehart, Inoué and Mabuchi, 1977; Mabuchi and Okuno, 1977) have been used to suggest that myosin, and therefore actomyosin interactions, are not responsible for chromosome movement (Kiehart *et al.*, 1977; Sakai, 1978). Antimyosin antibody was injected into starfish egg cells before nm breakdown. In five out of 17 cells studied no MA was formed. The remaining 12 MAs were "small and obscure" (Mabuchi and Okuno, 1977), and nine of these formed daughter nuclei. Put another way, eight out of 17 cells (47%) did not complete division and the remainder had small MAs. Sakai (1978, p. 44) makes the startling conclusion that Mabuchi and Okuno have "... clearly indicated that antistarfish egg myosin serum did not inhibit mitosis when it was injected into the starfish egg"! Clearly, these data could be used to argue for the presence and function of actomyosin in the spindle (Forer, 1978a). Antimyosin inhibits the formation of a cleavage furrow in the same organism and thus Mabuchi and Okuno (1977) suggest that actomysin is involved in the formation of the contractile ring.

Sakai, Hiramoto and Kuriyama (1975) and Sakai, Mabuchi, Shimoda, Kuriyama, Ogawa and Mohri (1976) report slow anaphase chromosome movement in isolated MAs. They found that antibodies against dynein blocked chromosome movement but antimyosin had no effect. They conclude that chromosome movement is most likely due to dynein ATPase interaction with MTs and not due to actomyosin interactions. Forer (1978a, p. 53) severely criticises these findings on the grounds of inaccurate measurements and he suggests that the antidynein result could be due to an effect on tubulin polymerization in the maintenance medium, rather than on the cells. With respect to the antimyosin results, Mabuchi and Okuno (1977) observed that antimyosin will only block cleavage in starfish eggs if it is injected before the actomyosin complex in the contractile ring is set up (i.e. before breakdown of the nm). If actin and myosin are involved in chromosome movement they are surely likely to be complexed into actomyosin by metaphase. Antimyosin was added in these experiments to isolated metaphase or anaphase MAs: thus one might even expect no effect on chromosome movement if actin and myosin are involved.

Even if myosin is found not to be involved in mitosis, there is no *a priori* reason to eliminate actin involvement, since some motile systems utilize actin without myosin as, e.g., in acrosome extension in echinoderm sperm (Tilney, 1975). The relevance of these data are uncertain since myosin has in fact been demonstrated in the spindle (Cooke, 1975; Fujiwara and Pollard, 1976a and b, 1978).

Actin filaments have been described in EM preparations of dividing cells after glycerination and addition of rabbit skeletal muscle heavy meromyosin (HMM) or the S1 subfragment of HMM (Benke, Forer and Emmerson, 1971; Forer and Behnke, 1972; for meiotic spindles, and Gawadi, 1971, 1974; Hinkley and Telser, 1974; Schroeder, 1973, 1976; Schloss *et al.*, 1977; Forer and Jackson, 1975, 1976, 1979; Forer, Jackson and Engberg, 1979, for mitotic spindles). Thin filaments in the spindle are decorated with characteristic arrowhead complexes and these decorated filaments are identical to HMM-decorated actin filaments in muscle. Filaments are found between the chromosomes and the poles at metaphase and anaphase. They are usually parallel to MTs (although see Hinkley and Telser, 1974), and sometimes are closely associated with MTs (Forer and Jackson, 1979). Filaments are also present in the interzone at anaphase. The binding of HMM to actin is prevented by pyrophosphate or ATP, as with muscle actin (Schloss *et al.*, 1977), and there seems little doubt that the HMM reaction is specific for actin (Forer, 1978a and b).

Prior glycerination of cells is necessary to make them permeable to the large molecules of HMM, but this treatment causes extensive cellular disruption and, therefore, there is a possibility of preparation artifact. In particular, actin might be translocated into the spindle (Nicklas, 1975). This possibility is especially likely in animal cells, which are often filled with cytoplasmic actin (Hinkley and Telser, 1974), but the very short (two to four minutes) extraction procedure of Schloss *et al.* (1977) indicates such translocation is unlikely. Also, plant cells do not have extensive cytoplasmic actin, yet HMM decorated filaments have been found in glycerinated endosperm cells of *Haemanthus* (Forer and Jackson, 1975, 1976, 1979; Forer *et al.*, 1979). Decorated filaments were found in each and every chromosomal fibre studied and, as far as could be determined, in roughly equal amounts. Of the arrowhead complexes that could be distinguished clearly, most pointed towards the kinetochore. These results argue against actin translocation into the spindle during glycerination, since, if it did occur, some kinetochore bundles might be expected to have no actin, and the polarity of the filaments should be random. Forer *et al.* (1979) therefore suggest that actin filaments are genuine spindle components.

The ability of HMM and S1 to polymerize actin is an issue which may have been understated in the past. Yagi, Mase, Sakaibara and Asai (1965) have shown that HMM or S1 can induce the polymerization of actin under conditions which, without the HMM, will not support polymerization, nor maintain polymerized filaments. Thus, Tilney (1975) notes that in general microfilaments are not seen at all in glycerinated spindles prior to HMM treatment (exceptions: Gawadi, 1974; Schloss *et al.*, 1977). Actin filaments can be seen in glycerinated cleavage furrows, but after HMM treatment these, and

many more decorated filaments, are seen both in the cleavage furrow and in the cytoplasm (Cooke and Morales, 1971; Tilney, 1975; see Schroeder, 1973). Tilney suggests that HMM induces unpolymerized actin to polymerize, and thus the increase in microfilament number and length after HMM is a crude measure of unpolymerized actin. On this interpretation spindle actin would be mostly unpolymerized, and observations on arrowhead polarity (Forer *et al.*, 1979) would then have no significance. Forer (1978a) argues that HMM stabilizes actin filaments, some or all of which might otherwise be lost during fixation.

Actin can also be revealed in the spindle by immunofluorescence. Heavy meromyosin conjugated with a fluorescent dye has been used to localize actin in the spindle of isolated MAs (Aronson, 1965) and in cells (Sanger, 1975; Sanger and Sanger, 1976; Schloss *et al.*, 1977; Herman and Pollard, 1978, 1979). Aubin, Weber and Osborn (1979) report no localization of actin in the spindle with fluorescent HMM. Fluorescence is seen in chromosomal spindle fibres at metaphase and anaphase, but not usually in astral fibres nor in the interzone. The technique is similar to HMM labelling for EM and prior glycerination is usually required, although Schloss *et al.* (1977) and Herman and Pollard (1978, 1979) report that extraction in acetone results in an identical staining pattern. Clearly, the objections raised above concerning the glycerol extraction procedure are valid here also.

There is a discrepancy between the EM and LM visualization of actin with HMM. Fluorescence is not usually observed in the interzone at anaphase, yet HMM decorated filaments can be found in this region in the EM (Schroeder, 1976). Schloss *et al.* (1977) suggest that the concentration of actin in the interzone is below the sensitivity of the fluorescent stain but estimates of the quantity of decorated actin in this region have not been attempted. Recently, Herman and Pollard (1979) reported diffuse staining in the interzone and with improved techniques Schroeder's objection may soon be eliminated.

Forer (1978a) questions the specificity of the fluorescent HMM technique. Heavy meromyosin binds to actin, specifically forming arrowhead complexes, but it could also bind to other cellular compounds (e.g. ribosomes) and not form the arrowhead complex. Thus, while EM localization of actin via arrowhead complexes is specific, fluorescence microscopy would reveal HMM wherever it bound, including possible non-specific sites.

Antiactin antibody coupled with fluorescence staining was first used to reveal actin filaments in interphase cells (Lazarides and Weber, 1974), in the spindles of lysed cells (McIntosh, Cande, Lazarides, McDonald and Snyder, 1976; Cande, Lazarides and McIntosh, 1977) and in whole cell spindles (Herman and Pollard, 1979). The staining pattern is similar to that obtained with HMM fluorescence. Herman and Pollard (1979) also found diffuse staining

in the interzone at anaphase.

Cande *et al.* (1977) found that detergent extraction of part of the cellular actin was necessary to detect differences in fluorescence intensity between the spindle and the cytoplasm. This procedure could alter the actin distribution within the spindle, although a similar staining pattern was also obtained by Herman and Pollard (1979) and more than 90% of cellular actin was retained in their procedure. Herman and Pollard suggest that both polymerized and unpolymerized actin is present in the cell and lysis (Cande *et al.*, 1977) extracts the non fibrous portion allowing clear staining of chromosomal fibres.

If the actin is polymerized in the natural state then there is a possibility that binding of antiactin antibodies to actin filaments might produce migration of filaments. Utter, Biberfeld, Norberg, Thorstensson and Fagraeus (1978) report that purified actin consists of randomly oriented filaments when examined in the EM, but if the actin is conjugated with anti-actin, bundles of parallel filaments are commonly observed. In particular, lateral associations between adjacent filaments can be seen and it appears that the manner of binding of the antibody is by the joining of adjacent filaments. Isolated single filaments in the same preparation invariably have no bound antibody. Since the spindle is composed of bundles of aligned MTs, bundles of actin could become aligned in the same direction. However, the consistent lack of stain in the aster region argues against such a possibility.

The procedure for immunofluorescence staining usually involves fixation in formaldehyde and extraction in acetone. Formaldehyde is known to depolymerize MTs at least (see earlier discussion) and is generally inadequate as an EM fixative (Pepper and Brinkley, 1977). Phase contrast photographs of cells fixed for immunofluorescence show that cells are poorly fixed and considerably different from living cells. Hence fixation artifact is a possibility in these studies: actin might be translocated into the spindle during fixation and extraction. Tubulin immunofluorescence of well preserved glutaraldehyde fixed cells (Eckert and Snyder, 1978) shows an identical pattern of fluorescence but a much sharper image than with formaldehyde fixed cells. This indicates that tubulin is not translocated through the cell by the formaldehyde procedure, but similar results with antiactin immunofluorescence have yet to be obtained.

The usefulness of immunofluorescence studies rests largely on the adequacy of control experiments. For example, Connolly and Kalnins (1978) have demonstrated immunofluorescence staining of centrioles and basal bodies using only non-immune serum. The sera from different rabbits varied in reactivity and sera from five out of 35 animals gave strong staining, while that from a further 20 showed some stain. Nenci and Marchetti (1978) report

specific staining of centrioles and basal bodies with antibodies raised against steroids and suggest that steroids are therefore present in centrioles and basal bodies. They do not state whether their non-immune control serum was from the same animal as the immune serum was taken, but it seems likely from their discussion that it was not. Their results are therefore still open to question. Non-immune sera from some animals might also be able to stain actin or tubulin, and in fact antibodies to tubulin have occasionally been reported in non-immune sera (Karsenti, Guilbert, Bornens and Avrameas, 1977).

A more obvious source of error is the production of antibodies against minor contaminants in the actin antigen. The actin antigen used by Cande *et al.* (1977) was stated to be only 85% pure and thus the non-specific staining that was observed was probably due to antibodies produced against impurities in the antigen. Herman and Pollard (1979) used 99% pure actin antigen and they report no non-specific stain.

The purity of the antibody preparation is also important. Fujiwara and Pollard (1976b, 1978) isolated and fluorescently labelled an immunoglobulin (IgG) fraction from immune serum produced against myosin and used this IgG fraction to localize myosin in the mitotic spindle (direct immunofluorescence). Aubin *et al.* (1979) point out that there is a possibility that more than one IgG antibody is present in the IgG fraction, although the crude immune serum showed only a single precipitin line in cross reaction with myosin. Other controls, including pre-absorption of the fluorescent IgGs with antigen, or pre-incubation of the cells with unlabelled antibodies, also indicate a single antibody (antimyosin) in the IgG fraction. However, when the IgGs were further purified by affinity chromatography with purified myosin, the spindle was not preferentially stained (Fujiwara and Pollard, 1976). Aubin *et al.* (1979) also report no preferential staining with indirect immunofluorescence using affinity purified antimyosin, and they suggest that Fujiwara and Pollard's use of an IgG fraction may have given non-specific staining.

Aubin *et al.* (1979) also used affinity purified anti-actin and anti-tropomyosin and report no increased accumulation of actin or tropomyosin in the spindle. They suggest that impure antibodies have resulted in erroneous actin localization in the spindle by Cande *et al.* (1977), but they did not conduct a positive control, showing spindle staining with impure antibodies, to support their view. Herman and Pollard (1979) have shown localization of actin in the spindle using affinity purified antiactin and they used both direct and indirect immunofluorescence in the same material (PtK₂ cells) as Aubin *et al.*, (1979). The main difference in technique appears to be the fixatives: formaldehyde (Herman and Pollard, 1979) versus methanol (Aubin *et al.* 1979). Aubin *et al.* (1979) imply, but do not state

directly, that formaldehyde and methanol fixation gave similar results with antiactin. If fixative differences account for the staining differences, it could be argued that methanol causes extraction or translocation of actin; but it also could be argued that formaldehyde causes translocation of actin into the spindle. The question is unresolved at present.

Forer (1978a) questions the specificity of the antibody-antigen reaction in immunofluorescence. He suggests that other proteins, or subunits of proteins, might have similar amino acid sequences to the target antigen, and these might also bind to the antibody. Control experiments in immunofluorescence are generally not directed towards this possibility.

Direct and indirect evidence for actin and myosin in the spindle has been described in this section. Many lines of evidence, from numerous laboratories, suggest that actin is present between the chromosomes and the poles and diffusely in the interzone. These are the positions where force production apparently occurs, and actin is a substance known to produce force. Even though there are criticisms of each line of evidence, each gives the same result, and it can thus be argued that actin is a genuine and functional spindle component.

1.3 FORCE(S) IN THE SPINDLE

Chromosome movements occur chiefly in prometaphase and in anaphase and they bring about the equal distribution of chromosomes to the daughter cells. The movements are very precise and "mistakes" occur only rarely. It is not necessary to give a detailed account of the movements of chromosomes on the spindle as this has been done many times (Luykx, 1970; Nicklas, 1971; Bajer and Molè-Bajer, 1971, 1972; McIntosh, Cande, Snyder and Vanderslice, 1975b), but a brief description will be useful. The end of prophase is marked by the breakdown of the nuclear membrane (nm) and the initially scattered chromosomes become associated with the spindle during early prometaphase. Each chromosome then individually moves to the equator of the spindle. The end of prometaphase is reached when all chromosomes have attained bipolarity and are situated approximately equidistant from both poles (metaphase). Anaphase begins by the sudden separation of all chromatids or half-bivalents and near uniform motion of daughter units to opposite poles ensues. Anaphase is characterized by (a) chromosome-to-pole movement and (b) polar separation. The relative extent and duration of these two components varies considerably (Mazia, 1961).

1.31 Position of force(s).

The forces producing prometaphase and anaphase chromosome motion act mostly at the kinetochore. This is shown by the shape of chromosomes during anaphase, where a chromosome with a single localized kinetochore has a characteristic I, J or V shape depending on kinetochore position (Nicklas, 1971). Thus the kinetochore leads in movement, dragging the rest of the chromosome passively. The shapes of multivalents at metaphase and anaphase (Bauer, Dietz and Röbbelen, 1961; Wise and Rickards, 1977) and of chromosomes with multiple or diffuse kinetochores (see e.g. Schrader, 1953) are also consistent with forces acting at the kinetochore. The saltatory motion during prometaphase and anaphase, of chromosome fragments lacking kinetochores (Bajer, 1958) and of chromosomes with experimentally damaged kinetochores (Brenner and Berns, 1978), also support this view. During the micromanipulation experiments of Nicklas and Staehly (1967) chromosomes were shown to be attached to the spindle only at the kinetochores and this attachment was necessary for movement.

1.32 Direction of force(s).

There is most probably a simple poleward directed force during prometaphase and anaphase. A pushing force from pole to chromosome (Darlington, 1937) would cause bivalents (or chromosomes) to lie across, rather than perpendicular to, the equator at metaphase (Östergren, 1951). Similarly, a pushing body action between separating anaphase chromosomes ("Stemkörper" Belar, 1929) is unacceptable, since it cannot explain prometaphase congression.

A Pushing body

Direct evidence for a poleward force comes from Forer (1965, 1966). Ultraviolet microbeam irradiation of chromosomal fibres usually caused anaphase motion to cease in living cells, but irradiation of the interzone had little effect. These experiments indicate that force is produced in the region between the chromosomes and the pole. Also, naturally occurring and experimentally introduced akinetochoric fragments will show directed movement only when they are in the half spindle and their movement is poleward, indicating poleward directed forces (Bajer and Molè-Bajer, 1972; Nicklas and Koch, 1972; Nicklas, 1975). Similarly, only a poleward directed force from the chromosome will explain the movement back to the spindle of bivalents displaced into the cytoplasm by micromanipulation (Brinkley and Nicklas, 1968; Nicklas, Brinkley, Pepper, Kubai and Rickards, 1979).

1.33 Magnitude of force(s)

Chromosomes move slowly ($0.2 - 5 \mu\text{m}/\text{min}$); thus the force required to move chromosomes in the spindle is correspondingly small (approximately 8-10 dyne; calculated by Taylor, 1965; Nicklas, 1965). Inoué and Ritter (1975) calculated that the force available through the depolymerization of one MT is 1.3×10^{-6} dyne, i.e. approximately 100 times greater than required to move a chromosome. The ATP molecules associated with a single short ($1 \mu\text{m}$) MT could produce flagella type shearing forces 10,000 times greater than necessary (Nicklas, 1975), and Forer (1978) estimates that one actin filament together with one myosin filament, operating as in skeletal muscle, could produce 3,000 times more force than required to move a chromosome.

1.4 FORCE PRODUCTION IN THE SPINDLE

There have been numerous hypotheses of force production in chromosome movement in the past, and many of them have been remarkably ingenious. Heidenhain (see Wilson, 1925) for example, constructed a complicated working model from several solid rings and a large number of rubber bands! Others devised strikingly spindle-like models with iron filings and bent wires in a magnetic field (Wilson, 1925). Modern theories only will be discussed here, and the reader is referred to Wilson (1925), Schrader (1953) and Mazia (1961) for excellent accounts of early views.

Professor Heidenhain constructing a complicated working model from several solid rings and a large number of rubber bands.

The mechanism of force production is still largely unknown and Wilson's (1896, p. 70) comment "... the mechanism of mitosis still lies before us as one of the most fascinating problems of cytology" is still valid today.

1.41 Microtubule length changes

The idea that chromosomal spindle fibres are in an equilibrium with subunits is not new, and it is still the basis of one modern theory of chromosome movement. Östergren (1949, 1951) and Wada (1950) separately proposed that spindle fibre subunits were in a dynamic equilibrium between an ordered state (the spindle fibre) and an unordered and inherently more unstable state. The ordered state is set up at the kinetochore and broken down at the pole and it has an inbuilt tendency to decrease in length. Shortening of the chromosomal fibre would draw the chromosomes poleward (Schrader, 1953). If "microtubules" are substituted for "fibres" above, the idea becomes quite similar to modern views (Inoué, 1964; Inoué and Sato, 1967; Inoué and Ritter, 1975; Dietz, 1972).

The evidence for a dynamic equilibrium between MT subunits and MTs comes from a variety of sources. In the UV microbeam experiments of Forer (1965, 1966), the poleward migration of an area of reduced BR in a chromosomal fibre suggests a flow of tubulin entering the fibre at or near the kinetochore and leaving predominantly near the spindle pole (Inoué and Ritter, 1975; the possibility of a second component to BR is not mentioned). Ciné films taken

using an interference contrast microscope show polewards migration of particles in individual spindle fibrils (Allen, Bajer and La Fountain, 1969; Hard and Allen, 1977). The effects of mitotic poisons, cold temperature, hydrostatic pressure etc. on mitosis and on MT numbers also support the concept of MT polymerization/depolymerization involvement in chromosome movement.

Other evidence comes from experiments on living *Chaetopterus* oocytes at metaphase I. Induction of slow depolymerization of spindle fibres with colchicine, slow cooling or moderate hydrostatic pressure, caused shortening of MTs and the chromosome-to-pole distance decreased. Since one aster is attached to the cell membrane the chromosomes were transported towards the surface of the cell. The process was reversible. Similarly, slow cooling of grasshopper spermatocytes in prometaphase or anaphase I caused some chromosome displacement towards the equator (Nicklas, 1975). It is evident from these experiments that movement of chromosomes can be produced by the depolymerization of MTs. In *Chaetopterus* both nkMTs and kMTs must shorten to produce the observed motion (Inoué and Ritter, 1975). In the grasshopper the more labile nkMTs (Brinkley and Cartwright, 1975) shorten, and this causes spindle shortening and chromosome displacement since kMTs are mechanically associated with nkMTs (Nicklas, 1975).

But is MT depolymerization actually used in the living cell to move chromosomes normally? Certainly kMTs become shorter during anaphase. Microtubules can be found in electron micrographs of metaphase cells that are longer than the final chromosome-to-pole distance in late anaphase (Lambert and Bajer, 1977) and therefore long MTs must either shorten or be completely depolymerized during anaphase. Weisenberg and Piazza (1978) show that calcium induced MT depolymerization *in vitro* is an all or nothing process: MTs are either completely depolymerized, or are unaffected. This is not compatible with the disassembly of kMTs at their polar ends as proposed by Dietz (1972) and Inoué and Ritter (1975), although these authors would argue that *in vivo* evidence is lacking.

But does MT assembly/disassembly directly provide the motive force for chromosome movement or does it govern the speed of movement by acting as a rate limiting step, controlling another force producer? The question remains unanswered since the inherent simplicity of the theory makes disproof extremely difficult. Perhaps the best method of disproof is to provide evidence for a different method of force production.

Depolymerization

1.42 Interactions between microtubules

1.421 Sliding hypotheses

The success of sliding hypotheses in many aspects of cell motility, especially skeletal muscle contraction (Huxley, 1973) and flagella and cilia motion (Satir, 1976), has prompted several workers to postulate MT/MT sliding mechanisms of force production in the spindle.

McIntosh, Hepler and Van Wie (1969) and McIntosh and Landis (1971) proposed that the direction of MT growth is polarized: kMTs grow away from the kinetochore and nkMTs grow towards the pole. Bridges between the MTs could produce force, in a direction opposite to the MT polarity, against either granules or adjacent MTs of opposite polarity.

McIntosh and co-workers have counted and traced MTs through serial transverse sections in an attempt to show redistribution and therefore sliding of nkMTs in the interzone during anaphase spindle elongation (McIntosh and Landis, 1971; McIntosh *et al.*, 1975a and b). McIntosh *et al.* (1975a and b) found that in stem bodies at anaphase, the ratio between the nkMTs in the region of MT overlap and nkMTs outside the region of overlap decreased during anaphase, indicating sliding between nkMTs. Brinkley and Cartwright (1971, 1975) used similar techniques in different mammalian cells but found no change in the above ratio during anaphase. Tracing of MTs from section to section showed that nkMTs in mammalian cells (McIntosh *et al.*, 1975a and b) and in diatoms (McDonald, Pickett-Heaps, McIntosh and Tippit, 1977; McIntosh, McDonald, Edwards and Ross, 1979) are made up of two families of tubules which interdigitate at the equator. There are very few, if any, nkMTs which run from pole-to-pole.

In contrast to the dynamic equilibrium hypothesis, McIntosh, *et al.*'s (1969) sliding hypothesis is readily testable. It predicts polewards and equatorial motion of granules in the half-spindle until early anaphase and only equatorial movement at later stages (Nicklas, 1971). In fact, mainly poleward granule motion is observed (Bajer and Molè-Bajer, 1972, 1975; Nicklas 1975). Also, an anaphase chromosome, detached by micromanipulation and pushed back to the opposite pole, should move back towards the original pole but stop at the equator on McIntosh *et al.*'s (1969) model (Nicklas, 1971; Forer and Koch, 1973). But in the experiments (Forer and Koch, 1973) motion was observed to continue all the way to the original pole, indicating that the model is inadequate as stated. However, Nicklas (1971) and McIntosh *et al.* (1975a) proposed alternative but more complicated hypotheses, which account for granule motion and the micromanipulation results. Other less specific sliding hypotheses are also tenable (Bajer, 1968; Subirana, 1968).

1.422 Microtubule sliding and opposite end MT assembly/disassembly

A mechanism combining both MT sliding and MT length changes has recently been proposed by Margolis, Wilson and Kiefer (1978). As noted earlier (section 1.12) these authors propose a parallel arrangement of nkMTs and kMTs in the half spindle, a situation, in fact, identical to that in the sliding model of McIntosh *et al.* (1971) described in the previous section. Margolis *et al.* (1978), however, further propose that

all MTs are in a equilibrium between assembly and disassembly. Thus, kMTs and nkMTs are assembled at the kinetochore or equator respectively and disassembled at the pole. Antiparallel nkMTs from opposite half-spindles therefore continuously slide against each other. After the separation of chromatids at anaphase the assembly of kMTs ceases and there is a net decrease in length of kMTs drawing the chromosomes poleward. Non-kinetochore MTs continue to slide against each other, and this, with the loss of chromatid attraction, contributes to spindle elongation. Sliding of kMTs against nkMTs of opposite polarity is proposed to account for prometaphase chromosome congression.

The micromanipulation experiment of Forer and Koch (1973) which was designed to test the McIntosh *et al.* (1971) model can also be applied to this model. The detached anaphase chromosome should not subsequently move at all since chromosomes detached from the spindle do not have any MTs attached to their kinetochores (Nicklas, Brinkley, Pepper, Kubai and Rickards, 1979) and, according to the model, MT assembly at the kinetochore ceases at the onset of anaphase. Reinitiation of MT assembly in detached anaphase chromosomes could be proposed to account for this observation but it detracts from the simplicity of the model.

1.423 Zipper hypothesis

The zipper hypothesis has been proposed to account for several features of MT distribution, especially in *Haemanthus* endosperm cells, which are unexplained in other hypotheses (Bajer, 1973, 1977; Bajer and Molè-Bajer, 1975), as follows. (i) Kinetochore MTs increase in divergence from prometaphase to late anaphase. Divergence starts at the periphery of the kMT bundle. (ii) Kinetochore MT numbers decrease during anaphase and there is an increase in the number of short lengths of MTs adjacent to and behind the advancing kinetochore during anaphase (Jensen and Bajer, 1973, 1974). Bajer and Molè-Bajer (1975) propose that these fragments arise from kMTs peeling off at the edges of the kMT bundles. (iii) Non-kinetochore MTs intermingle at oblique angles with kMTs.

The zipper model proposes that there are lateral connections between nkMTs and kMTs progressively along their length and that such "... mechanical connections between MTs are sufficient to produce the movement of the kinetochore" (Bajer, 1973, p. 146; and Bajer and Molè-Bajer, 1975, p. 90). If a kMT and a nkMT are a certain distance apart and the nkMT is anchored, the free end of the kMT will bend during zipping. If this free end is attached to the kinetochore and sideways movement restricted, the bending will result in a small poleward movement of the kinetochore. A final assumption is that MTs neither stretch nor contract.

Bajer (1973) and Bajer and Molè-Bajer (1975) concentrate their attention on anaphase movement, but prometaphase motion is equally important and must be explained by any hypothesis. If zipping causes prometaphase as well as anaphase motion then two events must occur:

(a) kMTs must increase in divergence from prometaphase through metaphase to anaphase, and this is observed (Bajer, 1973); (b) the numbers of kMTs per kinetochore must decrease especially from late prometaphase to, and during, metaphase. This is because once each zipping action is complete the kMT is lost from the kMT bundle. Jensen and Bajer (1974) report, however, that the average number of kMTs per kinetochore increases during prometaphase to a maximum at late metaphase (prometaphase average 46.5 kMTs, late prometaphase 61.5, metaphase 80.7, late metaphase 82.2, early anaphase 68.5). It might be argued that MT numbers are increasing during earlier stages at a rate faster than zipping uses them up. This may well be true but it brings into question the functionality of zipping. If increase in kMT numbers is the dominant feature of early division stages then perhaps MT assembly à la Inoué and Ritter (1975) is responsible for prometaphase congression and the metaphase equilibrium.

A more obvious criticism of the zipping hypothesis is given by Nicklas (1975). Some cells are known to have kMTs and nkMTs which are parallel to each other and yet exhibit normal anaphase movement (e.g., Jenkins, 1967). The zipper hypothesis requires non-parallel interactions between nkMTs and kMTs, and thus becomes untenable at least in some cells. Pickett-Heaps and Bajer (1977), however, argue that a single universal model for chromosome movement is unlikely. Certainly, the lower organisms display considerable diversity in spindle form and mitotic behaviour (see Heath, 1980, for a review) and it may be true that different mechanisms of chromosome movement apply in different species in these groups. However, a single theory explaining all "normal" chromosome movements in higher eukaryotes still has considerable appeal.

Zippering

1.43 Actin

The evidence for the presence of actin in the spindle has been reviewed in section 1.15. Since actin is apparently present, it is often argued that it also functions in moving chromosomes (Forer, Jackson and Engberg, 1979). Forer (1978a) marshalls a sizeable body of evidence for actin involvement in cell motility. Actin is present and probably involved in muscle contraction, amoeboid movement, protoplasmic streaming, animal cell cleavage, acrosomal movements, microvilli movements, and animal cell movements (see Forer, 1978a, for refs.). The implication from such a wide variety of data is that actin, if proven to be present in the spindle, is also functional in moving chromosomes.

If actin is present and functional in the mitotic spindle it is likely that it will function in a manner similar to muscle contraction (Forer, 1978a). If the kinetochore is equivalent to the Z line in muscle, then actin filaments should be attached to the kinetochore and arrowhead complexes (after HMM treatment) should point polewards. Conversely, if the kinetochore is not equivalent to the Z line and the actin filaments are attached to the spindle elsewhere, then the arrowheads should point towards the chromosome (Forer, 1978a, Fig. 3). Some evidence exists which supports the latter view (Forer, Jackson and Engberg, 1979; see also p. 14) but definitive evidence is sparse.

As noted earlier (p. 12), Rickards' (1975) studies on prophase chromosome movements suggested a two component system of force production. Rickards considers that all movements of chromosomes and granules within the spindle and in prophase nuclei are most likely brought about by variations on one and the same mechanism. Thus, Rickards (in press, 1980) proposes that for granule movement in the aster, actin filaments are aligned and polarized by aster MTs and particulate myosin is adsorbed onto the surface of the granules. The myosin interacts with an actin filament to propel the granule along the filament. In prophase, actin filaments are also aligned inside the nm parallel to aster MTs outside the nm. Myosin is adsorbed onto chromosome ends (and onto kinetochores), and interacts with actin filaments to propel the chromosome towards or away from the aster center. In prometaphase and anaphase, actin filaments are oriented in relation to the kMTs and are anchored to the rest of the spindle. Myosin is adsorbed onto kinetochores and kMTs (or just kMTs) and the kinetochores and kMTs are transported polewards through myosin-actin interactions. The motile mechanism is essentially the same as that in muscle (Huxley, 1973) and as with Forer's (1978a) interpretation (previous paragraph), the HMM arrowheads should point to the kinetochore.

This model is consistent with available data, but of course it is not proven. The presence of actin in the spindle has not even been shown unequivocally, and a great deal more research is required before we will be closer to the truth about chromosome movements.

Actin-Myosin-MTs

Chapter 2. MATERIALS AND GENERAL TECHNICAL DATA

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Chapter 2.

MATERIALS AND GENERAL TECHNICAL DATA

2.1 MATERIALS

Pollen-mother-cells of *Iris spuria* L. ($2n = 48 \pm 2$), *Allium triquetrum* L. ($2n = 18$) and *Tradescantia flumenensis* Vell. ($2n = 60$) were used in this study.

2.2 TECHNICAL DATA

2.21 General glassware cleaning

Glassware for living cell and for EM work was always cleaned in separate containers. Two methods of glassware cleaning were employed.

2.211 Nitric acid. Glassware was immersed in concentrated nitric acid and heated in a fume cupboard until the first bubble rose, indicating near boiling temperature. After cooling, the glassware was rinsed in distilled water, drained and then placed in a hot oven (200°C) to allow evaporation of all nitric acid. Each item of glassware was then covered with aluminium foil or parafilm and stored until use.

2.212 Detergent. This method was preferred because it was less time consuming. Glassware was soaked in 2-5% Decon 90 (Selby-Wilton) for at least one day (usually much longer), then rinsed in running tap water overnight, and drained and stored as above.

2.22 Coverslip cleaning

Coverslips were sonicated in 100% ethanol for 15 minutes using an L and Rultrasonicator. They were then stored in fresh 100% ethanol and flamed before use.

2.23 Living cell culture

2.231 Slide culture chamber. The chamber consists of a standard size aluminium or glass slide with a 18 mm diameter hole drilled in the centre (Nicklas and Staehly, 1967). A 22 x 22 mm No. 1 coverslip was sealed over the hole onto one side of the slide with hot petrolatum, so that it formed the base and viewing window of the chamber. Aluminium slides are adequate and they are cheaper and more durable than glass slides.

2.232 Humidity chamber. All living cell preparations were made in a humidity chamber (Fig. 1a) which was maintained at a humidity of 85-90% by feeding in steam from a remote electric jug. It was possible to sterilize the chamber before (not during!) use with UV light for about 30 minutes, and sterility could be maintained during slide preparation with a laminar

FIG. 1a

FIG. 1b

FIG. 2

flow of sterile air at constant temperature (Fig. 1b). However, sterile technique during specimen preparation is not important for short term cell culture and the unmodified system was used routinely.

2.233 Culture medium. The medium was a solution of sucrose (Analar grade, May and Baker), and sometimes 0.15% yeast extract was added to act as a weak buffer and to provide nutrients.

2.234 Oils. Living cell preparations were covered with an inert oil to prevent the dehydration of the cells. Oils used were: Paraffin oil (May and Baker); Halocarbon series 10-25 oil (Halocarbon Products Corporation, Hackensack, N.J., USA) (Nicklas and Staehly, 1967), and Voltalef Huile 10S (Ungine Kuhlmann, 25 Bd de l'Amiral, Bruix 75116, Paris).

2.235 Perfusion chamber. Figure 2 illustrates the perfusion chamber used in correlative living cell and EM studies. The design is based on that of McGee-Russell and Allen (1971) and has been modified for use with inverted microscopes. Short sections of hypodermic needles were glued into the small holes and each was connected to polythene tubing of the appropriate diameter. The inlet tube was connected to a hypodermic syringe containing fixative, and the outlet tube drained into a collection vessel.

2.24 Ciné micrography

Cell preparations were examined using a Zeiss phase contrast inverted microscope-D with a ^{0.63NA} long working distance condenser. Objectives were either a Zeiss Neofluar 40X (0.6 NA), a Plan Apochromat 40X (0.6-1.0 NA) oil immersion, or a Plan Apochromat 100X (1.3 NA) oil immersion. The light source, in either continuous or flash mode, was filtered with heat reflecting (Zeiss 46-78-30) and apple green interference (Zeiss) filters. The microscope was connected via a three way photochanger to a Bolex H-16 ciné camera. The photochanger was specially adapted by Zeiss to split 80% of the light to the camera and 20% to the binocular eyepieces. This 80/20 split provides for concurrent viewing and ciné-micrography. A Bolex-Wild Variotimer time-lapse control unit MBF-B, and timer MBF-C were connected to the camera and intervals from five seconds to two minutes were used between exposures. The exposure time was 0.2-0.3 sec, depending on light intensity. During filming, verbal comments were recorded on a small tape recorder and the comments were later transcribed onto protocol sheets. The film used was Kodak Plus-X, type 7231 negative film, and it was processed by the National Film Unit, Wellington, N.Z.

2.25 Ciné-film analysis

Films were analysed frame-by-frame using a Lafayette AAP-200 16 mm ciné-projector. The projected image was reflected onto a bench top with a 45° inclined mirror and the magnification was adjusted so that 1 mm on the image was equivalent to 1 μ m in the cell. Relevant parts of the cell were traced onto paper and measurements were recorded later. Exposures two minutes apart were usually chosen and this gives about 20 measurements for a typical anaphase.

2.26 Electron microscopy

2.261 Fixation. Coverslip preparations of cells were initially fixed in a small drop of 6% glutaraldehyde in 0.1 m cacodylate buffer and 1% sucrose (final pH of fixative was 6.9). Sucrose was sometimes added to the buffer to increase its osmolarity to a level of near isotonicity with the cells (Arborg, Bell, Brunk and Collins, 1976). The 6% glutaraldehyde was obtained by diluting a 12% aqueous stock just prior to use with 2X concentrated buffer and sucrose. The 12% stock solution was made up from a 2 cc vial of 70% glutaraldehyde (Polaron). After fixation the cells were secured to the coverslip (section 3.31) and covered with several drops of 3% glutaraldehyde in 0.1 m buffer and 1% sucrose (pH 6.9). The coverslip was then transferred to a mini-coplin jar containing 3% glutaraldehyde fixative, and fixed for 30 minutes. Subsequent treatment was as follows.

0.1 m cacodylate buffer plus 1% sucrose (pH 7.2) 4 changes in 5 min;

2% osmium tetroxide (Polaron) in 0.1 m cacodylate buffer plus 1% sucrose (pH 7.2), 30 min;

distilled water, 2 x 5 min;

25% ethanol, 3 min;

50% ethanol, 3 min;

70% ethanol, 5 min;

95% ethanol, 2 x 5 min;

100% ethanol, 2 x 10 min;

100% acetone, 2 x 10 min; all traces of water were removed from the acetone by including several large crystals of CaCl_2 in the reagent bottle (M.N. Loper, pers. comm.).

25% Araldite (Ciba) in acetone, 15-30 min;

50% Araldite, 30-60 min;

75% Araldite, 1 hr-overnight;

100% Araldite, 2 hr.

Coverslip preparations were then flat embedded in a thin wafer of Araldite and polymerized overnight at 60°C.

2.262 Photography of embedded cells. Embedded cells were examined with a Zeiss photomicroscope III using an oil immersion Plan Apochromat condenser (1.4NA) and either an oil immersion Plan Apochromat 40X (0.6-1.0 NA) or 100X (1.3NA) objective. Suitable cells were photographed on 35 mm Ilford Microneg black and white negative film, ASA 9-12.5.

2.263 Sectioning etc. The coverslip was removed from the wafer of araldite (section 3.34) and selected cells were cut out and mounted on pegs of araldite in LS or TS orientation. Serial sections were cut with a Du Pont diamond knife on an LKB III ultramicrotome, and were mounted on Formvar (Gurrs) coated single slot grids (Polaron 2 x 1 mm). Sections were stained in 13% Uranyl nitrate (10-15 min), then in lead citrate (10 min, Venables and Coggeshall, 1965); examined in a Zeiss EM 9A (upgraded to EM 9S); and photographed on 2½" square Ilford Line N4E-50 negative film.

Chapter 3. TECHNIQUES : DESCRIPTION AND DISCUSSION

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Chapter 3. TECHNIQUES : DESCRIPTION AND DISCUSSION

A considerable portion of this thesis involved the development of techniques for (a) short term (< 5 hr) culture and ciné-micrography of living PMCs in meiosis, (b) electron microscopy including serial sectioning at known stages and at known planes of section, and (c) correlative living cell and electron microscopy.

The development of these techniques and the techniques themselves are described and discussed in detail in this chapter.

3.1 LIVING CELL CULTURE : INTRODUCTION

The culture of living PMCs proved to be a difficult task. Pollen mother cells from different species vary considerably in their suitability for *in vitro* culture, and PMCs from some species could not be cultured at all, despite strenuous efforts. For example, PMCs of *Agapanthus umbellatus* invariably died within 15-30 minutes of explantation, regardless of the culture conditions. This was particularly unfortunate because *Agapanthus* PMCs appear otherwise to be excellent candidates for live cell studies.

Flower buds of monocotyledon species were much preferred to those of dicotyledons because they are large enough to be dissected easily without injuring the PMCs. Further, species in which PMCs are easily squeezed from the anther are distinctly advantageous. The PMCs of *Watsonia* sp. for example are unsuitable in this respect since they stay aggregated in a coherent filament of cells throughout meiosis, and cannot be easily spread.

A good quantity of tapetal fluid was found to reduce the chance of the cells drying out during the preparation of the culture, although oily and granular tapetal fluids such as those in *Lilium tigrinum* can obscure the cells.

Phase bright granules in the cytoplasm of PMCs of some species (as e.g. in *Lilium tigrinum*) were found to obscure chromosome detail. In *Tradescantia flumenensis* phase bright granules cover the polar regions, but the chromosomes are still visible. An attempt was made to remove the granules from *L. tigrinum* by centrifugation (Inoué, 1977; pers. comm. to G.K. Rickards), but the granules still covered the chromosomes after centrifugation.

Species with very small chromosomes are of limited use to the study of chromosome movements because the chromosomes are difficult to distinguish. The chromosomes in PMCs of *Vicia faba*, for example, are too small to distinguish clearly, even though *Vicia faba* root tip mitotic chromosomes are quite large.

Very small PMCs were also impractical. Apart from having small chromosomes, small PMCs tend to have a relatively large phase halo around their periphery and this can be disturbing (*V. faba*, *Crepis capillaris*). However, very large PMCs could be used (as e.g. *Iris*), although size may preclude the use of 100X objectives. This presents no difficulty so long as the chromosomes are of a sufficient size to be resolved with medium power objectives. If a high number of large PMCs were explanted from the anther, a monolayer of cells was not obtained (*L. tigrinum*) and a lower quality image ensued.

A long flowering season is of obvious advantage.

The factors described above severely limited the number of plants that could be used. Satisfactory species were: *Iris spuria*; *Allium triquetrum* and *Tradescantia flumenensis*. Unsuitable species were: *Agapanthus umbellatus*; *Crepis capillaris*; *Fuschia* sp.; *Hedycium monogynum*; *Lilium tigrinum*; *Phormium colensoi*; *P. tenax*; *Vicia faba*; *Watsonia* sp. and most dicotyledons studied.

In most of the above species the size of the anther correlates well with meiotic stage in any one individual plant, but this correlation does not usually extend to different plants of the same species.

3.2 CULTURE TECHNIQUES

The simplest method for the examination of living cells is that used for insect spermatocyte culture by Nicklas and Staehly (1967) and Nicklas, Brinkley, Pepper, Kubai and Rickards (1979). Briefly, testes are dissected from the animal in an isotonic or marginally hypotonic saline, spermatocytes are spread over the surface of the coverslip of the slide chamber, and the cells are quickly covered with an inert oil. This technique was modified for PMCs as follows. A flower was dissected in a drop of culture medium (see below) and an anther was transferred to the coverslip window of the slide chamber. The anther was cut open with a sharp scalpel under high humidity, the contents were gently squeezed out and spread over the surface of the coverslip. Finally the cells were quickly covered with an inert oil. After several minutes the thick callose wall of PMCs prepared in this way folds and puckers where it contacts the glass (Fig. 3). To a certain extent this may be due to the use of coverslips which are not scrupulously clean (Fuseler, 1975a), but even when coverslips were cleaned as Fuseler recommends, little improvement was gained.

Puckering was reduced in two ways. (a) By extruding the cells into a small drop of culture medium. With this procedure, however, the cells moved about freely and were unsuitable for timelapse ciné micrography. Furthermore, both cell survival and the image quality were poor. Alternatively, (b) a 1% aqueous solution of protamine sulphate (Steinhardt, Lundin and Mazia (1971); Calbiochem, A grade) was applied to one surface of a coverslip for one minute; the coverslip was then washed with distilled water and air dried. The protamine sulphate on the coverslip binds evenly to the callose wall of PMCs, reducing folding and actually sticking the cells weakly to the coverslip. Several preparations were made using this technique but the phase contrast image was only fair.

A sucrose polymer, Ficoll, has been used by Hard and Allen (1977) to permit high quality differential interference contrast microscopy of *Haemanthus* endosperm cells. As this article appeared after most of the living cells studies reported here had been completed, the effect of Ficoll in supporting PMC culture and reducing puckering was not determined.

The best method for culturing PMCs proved to be a modified version of the agar coverslip technique of Molè-Bajer and Bajer (1963). This technique results in an excellent phase contrast image of PMCs, as judged by the clarity and sharpness of chromosomes and cytoplasm. The phase halo around the cells is minimal with this technique (Fig. 7). Furthermore, the cells remain stationary, a longer dissection time is possible and cell health is better than with other techniques.

3.21 Agar-coverslip technique

A thin film (20-30 μm) of hot (70-80°C) culture medium containing 0.75-1.0% agar (Davis) was spread on the inside of the coverslip window of the slide chamber. This was done by placing a drop of hot agar/medium on the coverslip using a pasteur pipette. The slide was then quickly tilted and rotated to spread the drop over the coverslip surface, and at the same time excess liquid was removed with the pipette. The procedure is not difficult but it must be done quickly or else thick (0.5-1.0 mm!) and uneven films will be obtained. Concentrations of agar that are lower than 0.75% result in films that are too soft, and concentrations greater than 1.0% result in thick films. If the agar is insufficiently hot (< 70°C) it sets too quickly and gives a thick film. Uneven films are obtained if the agar is too hot (> 90°C).

The coated slides were stored in coplin jars lined with moistened tissue paper for several hours until use. Initially, a second coverslip was sealed onto the top side of the culture chamber to prevent evaporation from the film but this was found to be unnecessary so long as the coplin

jar was covered. Also, condensation droplets of water usually formed on the agar layer if the slide chamber was covered with a second coverslip. These droplets are, of course, anisotonic to the PMCs and they cause immediate cell death.

Pollen mother cells were gently spread on the agar surface of the slide chamber and quickly covered with an inert oil. It is important to avoid breaking the agar film since this re-exposes a glass surface and puckering of PMCs will result. The use of 100X objectives was possible if the agar coating had been made sufficiently thin. Very thin No. 0 coverslips were sometimes used to aid further the use of this objective.

Iris and *Tradescantia* PMCs were usually cultured at $20^{\circ} \pm 2^{\circ}\text{C}$ and *Allium* PMCs were usually cultured at $24^{\circ} \pm 1^{\circ}\text{C}$.

The phase contrast image of PMCs was greatly improved if the cells were flattened. Flattening could be achieved in two ways. (a) The cells were spread on the agar as above but with slightly more medium than usual. Then the cells were covered with a (heavy) No. 2 coverslip, which was then covered with oil. (b) The spread cells were covered with a thick, heavy film of agar/gelatine (see section 3.31 below). With both methods it is the weight of the overlying layer that achieves flattening. Flattening was successful only with small PMCs such as those of *Tradescantia*, where the degree of flattening relative to the cell size was high. Flattening always resulted in arrested or abnormal anaphase, and when cells did enter anaphase I the chromosomes in the centre of the group usually moved further poleward than the rest. This may be due to the central chromosomes on a metaphase plate having been moved laterally to a lesser extent than marginal ones: a well flattened metaphase plate is twice as wide as an unflattened one.

3.22 Culture medium

A solution of sucrose at an appropriate osmolarity was sufficient to maintain cells in short term (usually < 5 hr) culture. The pH of the medium was usually 6.2. A substantially higher or lower pH (< 5.4 and > 6.6) sometimes resulted in division abnormalities. Various defined media (White, 1963; Ringer, A, Sharma and Sharma, 1965; Earles; Penso and Balducci, 1963) were tried but none gave any advantage over a sucrose solution.

Iris spuria and *Tradescantia flumenensis* PMCs were best maintained in a 0.22 M (7.5%) solution of sucrose. *Allium triquetrum* PMCs required a 0.28 M (9.5%) solution. These concentrations of media were determined solely by trial and error, and are at best a compromise since it was often a case of "having to strike a happy medium ...". Relevant points are as follows.

- (a) Even with a standardized technique it was often difficult to reproduce a "good prep."
- (b) In some species it was difficult to get the large number of preparations at the same stage (usually metaphase I) necessary for comparisons of media.
- (c) The optimum osmolarity varied with the stage of meiosis (see also Shimakura, 1934). For example, although *Allium* PMCs at metaphase I survived best in 0.28 M (9.5%) sucrose, metaphase II cells were best in 0.32 M (11%) sucrose.
- (d) The response of PMCs to culture varied at different times in the same plant (see also Molè-Bajer and Bajer, 1963). The culture of *Iris* PMCs was sometimes more difficult towards the end of the flowering season (late May) than culture early in the season (April), when the weather is warmer. *Iris* PMCs were difficult to culture for the entire 1978 season and this may have been due to an unusually cold flowering season. The optimum osmolarity for PMC culture in the 1978 season was lower than in previous years (0.19 M vs 0.22 M sucrose).
- (e) Short flowering seasons also limit comparisons.

These factors usually meant that once a medium was found which supported continuing division, little attempt was made to improve it.

3.23 Inert oils

Paraffin, Halocarbon and Voltalef oils cause differences in the quality of the phase contrast image of PMCs, due mainly to variations in their ability to cover cells. A small pool of liquid (mostly tapetal fluid) surrounds PMCs covered in paraffin oil and this causes a bright phase halo around the cells. The amount of liquid is reduced when Halocarbon oil is used, and further reduced with Voltalef oil, with corresponding improvements in image quality. The reason for the difference in degree of covering cells between different oil types is not known at present, but it may be related to differences in weight per unit volume of oil. In conjunction with the improvement in image quality is, unfortunately, a reduction in cell viability. This may be due to the reduction in the amount of tapetal fluid around each cell and a corresponding loss of its protective buffering.

3.3 ELECTRON MICROSCOPY

Prior knowledge of both the meiotic stage, and the exact plane of section, must be known in advance for the detailed study of the ultra-structure of dividing cells. Standard EM techniques for tissue preparation are therefore inadequate. Anthers cannot be fixed whole: PMCs must be fixed in a monolayer and attached to a flat surface, usually a coverslip.

Several methods for the flat embedding of cells are available (Rose, Pomerat, Shindler and Trunnell, 1958; Molè-Bajer and Bajer, 1968; Forer, 1972; Brinkley and Cartwright, 1971; Pleschkewych and Levine, 1971; Rattner and Berns, 1976; Roos, 1973a, 1976; Nicklas *et al.*, 1979). Several of these techniques were tried and satisfactory results were obtained with a modification of the agar sandwich method of Molè-Bajer and Bajer (1968).

3.31 Agar sandwich technique

The following paragraphs firstly outline the preparation of agar sandwich cell cultures and then several important features are elaborated.

Individual 22 x 22 mm coverslips were coated with agar as above (3.21) and stored until required in small petri dishes lined with moistened tissue paper. The contents of an anther were extruded into a small drop of 6% glutaraldehyde on an agar coated coverslip, and the cells were spread over agar surface. A thin film of agar (0.75%)/gelatine (0.75%)/sucrose medium was then layered over the cells, sandwiching them between two layers of gel on a coverslip. The film was made as follows. Hot melted agar/gelatine medium was maintained at 90-100°C in a waterbath until required. Approximately 5 ml of the mixture was then poured into a small petri dish. An elliptic wire loop (20 x 30 mm) was dipped into the still liquid medium and carefully removed, supporting a very thin film of agar gel in the manner of a child's soap bubble toy. Before complete solidification of the gel, the loop was applied on top of the coverslip preparation in a rolling motion. The preparation was then further processed for EM with no danger of losing the cells. The procedure requires some practice and the following points are noteworthy.

3.311 Timing is crucial. If the operation is done too quickly the film is too hot and it usually bursts on application to the cells. If it does not burst the hot agar may affect the quality of fixation. Slow preparation results in cold solidified films which do not stick to the bottom agar layer and the sandwiched cells may be subsequently lost. An ideal preparation time is 15-20 seconds.

3.312 The small petri dish was used to cool the melted gel partially for ease of film making. It was difficult to get a film formed on the loop if the agar solution was hotter than 90°C.

3.313 The loop was made of 0.3 mm steel or platinum wire. Thicker wires result in thick (hot) films.

3.314 The optimum size of the loop was 20 x 30 mm; smaller loops result in thicker films.

3.315 Gelatine was added to the gel to facilitate the formation of a film on the loop. A film of agar gel bursts too readily.

3.316 Certain types of gelatine and agar, when mixed together, form an annoying milky precipitate, which is visible around the embedded cells. The least amount of precipitate was formed when "exquisitely purified" Calbiochem agarose was mixed with Difco purified gelatine.

3.317 The amount of fixative used on the cells initially was small, since large amounts can prevent the sticking of the top agar gel to the bottom one. And, large amounts of fixative can cause shrinkage of the cells similar to the puckering of living cells described in section 3.2. Also, since only small amounts of the initial fixative were used, it is diluted by the tapetal fluid. For this reason, the initial concentration of glutaraldehyde used (6%) was twice that used in the subsequent step (section 2.261).

3.32 Correlative light and electron microscopy

The technique described here permits the continuous filming of a living cell followed by rapid fixation and eventual examination of the same cell in the EM. Agar sandwich preparations were made on 22 x 40 mm coverslips in exactly the same way as described in 3.31 above except that a drop of culture medium was used instead of fixative. The coverslip preparation was then sealed onto the lower surface of a perfusion chamber (Fig. 2, section 2.235) with petrolatum. The chamber was not filled with culture medium as is done by Molè-Bajer and Bajer (1968), since this usually reduces cell health. The phase contrast image was generally poor due to an uneven air-agar interface, but cells suitable for ciné-micrography could be found in most preparations. These cells were filmed, and were then fixed by depressing the plunger of the hypodermic syringe until a small quantity of 6% glutaraldehyde fixative flowed over the cells. It is important that the exit tubing is of wider internal diameter than the inlet tubing, as this prevents the build up of hydrostatic pressure. The coverslip preparation was then removed from the perfusion chamber and treated as described in section 2.26.

The technique was difficult and there does not appear to be any simple explanation for the rapid death of PMCs in some agar sandwich preparations. The points listed in section 3.31 above are relevant; and particular care must be taken to ensure thin films. The application of medium over the sandwich preparation can sometimes cause cells to die, but this is not always

so. Perhaps the top layer of gel reduces the amount of the protective buffer (tapetal fluid) around the cell. This would reduce cell viability. If this is so, a possible technical improvement might be to lower the concentration of agar in the top gel. However, this gives a layer of agar that is not solid enough to hold the cells onto the coverslip.

3.33 Flat embedding and photography of fixed cells

Coverslip preparations were flat embedded in a thin wafer of Araldite using silicone rubber embedding molds. These were made from templates of glass rather than of aluminium as used by Chang (1971). Templates for the recesses in the mold were simply stacks of several coverslips (No. 2, 18 mm diam. round). The use of glass templates gives the mold, and therefore the Araldite, an optically flat surface, improving the quality of photographs of the embedded cells (system devised by Mr A. Harris).

Cells embedded in the wafer of Araldite were photographed before coverslip removal as this provides the best image quality. Optimum optics were obtained by using an aluminium slide chamber (2.231) without the coverslip window. The hole in the slide was made slightly wider (19 mm diam.) than the diameter of the embedding mold (18 mm diam.) so that the molded Araldite wafer recessed in the hole. The advantages of this system are that it eliminates a glass-Araldite interface, and it brings the condenser closer to the specimen; both of these factors improve the image quality.

3.34 Coverslip removal

The coverslip was removed from the wafer of Araldite by floating the wafer, coverslip down, in a small pool of hydrofluoric acid for 5-10 minutes until all of the glass coverslip was dissolved (Moore, 1975). Prolonged exposure to HF softens the Araldite and the phase contrast image is lost temporarily, but apparently there is no damage to the cells (Nicklas, *et al.*, 1979). The softening can be remedied by placing the wafer in an oven at 60°C for several hours or overnight. This procedure for the removal of the coverslip is simple and quick, and no prior coverslip preparation, such as carbon coating (Molè-Bajer and Bajér, 1968) or teflon coating (Chang, 1971), is necessary.

3.35 Blocking up

Selected cells were circled with a Zeiss diamond objective scribe and cut from the Araldite wafer with a fine jewellers saw or a sharp scalpel. The small piece of Araldite containing the cell was then glued with epoxy glue onto the top of a peg of Araldite, with the cell in either longitudinal or transverse orientation.

A LKB pyramitome was used to trim the sides of the block. A perspex "lens" was used to concentrate light onto the block face so that the light was reflected to the viewer, making the scribed circle visible (system devised by Mr M.N. Loper).

3.36 Serial sectioning

Serial sections were cut with a diamond knife and were picked up using an adaptation of the method of Kubai (pers. comm. to G.K. Rickards, 1977), as follows.

A clean slide was lightly greased by wiping with a finger and placed in a separating funnel filled with a solution of 0.75% Formvar in dried chloroform. The funnel was drained leaving a thin coating of formvar on the slide. The thickness and evenness of the film was varied by controlling the flow of liquid from the funnel. After drying for 10 minutes the slide was scored with a sharp razor blade into approximately 6 mm squares, and lowered, at an angle of about 45° , into a trough of water so that the formvar floated off. Removal of the formvar was facilitated by the prior greasing of the slide and by breathing heavily on the slide before lowering it into the water. Each formvar square was picked up on a 5 mm diameter loop of copper wire, blotted dry, and arranged around a piece of balsa wood impregnated with wax (Fig. 4). (I have been told that the latter arrangement closely resembles a wigwam for a goose's bridle!) The loops were then coated with a thin layer of carbon (5 nm) in an Edwards evaporator. The presence of wax in the balsa wood shortens the time taken to reach high vacuum.

The presence of carbon on one side of the formvar renders it hydrophobic. This undesirable quality was remedied by washing the loop in a vial of detergent (4 drops per 10 ml distilled water), rinsing in distilled water, and draining until dry. Only good quality detergent (e.g. "Agepon", Agfa-Gevaert or "Decon-90" Selby Wilton) should be used. A ribbon of sections was then picked up on the non-carboned side of the formvar loop using a "third hand" (Fig. 5). When dry, the sections were lowered onto a single slot grid, which was laid on top of a rotatable peg (Fig. 6). Attachment of the film to the grid was aided by breathing onto it (non-smokers only!) and by carefully pressing the edge of the grid with the fingers. The sections were then stained and examined in the EM.

This technique differs from that of Kubai in that the formvar loops are coated with carbon before the sections are picked up. This is only possible if the loops are rendered hydrophilic as above, and has the major advantage that there is no danger of losing vital sections during carbon coating; also, the sections can be examined immediately.

3.4 ANALYSIS OF SERIAL SECTIONS

3.41 Longitudinal sections

Consecutive serial sections were traced onto clear cellulose acetate film using different coloured, felt tipped pens. Ultrafine tipped, water-proof, overhead projection pens (Faber-Castell-23F-OH-Permanent) were the most suitable as they gave intense colour and a fine line. Different cellular components were traced in different colours: black for chromatin, blue for kinetochores, red for microtubules and green for membrane. Each tracing in a series was stacked in order and in register, and was separated by a sheet of perspex (1.5 mm thick) or glass (2.0 mm thick). Each sheet approximates the thickness of the sections at the magnifications used, and one between each tracing was used to obtain the best 3D effects. It was possible to rotate and examine the stack of tracings at all angles, when held against a strong light. (I am grateful to Prof. J.K. Heyes for suggesting this technique). A similar technique has recently been used by Fuge (1980). Perspex was inferior to glass in most respects, since it is easily scratched and tends to have a light scattering effect when a large number of sheets are used in a stack. Glass plates were normally used for photography. A photofloodlight was placed about 500 mm under a sheet of diffusing glass. The stack of tracings was placed on top of the glass and photographed on Kodacolour II negative film with an Asahi Pentax camera. The number of glass plates was reduced for photography to give a sharper image; and the order of the tracings was sometimes reversed to simulate focussing on the lower surface.

An alternative method involves arranging the tracings several millimeters apart on a framework and lowering the lot into a large tank of water (Jordan and Saunders, 1976). The system works well enough but it is cumbersome and the tracings cannot be easily rotated and examined from all sides. Other methods entail either the building of a physical model by cutting out the tracings in cardboard (Sotelo, Garcia, and Wettstein, 1973), polystyrene (Murray and Davies, 1979) or plywood (Pellegrini, 1980), or computer facilitated MT tracking (McDonald, Edwards and McIntosh, 1979).

3.42 Microtubule counts from transverse sections

Twelve to 15 transverse sections at various positions in the spindles were analysed for total MT numbers. Only clean, dirt free sections were chosen: in my hands a somewhat random occurrence! Large montage electron micrographs of each transverse section were taped onto a board and covered with a sheet of plastic, which was also taped to the board. A felt tipped

pen was used to put a dot on the plastic over each MT profile and counting was done using a hand tally counter. The face of the counter was masked off to avoid any bias in the final results, sections were counted out of order, and the counters were not unmasked until all counts for a particular spindle were completed. Several points arising from the analysis are as follows.

- (a) A MT is circular in transverse section (see Fig. 23) but if it is cut obliquely, as, for example, in regions of MT convergence to the pole, its profile can be figure 8 shaped. All such MTs were counted as one MT.
- (b) Although MTs are of uniform size and electron density in any one section, other structures, especially small vesicles can be confused with MTs. All MT counts were done by myself so that any errors in MT identification should be consistent from section to section.
- (c) Variations in section thickness and section staining can cause differences from micrograph to micrograph in the clarity and sharpness of MTs.
- (d) The negative magnification was usually 7-9000X and each negative was enlarged 3-4X. Photography at higher magnifications resulted in very large cumbersome montage electron micrographs which did not give greatly increased resolution.

3.43 Estimates of the relative area of membrane in the spindle

Two methods were used.

- (a) The outline of each profile of ER in the spindle was traced onto clear acetate film using a felt tipped pen, and the area enclosed by each profile was determined using a cartographers planimeter. The total area enclosed by membrane within the spindle was then expressed as a percentage of the total spindle area. The method is extremely laborious and probably inaccurate since the error in measuring each piece of ER is additive.
- (b) A much easier method is to use a leaf area meter (LAM) (I am grateful to the DSIR for the use of this equipment). The outline of all the spindle ER was traced as above, and the enclosed area was blacked in with a felt tipped pen. The tracing was then photocopied onto a transparency to give it a more uniform blackness. Similarly, the spindle outline was cut out in black paper to give an estimate of the total area of the spindle. The cut out was also photocopied onto a transparency so that it was composed of the same degree of blackness as the sample. The machine then measures the amount of blackness in both samples and the area of membrane in the spindle is given as a simple fraction. The accuracy of the scanner was estimated to be $\pm 2\%$; see section 4.232).

3.5 PROTOPLAST ISOLATION

Agar sandwich preparations of *Allium* PMCs were made as described in section 3.32, and were mounted in the perfusion chamber. The hypodermic syringe was filled with 0.75% Helicase (Industrie Biologique Francaise 5A, Gennevilliers, France) enzyme solution in sucrose medium, instead of fixative. The solution was perfused over the cells and protoplasts were isolated 15-30 minutes later. The enzyme solution was then replaced with fresh sucrose solution. Living protoplasts are easily distinguished since dead ones quickly lose their spherical shape and the cytoplasm becomes quite granular. Viability could also be determined with phenosafranine stain (Widholm, 1972), which specifically stains dead cells.

3.6 PROSPECTS FOR IMPROVEMENT IN TECHNIQUES

3.61 Living cell culture

The synthesis of a new, defined culture medium is a mammoth task even for somatic cells, which are available all the year round. Even the modification of existing defined media (e.g. White, 1963) for PMC culture is impractical for reasons listed under 3.22 above. The best approach at present seems to be to find species which can be cultured easily in sucrose solution, rather than to attempt to culture "uncooperative" cells.

Since osmotic requirements change during meiosis (3.223 above) it may be possible to change the medium concentration gradually by perfusion (3.32), if culture of PMCs through both meiotic divisions is required.

The sucrose polymer, Ficoll, could possibly be used instead of an agar layer, to eliminate the puckering of PMCs spread directly on a glass coverslip. This technique might also afford a higher quality phase contrast image than that obtained in the present studies.

Plants could be grown in controlled conditions in a glasshouse and, as with *Haemanthus* (Molè-Bajer and Bajer, 1963), it may be possible to induce flowering all the year round.

3.62 Electron microscopy

There is always scope for improvements in the quality of EM fixation. In particular, the newly developed PIPES buffer (Salema and Brandao, 1973; Baur and Stacey, 1977) could replace cacodylate buffer and may result in better fixation, especially of membranes. A lower concentration of osmium tetroxide (e.g. 0.025%) could reveal actin filaments in the spindle if any are present (Maupin-Szamier and Pollard, 1978). The dehydration procedure of Lin, Falk, and Stocking (1977) using 2,2-dimethoxypropane or 2,2-diethoxypropane could be used to dehydrate cells more rapidly than with a graded series of alcohols.

An improvement in the living cell culture medium may increase cell viability in agar sandwich preparations for correlative living cell and electron microscopy.

Chapter 4. USE OF THE NEW TECHNIQUES : SOME OBSERVATIONS ON LIVING AND
FIXED CELLS

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Chapter 4. USE OF THE NEW TECHNIQUES : SOME OBSERVATIONS ON LIVING AND FIXED CELLS

The approach taken in these studies was not to examine comprehensively one particular aspect of meiosis, but rather to use the techniques which have just been described to provide a base for future studies. Once this base is established, detailed studies of individual features of meiosis will then be possible. Future living cell study of meiosis in chromosome mutants, such as interchange heterozygotes (Rickards, 1977), clearly requires the prior establishment of both adequate technical procedures and the living cell study of meiosis in normal cells. Similar requirements also apply to the study of the spatial relationships between kinetochores and the rest of the bivalent in the first division of meiosis, for example. Again, the basic arrangement and structure of kinetochores must first be described. The relationship between kinetochores and the rest of the bivalent is a fascinating but unanswered question, and it will be examined in more detail later (section 5.3) to provide a specific example of the direction along which subsequent studies might be focussed.

4.1 LIVING CELL STUDIES

The maintenance of living PMCs in slide culture is an exacting business, although once the techniques have been thoroughly practised good preparations of dividing PMCs from favourable species can often be obtained. Three species were found to be satisfactory for living cell culture: *Iris spuria*, *Allium triquetrum* and *Tradescantia flumenensis*.

4.11 Anaphase I and II

4.111 Anaphase I and II in *Iris spuria*

A number of PMCs were filmed during various stages of meiosis and two representative series of still photographs taken from ciné-films of *Iris* PMCs are shown in Figs. 7 and 8. The cell in Fig. 7 progressed from metaphase I to tetrad formation over a 20 hr culture period. The spindle region is commonly seen as a zone of uniform grey, somewhat darker than the surrounding cytoplasm (Fig. 7, 0 min print). Anaphase I lasted approximately 40 minutes in this cell and there was no apparent polar separation (spindle elongation) when the film was projected at ordinary speed. The daughter nuclei rounded up during telophase I and granular cytoplasm moved into the interzone region between the nuclei. The end of a four and a half hour interphase was marked by the rapid (two to four minutes) recondensation of chromosomes at prophase II (5 hr 34 min print). The second division of

Fig. 7. Living PMC; *Iris spuria*. Metaphase I to tetrads. Time in hours and minutes. Arrowheads in 0 min print outline the edge of the spindle. Scale = 10 μ m.

Fig.8. Living PMC; *Iris spuria*. Prophase II to telophase II. A nucleolus is arrowed in 0 min print. Scale = 10 μ m.

meiosis was not particularly clear in this cell since chromosomes tend to lose contrast after three to four hours in slide culture. Cell walls appeared around the four tetrad nuclei some five hours after telophase II and complete walls are visible in the 20 hr print.

A clearer view of the second division of meiosis can be obtained by initiating slide culture at prophase II (as in Fig. 8). A nucleolus is arrowed in the 0 min print and, as far as could be determined, it faded away before prometaphase II chromosome movements began. Attempts to verify this point in other cells have been unsuccessful due to difficulties encountered in culturing prophase II cells. If more positive results had been obtained here, the presence of a nucleolus in electron micrographs of a prometaphase II spindle would unequivocally indicate that chromosome movements had not begun in that cell at the time of fixation (section 4.34). The chromosomes in the lower spindle in Fig. 8 became aligned at the equator much earlier than those in the upper spindle (1 hr, 17 min print), but both spindles entered anaphase II synchronously. Anaphase II lasted about 30 min in this cell.

4.1111 Ciné-film analysis of anaphase I

Graphs of anaphase chromosome movements in the cell in Fig. 7 are presented in Figs. 9a and b (see section 2.25 for details of analyses). Anaphase chromosome movement can be represented graphically in two ways. The first is to plot the distance between the kinetochores of each pair of half-bivalents during anaphase I with time (Fig. 9a). The second method is to graph the positions of the two poles and then to plot pole-to-kinetochore distance (Fig. 9b).

The first method of presentation is particularly useful in providing velocity data when the positions of the spindle poles are difficult to determine (as they are in the cell in Fig. 7). However, it does not provide information on the relative contribution (if any) of spindle elongation to chromosome separation during anaphase.

The second method of presentation shows both kinetochore-to-pole movement and the position of the poles during anaphase. Hence spindle elongation data can be obtained from graphs of this sort. In this analysis, a spindle pole was defined as: a line parallel to a line drawn through the approaching anaphase kinetochores, and this pole line was positioned at the point of the change in contrast between the spindle and the surrounding cytoplasm. This definition was considered the most appropriate since there is no single point of focus in the pole of anastral spindles. The spindle pole actually consists of an aggregation of membrane

Fig. 9. *Iris spuria*. Graphs of anaphase I movement in cell 10-1 (Fig. 7).

- a. Interkinetochore distance. Graphs are drawn for four of the six bivalents analysed. Two of the four bivalents were in the centre of the metaphase plate and two were on the edge of the plate.

Fig. 9. b. Pole-to-kinetochore distance. Data for six half-bivalents pooled.

FIG.9a, IRIS. INTERKINETOCHORE DISTANCE. ANAPHASE I FIG.9b, IRIS. POLE-TO-KINETOCHORE DISTANCE. ANAPHASE I

(Hepler, 1980); therefore the assignation of a single point as the pole would be meaningless. Also, of course, even in astral spindles, the chromosomes do not completely converge on the aster centre as if it were some sort of black hole! In the film analysis there are unavoidable fluctuations in the localization of the poles, due mainly to differences in image clarity from photograph to photograph. Any error in the identification of the spindle poles would have been translated into both halves of the spindle during the plotting of the pole graphs, since the graphs were drawn as mirror images about the equator (the equator was defined as a straight line graph). At the same time, however, the error would be halved. In Fig. 9b, deviation from a line drawn by eye through the pole points is slight, and identification errors are thought to be negligible.

The distance from the kinetochore to the pole line was measured along the actual path of kinetochore movement. The path of movement was found by projecting the film of the anaphase backwards and forwards several times; thus this measurement gives the actual velocity of the movement. If the measurements had been made to a single pole point, the data would have had to be corrected (Fuseler, 1975).

Graphs of the kinetochore-to-kinetochore distance for four bivalents at various positions on the metaphase I plate are shown in Fig. 9a. All four graph lines have the same slope; i.e. each kinetochore pair separates at the same velocity. The slopes of all graphs were always measured over a five to ten minute period during mid-anaphase when the chromosome velocity was more-or-less constant. The mid-anaphase I separation velocity for each pair of half-bivalents in this cell was $1.1 \mu\text{m}/\text{min}$, and the actual velocity of each half-bivalent is therefore $1.1 \div 2 = 0.55 \mu\text{m}/\text{min}$.

The positions of the kinetochores were averaged for each half-spindle in Fig. 9b. This procedure was considered to be valid because all half-bivalents moved at the same rate, and variation in the size of the bivalents at metaphase I was slight (Fig. 7, 0 min print). Other *Iris* PMCs which have been studied also show no variation in anaphase I chromosome velocity from chromosome to chromosome (see appendix 1). There is a small difference in kinetochore-to-pole velocity in the sister half-spindles in Fig. 9b (0.60 versus $0.50 \mu\text{m}/\text{min}$), but the mean velocity for the whole spindle is the same as that derived from Fig. 9a ($0.55 \mu\text{m}/\text{min}$). A small difference in velocity between sister half-spindles also occurred in another of the cells analysed (Table 1).

As can be seen in Fig. 9b, there was no change in pole-to-pole distance during the first 30 minutes of anaphase I, but during the last 10 minutes there was a 15% increase in the interpolar distance in association with the slowing of chromosome movement in late anaphase I. Minor (16%) polar elongation can also be seen in cell 16-10 (Table 1, and appendix 1); polar elongation could not be estimated in cells 10-9 and 17-10 (cell 10-9 died at late anaphase I and the poles could not be distinguished in cell 17-10).

Chromosomes converged laterally as they approached the poles during anaphase I (compare the width of the chromosome group in the 0 min and 1 hr prints in Fig. 7), yet, as has been noted above, all chromosomes moved at the same velocity. Since laterally positioned chromosomes kept level with chromosomes in the centre of the anaphase group (Fig. 7), even at late-anaphase I, it follows that a laterally positioned chromosome must move faster than a chromosome on the spindle axis. All the chromosomes in Fig. 7 could in fact be marginal chromosomes, if the level of focus was through the edge and not through the centre of the spindle. However, it is unlikely for six to seven bivalents to all be at the same level of focus in other than median optical sections in spindles which only have 24 bivalents. Also, in all *Iris* cells studied, chromosomes on the side of the anaphase group always kept up with central ones, and it is unlikely for the level of focus in all these cells to always be on the edge of the spindle.

How much faster than a central chromosome might a lateral one move? An estimate was derived from the graphs of the decrease in width of the chromosome group during anaphase I (Fig. 9c). The rate of decrease in width in the upper half-spindle is $0.12 \mu\text{m}/\text{min}$. A chromosome on the edge of the anaphase I group therefore has a lateral velocity vector of $0.06 \mu\text{m}/\text{min}$, which is an order of magnitude less than the observed poleward velocity. Assuming a poleward velocity of $0.6 \mu\text{m}/\text{min}$, then, by simple trigonometry, the increase in poleward velocity which would be required for a lateral chromosome to keep level with central chromosomes is only $0.002 \mu\text{m}/\text{min}$. The methods of ciné-film analysis used here could not possibly resolve a variation in velocity as small as this. These observations confirm that no bias was introduced by averaging kinetochore position in constructing Fig. 9b.

Mid-anaphase I chromosome velocities vary between 0.45 and $0.60 \mu\text{m}/\text{min}$ (Table 1a), with a mean for all cells of $0.55 \mu\text{m}/\text{min}$. Cell 16-10 was prepared with the agar sandwich technique (Ch. 3, section 3.31), but was not fixed and was allowed to progress to telophase I. This cell, therefore, serves as a control for correlative living cell and EM studies to be described later. In this cell chromosome velocities (0.45 and $0.55 \mu\text{m}/\text{min}$) and spindle elongation (16%) (see also appendix 1) are similar to those obtained from cells in standard preparations, suggesting that the agar

Fig. 9. *Iris spuria*; anaphase I in cell 10-1 continued.
c. Width of chromosome groups.

Fig. 10. *Iris spuria*. Graph of anaphase II movement in
spindle 19-3.2 (Table 16).

FIG.9c, IRIS. WIDTH OF CHROMOSOME GROUPS. ANAPHASE I FIG.10, IRIS. POLE-TO-KINETOCHORE DISTANCE. ANAPHASE II

Table 1a. Anaphase I chromosome velocities in *Iris* PMCs.

Cell No.	Mid-anaphase chromosome velocity ($\mu\text{m}/\text{min}$)		Percent spindle elongation	Time in culture before anaphase onset	Culture temperature	see Figure
	first half spindle	second half spindle				
10-1	0.60	0.50	15	2 min	20°C	7, 9a-c appendix 1
10-9	0.55	0.55	-	13 min	20°C	
16-10	0.45	0.55	16	30 min	20°C	
17-10	- 0.60* -		-	4 hr	22°C	

Table 1b. Anaphase II chromosome velocities in *Iris* PMCs.

Cell No.	Mid-anaphase chromosome velocity ($\mu\text{m}/\text{min}$)		Percent spindle elongation	Time in culture before anaphase onset	Culture temperature	see Figure
	first half spindle	second half spindle				
10-1	- 0.28* -		-	10 hr	20°C	appendix 2
16-1.1	0.50	0.45	0	3 hr	19°C	
16-1.2	0.50	0.35	0	3 hr	19°C	
19-3.1	0.55	0.60	0	1½ hr	20°C	10
19-3.2	0.80	0.70	0	1½ hr	20°C	

* Measured from a graph of the rate of kinetochore separation.

sandwich technique does not significantly alter the process of anaphase I from that in standard preparations.

Culture temperature was kept constant to within 2°C (Table 1a) and thus variation in temperature is not a source of variability in chromosome velocities. Similarly, the time in culture before anaphase I onset varied considerably in these cells (Table 1a) but there is apparently no corresponding effect on anaphase chromosome velocities.

4.1112 Ciné-film analysis of anaphase II in *Iris spuria*

A typical anaphase II is graphed in Fig. 10, and Table 1b summarizes observations of anaphase II in *Iris*. Individual anaphase II kinetochores are usually difficult to distinguish, especially during early anaphase II. This is because chromatid arms extend polewards and tend to obscure kinetochores. Thus, in the analysis of this cell, a line was drawn through the estimated kinetochore positions, and pole-to-kinetochore distance was measured along the interpolar axis. This method of analysis was considered valid since a straight line could always be drawn through all the kinetochores in the anaphase group. Also, no variations in velocity, such as chromosome lagging, could be detected when the film was projected. Further support for this assumption comes from cell 16-1 (Table 1b and appendix 2), which was cultured with the agar sandwich technique. Individual kinetochores were clear enough in this cell to permit the movement of several chromosomes to be graphed. Velocities were the same for each chromatid in each half-spindles, and the lateral vector was negligible.

Anaphase II velocities were of a similar rate to those of anaphase I (mean for all anaphase II cells = $0.50\ \mu\text{m}/\text{min}$), but were generally more variable than in anaphase I (compare Tables 1a and 1b). As noted above for anaphase I, there was often some variation in mean mid-anaphase II velocities between sister half-spindles. No polar elongation has been observed in any anaphase II spindle studied, and the chromosomes do not move as close to the poles as they do in anaphase I.

Cell 16-1, as mentioned above, was prepared with the agar sandwich technique and no significant difference in mean velocity was detected in either spindle from similar cells prepared with the standard technique.

Culture temperatures were not a source of variability in chromosome velocities (Table 1b). The time in culture before anaphase II onset, with one exception, had no apparent effect on velocities. However, cell 10-1 (Fig. 7) was cultured for 10 hours before anaphase II began, and in this cell the mean anaphase velocity was less than in other cells.

4.112 Anaphase I in *Allium triquetrum*

Figure 11 illustrates anaphase I in a living *Allium* PMC and Fig. 14a is a graphical presentation of the chromosome movements in the same cell. For clarity, both the vertical and horizontal axes have been doubled in size from those used in Fig. 9b.

The spindle poles are situated close to the cell wall at metaphase I (arrow at lower pole, 5 min print, Fig. 11; see also LP electron micrograph, Fig. 40a). Spindle elongation cannot, therefore, occur during anaphase I in *Allium*, and none was observed in any of the cells studied (Table 2).

The culture temperature was maintained at 23-25°C in all but one of the cells studied (cell 11-3; 18°C). The mean mid-anaphase velocities in cell 11-3 were similar to those in other cells cultured at 23-25°C (e.g. cell 8-5b), and a much greater difference in velocity was observed among the first four cells in Table 2 (all cultured at 25°C) than was observed between cells 11-3 and 8-5b. Thus, if culture temperature does have an effect on anaphase chromosome velocities (Fuseler 1975b), its effect must be trivial in comparison to the natural variation observed among different cells.

There is no relationship between anaphase I velocities and time in culture before anaphase onset (Table 2). Cells 8-3 and 8-5a had similar chromosome velocities yet the former was cultured for 1½ hours before anaphase I began, while the latter was cultured for only 3 minutes. Similarly, cell 7-1 was cultured for 4½ hours before anaphase onset yet chromosome velocities in this cell are in the centre of the observed range.

Comparison of Figs. 9b and 14a, and Tables 1 and 2, show that anaphase I chromosome velocities are of a similar rate in both *Allium* and *Iris* (mean mid-anaphase I velocities were both the same: 0.55 µm/min.), although, as in *Iris*, there is often some variation (0.1 - 0.2 µm/min.) in velocities between sister half-spindles. In *Allium*, anaphase I lasts about 15-25 minutes and the chromosomes travel about 7-10 µm, while in *Iris* the comparable figures are 30-40 minutes and 15 µm respectively.

Anaphase I chromosome velocity varied with the position of the chromosomes on the metaphase plate in the cell in Fig. 11. This is shown in Table 3 below.

Fig. 11. Living PMC; *Allium triquetrum*. Metaphase I to telophase I. Four bivalents are numbered in the 5 min print; and in the 15 min print the two arrows indicate the positions of the kinetochores of half-bivalents 3 (upper arrow) and 4 (lower arrow). The position of the lower spindle pole is arrowed in the 5 min print. Scale = 5 μ m.

Fig. 12. Living PMC; *Allium triquetrum*. Metaphase II to telophase II. Scale = 5 μ m.

Fig. 13. Living PMC; *Tradescantia flumenensis*. Metaphase I to interphase. The dark spot to the right of the spindle equator is a speck of dust on the camera lens. Note the phase bright granules surrounding the spindle area. Scale = 5 μ m.

Fig. 14. *Allium triquetrum*. Graphs of anaphase I in cell 3-1 (Fig. 11).

- a. Pole-to-kinetochore distance. Individual graphs for bivalents 1 to 4 of Fig. 11 are shown.

FIG4a, ALLIUM POLE-TO-KINETOCHORE DISTANCE ANAPHASE I

Table 2. Anaphase I chromosome velocities in *Allium* PMCs.

Cell No.	Mean mid-anaphase velocity ($\mu\text{m}/\text{min}$), range () where applicable		Percent difference in velocity between marginal and central chromosomes		Anaphase group bowing	Percent spindle elongation	Time in culture before anaphase onset	Culture Temp.	Figure
	first half spindle	second half spindle	first half spindle	second half spindle					
3-1	0.95 (0.75-1.10)	1.05 (0.75-1.40)	27% slower	9-46% (mean 27%) slower	away from pole	0	14 min	25°C	11,14
3-3	0.25	0.25	-	-	none	0	20 min	25°C	
7-1	0.60	0.85	-	-	none	0	4½ hr	25°C	
8-3	0.35	0.40	-	-	none	0	1½ hr	25°C	
8-5a	0.35 (0.30-0.45)	0.55 (0.40-0.65)	50% faster	42% faster	towards pole	0	3 min	23°C	
8-5b	0.50	0.50	-	-	none	0	20 min	23°C	
11-3	0.50	0.40	-	-	none	0	2 min	18°C	

Table 3. Anaphase I chromosome velocities. *Allium* cell 3-1*

Half spindle	Chromosome No.**	Velocity ($\mu\text{m}/\text{min}$)	Mean velocity ($\mu\text{m}/\text{min}$)
Upper	1	0.75	0.95
	2	1.10	
	3	1.10	
	4	0.80	
Lower	1	1.00	1.05
	2	1.10	
	3	1.40	
	4	0.75	

* Data from Fig. 14a

** See Fig. 11, 5 min print.

The laterally positioned chromosomes 1 and 4 (see Figs. 11 and 14a) moved more slowly than the centrally positioned ones (2 and 3) in both half spindles. Since measurements were made along the actual paths of movement, these data reflect real differences in velocity. Indeed, the differences are visible when the film is projected because laterally positioned chromosomes lag behind the central ones (bow away from the pole; Fuseler, 1975a). In Fig. 11, 5 min print, the kinetochores of bivalents 3 and 4 are at approximately the same levels in both half-spindles, but at mid-anaphase I half-bivalent 3 is about 2 μm in front of half-bivalent 4 in the upper half-spindle (arrow 15 min print). Note also that the short half-bivalent 2 catches up and passes half-bivalent 4.

As was noted in the analysis of anaphase I in *Iris*, all the chromosomes in Fig. 11 could actually be marginal in position if the level of focus were through the edge of the spindle. But four bivalents (from a total of nine), are visible in the one plane of focus in this cell (Fig. 11). This fact suggests that, as with *Iris*, a median optical section has been obtained, and the "central" chromosomes above are indeed central in the spindle.

The lateral velocity vector derived from chromosome convergence during anaphase I, will, in theory, make some contribution to the observed chromosome bowing away from the pole. The rates of convergence are 0.70 and 0.60 $\mu\text{m}/\text{min}$ for the upper and lower spindles of this cell (Fig. 14b), i.e. lateral vectors of 0.35 and 0.30 $\mu\text{m}/\text{min}$ respectively. These values are about five times as great as those in *Iris*. If we assume for the moment that all chromosomes move at the same velocity during anaphase I in this cell (say 0.95 $\mu\text{m}/\text{min}$, Table 3 above), then a vector diagram incorporating both poleward and lateral vectors can be drawn (Fig. 14c). From this diagram it can be seen that a laterally positioned chromosome should lag behind a central chromosome by 0.05 μm every minute. Anaphase I begins at about +7 minutes in

Fig. 14. *Allium triquetrum*, anaphase I in cell 3-1 continued.
b. Width of chromosome group.

Fig. 14. c. Vector diagram. See text for details.

FIG.14b, ALLIUM ⁶⁵.

WIDTH OF CHROMOSOME GROUP
ANAPHASE I

FIG.14c, ALLIUM. VECTOR DIAGRAM, ANAPHASE I

the cell in Fig. 14a, so that by +15 minutes a lateral chromosome should be $0.05 \times 8 = 0.40 \mu\text{m}$ behind a chromosome on the spindle axis. This is only 20% of the observed $2.0 \mu\text{m}$ lag indicated in Fig. 11. Thus, it is obvious that the major contributor to the bowing of chromosomes away from the pole in anaphase I in this cell is variation in chromosome velocity.

Table 2 includes a summary of comparable observations of anaphase I in other *Allium* PMCs. In one cell (8-5a) the lateral chromosome moved faster than the central ones and this resulted in bowing of the chromosome groups towards the pole, not away from it, as in the cell just described. In the five other PMCs studied, no differences in anaphase I velocities were observed between marginal and central half-bivalents (Table 2 and appendix 3). Hence, there may be no relationship between chromosome velocity and position on the spindle.

4.113 Anaphase II in *Allium triquetrum*

Anaphase II in a living PMC of *Allium* is illustrated in Fig. 12 and a graph of chromosome movements in the same cell is shown in Fig. 15. For clarity, data from three chromosomes were pooled in this graph, since no differences in mid-anaphase velocity were detected when each graph was drawn individually (see appendix 4).

The spindle pole in the second division of meiosis in *Allium* is located in the cytoplasm and approximately 40% spindle elongation was observed during mid-to-late-anaphase in this cell. Chromosome velocity (mean = $0.55 \mu\text{m}/\text{min}$) was of a rate similar to that in anaphase I, and anaphase II lasted approximately 15 minutes.

4.114 Anaphase I in *Tradescantia flumenensis*

Pollen mother cells of *Tradescantia* were cultured and filmed during anaphase I (Figs. 13 and 16). The positions of the spindle poles were impossible to determine in this material due to the large cytoplasmic granules which surround and partially cover the spindle, and hence only graphs of kinetochore separation can be presented. This meant that the extent of polar elongation could not be determined in *Tradescantia*. As in anaphase II of *Iris*, the positions of individual kinetochores were difficult to distinguish, and consequently kinetochore positions were averaged (Fig. 16).

Fig. 15. *Allium triquetrum*. Anaphase II in cell 5-3 (Fig. 12). Pole-to-kinetochore distance. Data from three chromosomes pooled.

Fig. 16. *Tradescantia flumenensis*. Anaphase I in cell 14-5 (Fig. 13). Interkinetochore distance. Data from four bivalents pooled.

FIG.15, ALLIUMPOLE-TO-KINETOCHORE DISTANCE
ANAPHASE II FIG.16, TRADESCANTIAINTERKINETOCHORE DISTANCE
ANAPHASE I

The mid-anaphase I velocity calculated from Fig. 16 is $0.6 \div 2 = 0.30 \mu\text{m}/\text{min}$. In the only other cell with a sufficiently clear anaphase I, the mid-anaphase I velocity was $0.35 \text{ m}/\text{min}$. These values are again similar to those obtained in *Iris* and *Allium*. Anaphase I in *Tradescantia* lasts about 20 minutes. Anaphase II was not studied in this species.

4.114 Summary

A summary of anaphase I and II in *Iris*, *Allium* and *Tradescantia* are presented in the following tables.

Table 4a. Anaphase I.

Species	Mean Velocity ($\mu\text{m}/\text{min}$)	Spindle Elongation	Anaphase Duration(min)	Distance Travelled (μm)
<i>Iris</i>	0.55	15%	40	15
<i>Allium</i>	0.55	0	15-25	10
<i>Tradescantia</i>	0.35	n.a.	20	5

Table 4b. Anaphase II

Species	Mean Velocity	Spindle Elongation	Anaphase Duration(min)	Distance Travelled (μm)
<i>Iris</i>	0.50	0	35	15
<i>Allium</i>	0.55	40%	15	7

Mean mid-anaphase velocities are quite similar, both between the first and second divisions within each species, and between different species.

There is no spindle elongation in anaphase I of *Allium* but 40% elongation in anaphase II. In *Iris*, spindle elongation has a minor role in chromosome separation in both divisions.

The duration of anaphase is of course proportional to the distance travelled and to chromosome velocity. Thus, when velocities are the same in *Iris* and *Allium* the duration in the latter is half that of the former because the distance travelled is half.

4.12 Prophase and prometaphase I

Pollen mother cells at prophase and prometaphase I were difficult to culture successfully in all species studied, but it was occasionally possible to keep some cells alive for several hours. Prophase I was recorded in *Allium* PMCs in four cases. Two of these cells remained alive for approximately four hours, but both died before they entered prometaphase I. The other two cells entered prometaphase I after 30 and 60 minutes respectively, and both subsequently entered anaphase I. Prophase I was examined in all four cells for prophase chromosome movements (Rickards, 1975, 1981 in press) but no movements other than Brownian motion of chromosomes and saltatory (stop-start) rotation of the whole nucleus were observed (compare the arrowed bivalents in the 0 and 21 min prints, Fig. 17). A detailed description of prometaphase I, based on only two cells, is obviously impossible (compare, for example, the extensive studies of Roos, 1973a, 1976, on prometaphase in mitotic PtK₂ cells); so a brief description only is given.

After the breakdown of the nuclear membrane (nm) (34 min print, Fig. 17) the bivalents moved to the central region of the developing spindle (50 min print). Most bivalents achieved immediate bipolarity but in both cells some bivalents moved firstly to a pole and back to the equator at approximately anaphase velocity (52 to 90 min prints). Most bivalents moved a short distance back and forth along the interpolar axis during mid- and late-prometaphase I (congressional movements, Nicklas, 1971) and metaphase was reached in both cells some 50 minutes after nm breakdown. Filming was abandoned in the cell in Fig. 17 after about two and a half more hours due to fading chromosome contrast and anaphase I was not recorded, although the cell was observed to be arrested in interphase 12 hours later. Filming continued in the second cell despite the loss of chromosome contrast, and a very faint anaphase I can be observed in the projected film after four hours in metaphase I. Parenthetically, lengthy (and frustrating) delays of up to four hours before anaphase I were often encountered when culture and filming was initiated at metaphase I.

4.13 Protoplast Isolation

Protoplasts of PMCs were isolated and cultured in a perfusion chamber as described in section 3.6. Protoplasts of tetrad cells could be easily and reproducibly isolated with 80-100% yield and it was possible to observe the isolation process continuously (Fig. 18a). Once the protoplasts were free of their enclosing cell wall they immediately assumed a completely spherical shape.

All attempts to isolate PMCs at earlier meiotic stages failed (Fig. 18b).

Fig. 17. Living PMC; *Allium triquetrum*. Prophase I to metaphase I. Two bivalents (white arrows, 0 min to 34 min prints) remain in the same position until the breakdown of the nm (34 min). The right hand bivalent moves to the upper pole (double arrows 52 and 62 min prints) and then moves back to the equator (65 min). The left hand bivalent moves out of the plane of focus. Scale = 5 μ m.

Fig. 18. Protoplast isolation; *Allium triquetrum*.

a. PMC at tetrad stage.

b. PMC at metaphase I. The cell "explodes" at 4.7 min.
Scale = 5 μ m.

4.14 Living cell and correlative electron microscopy in *Iris*

The technique for ciné-micrography of a particular living cell, followed by *in situ* fixation and examination of the same cell in the EM, is described in chapter 3, section 3.32. The technique is difficult but it is possible to fix a PMC in mid-anaphase I and examine it in the EM (Fig. 19a and b). This cell was fixed rapidly and anaphase I chromosome motion ceased within one minute. A control cell, prepared in exactly the same way but not fixed in mid-anaphase I, continued to divide in a manner that was indistinguishable from anaphase I in standard preparations (section 4.1111). Correlative LM and EM of this nature is no more advantageous to the EM study of meiotic stages than the selection of particular cells from a coverslip preparation of fixed cells (sections 3.33-3.36). However, the method does provide cells at an otherwise rare stage: early anaphase I, and there is the certain knowledge that the PMC was healthy and actively dividing immediately prior to fixation. The advantage of correlative LM and EM lies in its potential for the study of individual chromosome movements, particularly in prometaphase I cells. An attempt to realize this potential was not pursued because of a lack of detailed documentation of prometaphase I in living *Iris* PMCs.

4.2 ELECTRON MICROSCOPY OF PMCs

4.21 Fixation

Fixation was variable in *Allium*. In the metaphase I cell in Fig. 40a the ER is in a closed tubular or plate-like form and MT numbers are low. There are very few nkMTs, and kMTs are surrounded by a cloud of electron dense material. Another metaphase I cell of *Allium* (Fig. 40b) has open ER and many more MTs than the cell in Fig. 40a. Both nkMTs and kMTs are present in the second cell, the MTs are more lightly stained and there is no perimicrotubular cloud of dense material. The general fixation of this cell appears to be poor. Other limited observations in *Allium* support a correlation between well fixed (closed) ER and low numbers of MTs (especially nkMTs, which are more labile; Brinkley and Cartwright, 1975); and poorly fixed (open) ER with higher numbers of MTs.

In *Iris*, fixation was more uniform with high numbers of MTs of both types, and open ER. General cell preservation was good. One cell received a fixation treatment different from the rest, as it was filmed in the living state for a short time before fixation (Fig. 19b). Profiles of ER in this cell are unaligned and MTs are conspicuous.

Fig. 19. Living cell and correlative EM, *Iris spuria*.

- a. The cell was cultured in a perfusion chamber (Fig. 2) and was fixed after 25.5 min by perfusion with glutaraldehyde fixative (see section 2.235 for details). All chromosome movement ceased within one minute.
- b. Low power electron micrograph of the same cell. For key to abbreviations see List of Figures, p. ii. Scale = 1 μ m. Insert shows the same cell embedded in plastic prior to sectioning. Scale in LM photographs = 10 μ m.

4.22 Kinetochore structure

At all stages of division examined kinetochores are distinct regions on chromosomes; they are less electron dense than chromatin and more finely granular (Figs. 20, 26, 36). Kinetochore MTs, when present, penetrate at least half of the depth of the kinetochore and often penetrate almost right through it (Fig. 21). The kinetochore is attached to chromatin over most of the area of the kinetochore base, but in grazing sections it can appear to be free or almost free from chromatin in a cup shaped housing (Fig. 41a).

4.221 Meiosis I

The kinetochore, from prometaphase to late anaphase I, consists of two closely adjacent, but usually distinct, sister kinetochores, which are connected by MTs to the same pole ("syntely"; Bauer, Deitz and Röbbelen, 1961). The double structure is evident from prometaphase I to anaphase I (Fig. 20a-d) and has been observed in other species (Fig. 41b).

Fig. 20. Sister kinetochores during the first division of meiosis.

a-c, *Iris spuria*, d, *Allium triquetrum*. Sister kinetochores are arrowed in all prints.

- a. Prometaphase I; a LM photograph of the same cell embedded in plastic is shown in the insert to Fig. 38a.
- b. Metaphase I; a composite photograph of a single section of one bivalent. Four kinetochores are visible. A possible MT cross bridge is also present. See Fig. 39, insert for an LM photograph of the same cell.
- c. Anaphase I; see Fig. 28c, insert for an LM photograph of the same cell.
- d. Metaphase I; see Fig. 40b, insert for an LM photograph of the same chromosome. All scales = 0.5 μ m.

20a

20d

20b

20c

Fig. 21. Selected serial longitudinal sections of anaphase I sister kinetochores; *Iris spuria*. Separate sister kinetochores are arrowed in section 7. Scale = 0.5 μm .

Fig. 22. *Iris spuria*. Diagrammatic interpretation of the 3-D structure of anaphase I sister kinetochores. The dotted lines represent the chromatin between the kinetochores.

a-c. From LS. d-e. From TS. Lines AB, CD and EF represent three of the many different planes of LS through a pair of kinetochores.

FIG. 22 IRIS. ANAPHASE I SISTER KINETOCHORES
a-c from LS

a front view

b side view

c face views

d-e from TS

d front view

e side view

Fig. 23. Selected serial transverse sections of anaphase I sister kinetochores; *Iris spuria*. Separate sister kinetochores are arrowed in section 698. Kinetochores MTs and nkMTs are outlined by dotted lines. Scale = 0.5 μ m.

Eight metaphase I sister kinetochores were also examined in serial LS and TS (e.g. Fig. 24), and they tended to be more closely fused than at anaphase I. It was often only possible to distinguish sister kinetochores at their bases. A projection of chromatin was sometimes found piercing the region of fusion of the kinetochores, as in anaphase I.

The single pair of prometaphase I sister kinetochores examined (Fig. 25; this kinetochore region is shown in Fig. 38a) were more closely fused together than at anaphase I, although the two kinetochores were more easily distinguished than at metaphase I. Chromatin was found on the poleward side of the kinetochore region, but serial section analysis revealed that the chromatin was connected to the chromatin below the kinetochores and not to the small kinetochore chromatin projection. The chromatin on the poleward side of both kinetochore pairs in the metaphase I bivalent in Fig. 20b is probably also connected to chromatin below the kinetochores in a similar manner, but serial sections were not available to confirm this point.

4.222 Meiosis II

Sister kinetochores in the second division of meiosis are oriented to opposite poles ("amphitely" Bauer *et al.*, 1961), and are separated by the thickness of two chromatids. A kinetochore is seen as a single, slightly flattened ball, often in a shallow depression in the chromatin (Figs. 26b, 41a). A ball and cup kinetochore structure has also been described by Wilson (1968) for *Tradescantia* metaphase II chromosomes, and by Bajer and Molè-Bajer (1972) for *Haemanthus* mitotic chromosomes.

4.223 Summary

Sister kinetochores were arranged side-by-side in the first division of meiosis. The degree of fusion between adjacent sister kinetochores varied with meiotic stage and the closest fusion tended to be at metaphase I, while the least amount of fusion was observed at anaphase I.

Sister kinetochores were arranged back-to-back in the second division of meiosis.

Some regions resembling kinetochores were observed in prophase I and in interphase nuclei, but these data are not presented due to their incompleteness.

4.23 Microtubule distribution

4.231 Three-D reconstructions from serial longitudinal sections

Serial sections of metaphase and anaphase chromosomes from both divisions of meiosis were analysed as described in Chapter 3, section 3.41. An analysis of MT distribution during prometaphase I and II was not attempted since this would require the detailed study of many cells, all at slightly different stages.

Fig. 24. *Iris spuria*. Diagrammatic interpretation of the 3-D structure of metaphase I sister kinetochores.

a-b. From LS. c-f. From TS.

Fig. 25. *Iris spuria*. Diagrammatic interpretation of the 3-D structure of prometaphase I sister kinetochores.

FIG. 24 IRIS. METAPHASE I SISTER KINETOCHORES

a-b from LS

a front view

b side view

c-f from TS

c front view

d side view

e

f

FIG. 25 IRIS. PROMETAPHASE I SISTER KINETOCHORES

a-b from LS

a front view

b side view

Figure 27a is a 3-D reconstruction of the metaphase I bivalent in Fig. 26a. The kMTs attached to the upper kinetochore region are arranged in two parallel, cylindrical bundles, one to each sister kinetochore. The MTs in these bundles do not diverge greatly, and are still distinguishable a considerable distance polewards from the kinetochore. This double arrangement of kMTs was not noted, nor even suspected, until the 3-D analysis was done, suggesting that there was no bias in the alignment of the tracings. Other evidence for the lack of bias in the analysis is as follows. If one or several tracings were purposefully put out of alignment, the overall pattern of MT distribution still remained, and this was probably due to the large numbers of MT still in the other tracings. Furthermore, the pattern was still the same even when every second tracing was removed.

A bundle of nkMTs passes along the length of the bivalent and passes alongside, but does not come in contact with, each sister kinetochore pair at either end of the bivalent. The nkMT bundle extends polewards in both directions parallel to the kMTs, and there is some intermingling of kMTs and nkMTs along the edges of each bundle. This intermingling occurs only between nkMTs and kMTs of the left hand kMT bundle; kMTs from the right hand bundle are not involved. The MTs of the nkMT bundle appear to be more tightly packed in the interzone region than in the half spindle.

Complete 3-D analysis of MT distribution in anaphase I was partially unsuccessful due to difficulty in getting a completely longitudinal section through the exceptionally large spindles of *Iris* PMCs (80-100 μ m, pole-to-pole). Even if a section is only slightly oblique to the interpolar axis, separating sister half-bivalents at anaphase I will be many sections apart, and consequently analysis of MT distribution from dyad to dyad was not possible. But serial sections of the half-bivalent and associated MTs shown in part, in Fig. 21, were analysed as above and a 3-D reconstruction is given in Fig. 27b. Although this half-bivalent has double kinetochores (Fig. 21), there is no evidence that there are two adjacent kMT bundles, as was seen in metaphase I. Kinetochore MTs are associated in a single bundle, which does not diverge, at least in the region analysed. A nkMT bundle runs parallel to the kMTs and there is virtually no intermingling of MTs. The nkMT bundle has a pronounced kink in the region of the kinetochore (but this is not always so; Fig. 28b) and the bundle extends into the interzone. Bundles of nkMTs cross the interzone at various stages of anaphase I (Figs. 19b, 28b and c) but it has not been determined if the same bundle passes the kinetochore regions of sister half-bivalents.

Fig. 26. Chromosomes used for the 3-D reconstructions of MT distribution in Fig. 27; *Iris spuria*.

- a. Metaphase I; kinetochore regions visible at the upper and lower ends of the bivalent. A possible MT cross bridge is also present.
- b. Metaphase II; the circled region shows interaction between nkMTs and kMTs. A LM photograph of the same cell embedded in plastic is shown in Fig. 36, insert.
- c. Anaphase II; see LM photograph of the same cell in Fig. 37, insert. Scales = 1 μ m.

Fig. 27. Three-D reconstructions of MT distribution; *Iris spuria*. Chromatin is shown in black; MTs, red; kinetochores, blue; and membrane, green. See text for details.

- a. Metaphase I.
- b. Anaphase I, this chromosome is the same as that in Fig. 21.
- c. Metaphase II.
- d. and e. Anaphase II. Scale = 1 μ m.

Fig. 28. Microtubule distribution; *Iris spuria*.

- a. Prometaphase I, see LM photograph of the same cell in Fig. 38a, insert. Scale = 0.5 μm .
- b. Anaphase I, note the possible MT cross bridge (b), and possible interaction between chromatin and an nkMT is indicated. See LM photograph of the same cell in Fig. 19b. Scale = 0.5 μm .
- c. Late-anaphase I, low power electron micrograph. Scale = 1 μm . Insert shows the same cell embedded in plastic. Scale = 10 μm .

A 3-D reconstruction of the metaphase II chromosome in Fig. 26b is shown in Fig. 27c. The series was split in half because one chromatid arm obscured some of the MTs. This arm (left photograph) lies lower than the kinetochore region (right photograph). The long arm of the other chromatid lies lower still and extends to the opposite (upper) pole, but for clarity it was not included in this series. The outline of the chromosome on the right in Fig. 26b was not traced, nor were the kMTs of the lower chromatid of this chromosome. The kMTs from the upper chromatid were traced in the analysis in order to show their arrangement with respect to the kMTs of the traced chromosome.

Kinetochore MT bundles at metaphase II are composed of a single cylindrical array of MTs. There is no divergence polewards of the MTs of the kMT bundles and closely adjacent kMT bundles remain distinct from each other, with little interaction for a considerable distance polewards. The two adjacent kMT bundles do join together at their margins further polewards, but this is more likely to be due to the convergence polewards of all MTs than to divergence of each kMT bundle. A single nkMT bundle spans the interzone between the sister kinetochores and extends polewards in opposite directions parallel to the kMT bundles. There is only minor intermingling of nkMTs and kMTs, although some interaction is evident in the circled region of Fig. 26b. The nkMT bundle diverges slightly towards the upper pole.

An anaphase II cell was fortuitously sectioned exactly in the longitudinal plane so that separating sister chromatids, and even sister kinetochores, are present in the same section (Fig. 26c). This greatly facilitated 3-D analysis (Figs. 27d,e). A nkMT bundle spans the interzone between separating sister kinetochores. The bundle bends around the chromatids, and extends polewards in opposite directions, parallel to kMTs. The MTs of the kMT bundles do not diverge any more than they did at metaphase II, and there is no major intermingling of nkMTs and kMTs. The second anaphase II series (Fig. 27e) illustrates the convergence of adjacent kMT bundles as they approach the pole. Note also that the nkMT bundle associated with the left hand pair of sister chromatids branches, and some nkMTs are associated with kMTs from the middle chromosome (arrow Fig. 27, LH photograph). And, in Fig. 27e, RH photograph, which is taken from a few sections higher than the main series, there are two more small bundles of nkMTs which lie above the kinetochores of the left hand chromatids. Branching of nkMT bundles and extra, small nkMT bundles have not been observed at other stages.

28a

28b

28c

Other stages of meiosis have also been examined briefly for MT distributions. Non-kMT bundles are present at prometaphase I and run around the edge and through the body of the spindle (Fig. 38a). These bundles often run alongside bivalents. As at anaphase I they can show a pronounced kink in the region of the kinetochores (Fig. 28a), but this is not always so (see Fig. 20a). Compared with later stages the ratio of kMTs to nkMTs at prometaphase I appears to be quite low, although this ratio has not been determined statistically. The relationship of the broad nkMT bundles to the whole prometaphase I bivalent has not yet been firmly established.

The centre of the interzone at late anaphase I is devoid of MTs (Fig. 28c). Some nkMTs have a marked lateral bend, similar to that observed in *Haemanthus* during the development of the phragmoplast (Lambert and Bajer, 1972). However, there is no phragmoplast, nor cell plate, formed in *Iris* at telophase I.

Short ($< 1.5 \mu\text{m}$) MTs are present around the nuclei at interphase (Fig. 33b, section 19), but no MTs have been observed within the nucleus. Short ($< 1.5 \mu\text{m}$) MTs are found within and around the developing spindle at early prometaphase II (Figs. 34a; 34b, section 23). Most kinetochores have one to six short kMTs attached at this stage, and even the shortest MTs are aligned along the future interpolar axis. At the slightly later stage in Figs. 35a and b, the number and length of MTs have increased.

Bundles of nkMTs are present in the interzone at late anaphase II (Fig. 29a), but by telophase II (Figs. 29b) all MTs have disappeared. There is no phragmoplast formation at telophase II in *Iris*, and the four nuclei are not separated into tetrad cells until several hours after telophase II (section 4.111).

Chromosome associated nkMTs have also been observed in *Allium* and *Tradescantia*. Figure 30a shows an *Allium* metaphase I bivalent with associated kMTs and a single nkMT passing alongside the kinetochore region. Several nkMTs can be also seen in Fig. 30b in the interzone between two separating half-bivalents at early anaphase I. From a preliminary study of low power electron micrographs of *Allium* spindles at metaphase I and anaphase I, it is apparent that nkMTs are not organized into bundles as distinctly as they are in *Iris*.

Two adjacent serial sections of three prometaphase I bivalents of *Tradescantia* are shown in Figs. 31a and b. In the middle bivalent, several nkMTs lie alongside the upper kinetochore (lower kinetochore not visible) and its associated kMTs. Also, a single nkMT can be traced across the right bivalent, past the kinetochore and into the upper half-spindle. The presence or absence of distinct nkMT bundles has not been established for *Tradescantia*.

Fig. 29. Microtubule distribution; *Iris spuria*.

- a. Late anaphase II, insert shows the same cell embedded in plastic.
- b. Telophase II, insert shows the same cell embedded in plastic, the upper two nuclei are shown in the EM photograph. Scales in EM photographs = 1 μm . Scales in LM photographs = 10 μm .

Fig. 30. Microtubule distribution; *Allium triquetrum*.
(a) and (b) Metaphase I. Scales = 0.5 μm .

Fig. 31. Microtubule distribution; *Tradescantia flumenensis*.
a and b. Adjacent serial sections of a prometaphase I PMC.
Three bivalents are visible. Scale = 0.5 μm .

Cross bridges can occasionally be found between MTs (Figs. 20b, 26a and 28b), but these were never a regular feature of PMC meiotic spindles in any of the hundreds of electron micrographs examined in this study. Indeed, some of the cross bridges observed here may, in fact, be artifacts caused by a small piece of granular spindle matrix fortuitously lying between two MTs (as e.g., in Fig. 20b). Similarly, microfilaments were not observed in meiotic spindles in this study.

4.232 Microtubule counts from transverse sections

The analysis of transverse sections from one pole of the *Iris* spindle to the other involves the cutting of 1000 or more sections for each spindle; therefore only four cells were sectioned: one each of metaphase I and II, and anaphase I and II. It would be useful to be able to track MTs from section to section, as has been done with some diatom spindles (e.g., McIntosh, McDonald, Edwards and Ross, 1979). This technique requires that each section in a series must be the same size so that tracings of consecutive sections can be superimposed one on the other, in exact register. However, there is always some variation in the degree of section compression even when a diamond knife is used to cut sections, and therefore sections always vary slightly in size. In very small spindles, which have low MT numbers, these variations are negligible, but when the spindle cross section area is large, as in *Iris* (approx. 1000 sq. μm), and MT numbers are high (up to 13,000 MTs/section), MT tracking experiments are not possible. Thus no attempt was made in this analysis to track MTs from section to section (nor, heaven forbid, from pole to pole).

The total number of MTs in a number of transverse sections, taken from various points along the interpolar axis, was obtained for each cell as described in section 3.42; and the results are graphed in Figs. 32a-d. The error has been estimated in two ways. Microtubule counts were made from two, near-adjacent sections (sections 361 and 364 from a metaphase II cell) which had received the same staining regimen, but were photographed at different magnifications; and this reflects the differences encountered in the general analysis from photograph to photograph. The assumption was made that the total number of MTs was the same in both sections. The counts were 6494 and 6149, a variation of 5.3%. Another section (203, from the anaphase II cell) was recounted after several months, when the initial count had been forgotten. The MT counts were 11450 and 11790, a difference of 3.0%. Similarly for section 562 from the metaphase II cell, two MT counts were 5722 and 6211, a difference of 7.9%.

These three estimates all fall safely within an error of $\pm 10\%$. Other workers (e.g. McIntosh and Landis, 1971; Brinkley and Cartwright, 1971; see also section 1.23) have discussed this error and both groups consider

that a 10% confidence limit is reasonable, and therefore this percentage has been used in constructing Figs. 32a-d.

Each of the graphs in Fig. 32 have maximum numbers of MTs immediately polewards of, and possibly also to some extent at, the kinetochore regions. The graph lines all fall to low levels at the poles. A portion of the anaphase II graph has been left open since the sections from this region were lost during preparation. Each graph is more-or-less symmetrical, although one peak is lower than the other in each of graphs a-c (the height of the right hand peak in d could not be determined). This asymmetry may simply be due to experimental error since all the graphs could have been drawn symmetrically within the 10% confidence limits.

At metaphase I there is a plateau in MT numbers about 100 sections (approx. 5-6 μ m) on the poleward side of the kinetochores in each half-spindle, but no plateaus were visible at anaphase I. How much these plateaus in Fig. 32a are a reflection of the actual MTs distribution, and to what extent they are due to experimental error, is difficult to determine. An almost smooth curve can be drawn within the confidence limits of the plateau regions, and the plateaus could therefore be due to chance variation. However, they occur in both half-spindles, and at approximately the same distance from the kinetochores; and it is therefore argued that they reflect the actual MT distribution in this cell. Similarly, there are secondary peaks (or at least plateaus) 100 sections (5-6 μ m) poleward of the kinetochores in both half-spindles in the metaphase II cell in Fig. 32c. Again, it is argued, on similar grounds to those above, that real MT distributions are being revealed. No plateaus were observed at anaphase I and II.

At metaphase I the two plateaus are at approximately the same level as the trough in the middle of the interzone. This may indicate that nkMT numbers are constant across the interzone and 5-6 μ m into each half-spindle, as suggested by the 3-D reconstruction (Fig. 27a). However, chromosome associated nkMTs extend further into the half-spindle (> 10 μ m) than this, and they may not, therefore, be responsible for the plateaus. Mantle nkMTs (non-chromosome associated nkMTs around the edge of the spindle; Jensen and Bajer, 1973; see Figs. 36, 38) may instead be the cause of the plateaus observed at metaphase I, since they cover approximately the middle two-thirds of the spindle. The distribution of mantle MTs has not yet been studied in detail and further speculation is pointless.

The two secondary peaks (or plateaus) at metaphase II contain more MTs than are observed in the centre of the interzone, and their relationship to MT distribution is at present not clear.

Fig. 32. Microtubule distributions; *Iris spuria*. Microtubule counts from transverse sections. The sections containing kinetochores are indicated ($\leftarrow K \rightarrow$). The confidence limits at each point are $\pm 10\%$.

a. Metaphase I. b. Anaphase I. c. Metaphase II. d. Anaphase II.

FIG.32, IRIS, MT DISTRIBUTIONS

a METAPHASE I

FIG. 32b ANAPHASE I

FIG. 32c METAPHASE II

FIG. 32d ANAPHASE II

The overall flatness of the curves across the interzone region (with the exception of anaphase II) indicates relatively constant numbers of nkMT between the kinetochores. A slight peak could be drawn in the centre of the interzone at metaphase I and this might indicate a small amount of nkMT overlap, but this "peak" is more probably due to experimental variation. If we assume that the numbers of nkMTs remain constant, not only across the interzone, but also past the kinetochore region for a short distance (see above and Figs. 27a-e), then the number of kMTs at the kinetochores may be obtained by subtraction (Table 5). The average number of kMTs per sister kinetochore pair (meiosis I) or per kinetochore (meiosis II) is then found by dividing by the haploid number of *Iris* ($n = 24 \pm 1$). For metaphase and anaphase I this value is then divided by two to give an estimate for kMTs per kinetochore.

An estimate was not made for anaphase II because the number of nkMTs was not constant across the interzone.

Counts of the MTs associated with one particular anaphase I double kinetochore can be obtained from Fig. 23. A nkMT bundle can be seen to one side of the kinetochore region and it contains 73 nkMTs (mean from nine sections). The number of kMTs is 148 (mean from four sections) giving a total MT bundle size of 221 MTs. This individual count (148) is also close to the mean kMT number per sister kinetochore pair in Table 5 (18.4% difference).

Some corroboration of these estimates can be obtained as follows. Individual bundles of MTs can be seen in the transverse sections of the spindle just poleward of the kinetochore region. These bundles consist of both kMTs and nkMTs (see Fig. 23, Sections 706, 702). Average counts of total MTs per bundle were made from several sections and an estimate of the contribution of nkMTs was provided by counts of the numbers of nkMTs in bundles in the interzone (Table 6).

Column (e) was divided by two as in column (d), Table 5. The assumptions made in this analysis were: (a) each chromosome associated nkMT bundle has the same number of nkMTs just poleward of the kinetochore region as in the interzone (discussed above); (b) there is an average of one nkMT bundle per kMT bundle in each half-spindle. The anaphase II spindle was excluded from the analysis on this ground (also by it not conforming to assumption (a) above). Metaphase II was not analysed because the bundles of MTs were difficult to delineate in this spindle.

Comparison of column (e) in Table 5 and column (f) in Table 6 show a close agreement in kMT numbers per kinetochore for metaphase I and anaphase I between each method. The differences are not statistically significant using the χ^2 test ($\chi^2_1 = 0.96$, $0.3 < p < 0.5$ for metaphase I; $\chi^2_1 = 0.10$, $0.75 < p < 0.8$ for anaphase I).

Table 5. Estimates of kMT number from total MT counts.

Meiotic stage	Total no. of MTs at the kinetochore (a)	Total no. of nkMTs at the interzone (b)	Total no. of kMTs at the kinetochore (= a - b) (c)	No. of kMTs per kinetochore region (= c ÷ 24) (d)	No. of kMTs per kinetochore (= d ÷ 2 for MI) (e)
Metaphase I	8000	5000	3000	125	62.5
Anaphase I	7000	4000	3000	125	62.5
Metaphase II	7000	5000	2000	83	83

Table 6. Estimates of kMT number from MT bundle counts.

Meiotic stage	half-spindle		interzone		No. of kMTs per bundle (= b - d) (e)	No. of kMTs per kinetochore (= e ÷ 2) (f)
	No. of bundles counted (a)	Mean no. of MTs per bundle (range) (b)	No. of bundles counted (c)	Mean no. of nkMTs per bundle (range) (d)		
Metaphase I	6	167 (149-186)	26	63 (30-133)	104	52
Anaphase I	54	179 (108-258)	34	61 (38-97)	118	59

The number of kMTs per kinetochore does not change from metaphase I to anaphase I in the cells studied. In Table 5 there is no difference between the estimates, while in Table 6 the small difference is not statistically different ($\chi^2_1 = 0.44$, $0.5 < p < 0.7$).

The number of kMTs per kinetochore does not differ significantly from metaphase I to metaphase II ($\chi^2_1 = 2.88$, $0.05 < p < 0.1$).

The number of mantle MTs can be estimated using data from both Tables 5 and 6. Thus, the number of mantle MTs equals the total number of nkMTs at the interzone (column (b), Table 5) minus the total number of chromosome associated nkMTs (column (d), Table 6, multiplied by the haploid number, 24). These calculations give 3,500 and 2,500 mantle MTs for metaphase I and anaphase I respectively.

4.233 Summary

Chromosome associated nkMT bundles span the interzone between the kinetochores at either end of a bivalent (metaphase I) or between the kinetochores of sister chromatids (metaphase and anaphase II). The nkMT bundles extend into both half-spindles and run parallel to kMTs. Kinetochore-MTs are also associated into bundles and they do not increase in divergence at any stage of division.

Microtubule distributions from pole-to-pole showed maximum numbers of MTs at the regions immediately polewards of the kinetochore region at all stages studied. At metaphase of both meiotic divisions, plateaus (metaphase I) or secondary peaks (metaphase II) were present about 100 sections (6 μ m) poleward of the kinetochore region.

Estimates of kMT numbers were obtained by two different methods and these showed that the number of kMTs per kinetochore was not significantly different between metaphase I and anaphase I, nor between metaphase I and metaphase II.

4.24 Membrane distribution

4.241 Endoplasmic reticulum (ER)

The most complete data of ER distribution in the spindle were obtained from *Iris* PMCs in the second division of meiosis, so these will be described first.

At interphase there is a zone around each nucleus consisting mostly of ER (Fig. 33a). These open and irregularly shaped profiles of ER are commonly seen as being continuous with the outer nuclear membrane (sections 20, 21, Fig. 33), and it is possible that all ER elements in this region are connected to the nuclear membrane. Similarly, inclusions of membrane within the nucleus are found to be continuous with the nm, probably the inner nm (Section 20, Fig. 33). In subsequent sections the membrane

Fig. 33. Membrane distribution; *Iris spuria*.

- a. Interphase, showing both nuclei. Serial sections of the region outlined in the right nucleus are given in b.
- b. The white arrows in sections 20, 16, 10 and 3 indicate a small invagination of the (inner?) nm. The small arrow in section 21 shows an evagination of the outer nm. Scale in (a) = 1 μm . Scale in (b) = 0.5 μm .

inclusion can be seen to penetrate deeply into the nucleus as a long cylindrical invagination.

The spindle in Fig. 34a was initially judged to be in late prophase II on the basis of LM observations (insert, Fig. 34a), but it is actually in very early prometaphase II since the nm has broken. Open and irregularly shaped cisternae of ER are present around the edge of the incipient spindle, as at interphase; and some ER is also found in the centre of the future spindle. Careful examination of the edge of the spindle area reveals nm remnants, which still have nuclear pores. In many places remnants of the nm are continuous with profiles of membrane indistinguishable from ER, and serial section analysis has shown that most (and probably all) of the ER cisternae within the spindle region are invaginations of the inner nm (sections 17, 18, Fig. 34b). Evaginations of the outer nm are also present (sections 17, 24, Fig. 34b) and these may comprise most of the ER around the spindle.

The upper spindle in Fig. 35a is at a stage of prometaphase II slightly later than the cell in Fig. 34. This was judged by the lack of recognisable nm remnants and the increased length and number of MTs. This spindle is completely filled with irregularly shaped ER and the zone of ER around the edge of the spindle is still present. In the lower spindle, some of the ER is oriented parallel to the spindle interpolar axis, and the number and length of MTs has increased (Fig. 35b) from earlier stages.

At metaphase II there is no longer a zone of ER around the outside of the spindle and most of the ER is concentrated in the large, diffuse poles (Fig. 36). Spindle ER not at the poles is aligned along the interpolar axis and, in particular, is often parallel to kMT bundles (Figs. 36 and 27c, d). Some ER is also found between adjacent chromosomes on the metaphase plate. The distribution of ER at anaphase II is essentially the same as at metaphase II, with the exception that large open profiles of ER are present in the interzone (Figs. 37 and 27 d-e).

The distribution of ER in the first division of meiosis was not well documented as only a single prometaphase I spindle has been examined. Most of the ER in this prometaphase I spindle (Fig. 38a) is concentrated around the edge of the spindle and only a small amount is found in the centre. Endoplasmic reticulum within the body of the spindle tends to be close to bundles of MTs: note the large regions devoid of both ER and MTs. An *Allium* prometaphase I spindle is provided for comparison in Fig. 38b, where very little ER is evident in the spindle, although it is abundant in the cytoplasm.

34a

34b

section 17

37

38a

38b

The overall distribution of ER at metaphase I in *Iris* (Fig. 39) is similar to that in metaphase II. There are aggregations of membrane at the pole in metaphase I and spindle membrane is aligned alongside MTs. The amount of membrane in metaphase I is less than in metaphase II (compare the LP electron micrographs in Figs. 39 and 36, and the 3-D reconstructions in Figs. 27a and c).

A metaphase I spindle of *Allium* is shown in Fig. 40a. An accumulation of membrane can be clearly seen in the left hand pole and long thin profiles of ER are aligned alongside kMTs in the spindle.

Endoplasmic reticulum is aggregated at the pole in anaphase I of *Iris* PMCs (Fig. 28c) and is aligned alongside kMTs in the spindle. Profiles of ER are also found around the nkMT bundles in the interzone. The large open profiles of ER seen at anaphase II (Fig. 26c) are absent in anaphase I, although some small pieces of ER are present.

4.242 Relative amounts of ER in the spindle during the second meiotic division.

Estimates of the relative area enclosed by ER cisternae within the spindle were obtained with the leaf area meter (LAM) as described in section 3.5. The relative surface area of membrane is the best parameter to represent the amount of membrane in the spindle, but it is impossible to estimate surface area in thin sections. The area enclosed by ER is the next best parameter since, like the surface area, the cross section area of a cylinder is proportional to the square of the radius. The circumference of a cylinder in TS, on the other hand, is proportional only to the radius.

The estimates of relative areas of membrane in meiosis II spindles are presented in Table 7. All cells, with the exception of 063a and 063b (late anaphase II and telophase II), were taken from the same coverslip preparation. This ensures that all cells were fixed in the same way and the assumption was made that if the membrane is altered during fixation, then all membrane should be altered to the same degree in these cells. No cells in the first division of meiosis were available from this coverslip. Analysis was therefore limited to second division stages.

The accuracy of the estimates in Table 7 was determined as follows. The outlines of each of eight spindles were traced onto paper, cut out, and weighed accurately with a balance. Since weight is proportional to paper area, accurate measurements of the actual spindle areas were obtained. These measurements were then compared with the LAM estimates of the total area of the same spindles. Thus, for example, the relative area of membrane in the spindle of section 0456-27 was 35.2% (64 cm^2 membrane divided by 182 cm^2 total spindle). The total area estimated by weight as above was 184.1 cm^2 . Thus the LAM measurement differs from this by -1.1%. If this last

40a

40b

Table 7. Relative area of ER in the spindle.

Meiotic stage	Spindle No.	Section No.	Percent ER in spindle*	Mean %	See Fig.
Interphase	044c	10	22.2	19.4	33
		10	16.6		
Breakdown of the nm	045a	1	24.9	24.7	34
		17	25.4		
		27	23.8		
Post-breakdown of the nm	045b	27	35.2	32.9	
		22	33.1		
		16	31.7		
		66	31.6		
Early Prometaphase II	036a	80	30.7	30.8	
		111	30.9		
Early Prometaphase II	036b	111	32.9	32.9	
Early/mid-prometaphase II	044a	174	40.9	38.6	35
		156	38.3		
		183	36.7		
Mid-prometaphase II	044b	174	36.8	39.1	35
		156	36.6		
		183	39.7		
		187	43.4 (33.0)		
		165	39.1 (28.5)		
Metaphase II	034	53	28.6 (23.0)	24.3	36
		48	27.0		
		68	20.4		
		12	22.8		
		5	22.5		
Mid-anaphase II	035	32	33.2	29.0	37
		50	27.5		
		86	26.2		
Mid-anaphase II	039	55	31.8	31.4	
		42	30.9		
Mid-anaphase II	063a	6	28.8	28.8	
Telophase II	063b	10	24.0	23.4	
		34	22.9		

*Percentage estimates using a planimeter are bracketed.

measurement had been used in the calculation above, the percentage of membrane would have been $64 \div 184.1 \times 100\% = 34.8\%$, a difference from the observed of only 0.4%. It was not possible to determine the error of the membrane measurements (e.g. 64 cm^2 above) and the assumption was made that it is of no greater magnitude than those for total spindle area. In fact, if it is exactly the same as the error for the total spindle (e.g. -1.1%), the errors cancel and the same estimate of relative area is obtained.

The mean difference between the LAM and weight determinations of total spindle area in the eight spindles was 2.4%. The largest difference in this sample was +4.6% (section 034-68). As above, this might give a relative area of membrane in this spindle of 21.3% instead of the 20.4% observed, a percentage error of 0.9%. In a spindle with more membrane (e.g. 43.4% in section 044b-187) the percentage error would be 2.0%.

From the above calculations the maximum error in the estimates of relative area of membrane in the spindle is $\pm 2\%$.

The planimeter readings (bracketed in column 4) are about 20-25% too low and this reflects the high degree of inaccuracy associated with this method.

The limit of the spindle (or future spindle) was defined as the chromosome-containing region bounded by the zone of mitochondria and large cytoplasmic vesicles. Thus at interphase and early prometaphase II, the spindle region includes both the nuclear region and the zone of ER surrounding the nucleus. This definition was chosen because the zone of ER around the nucleus or future spindle is connected, via evaginations, to the outer nm; and also these ER cisternae become incorporated into the spindle at later stages (section 4.241).

All photographs were taken from sections of the central region of the spindle such that, where applicable, both spindle poles were sectioned (see Fig. 36). Differences between a single group of sections in Table 7 probably reflect chance variation.

As can be seen in column 5, Table 7, the quantity of membrane in spindles at the second division of meiosis increases until mid-prometaphase II, where it is double that at interphase. The amount of membrane at metaphase II is approximately the same as that at the beginning of pro-metaphase II. There is slightly more membrane at anaphase II than at metaphase II and the amount drops by telophase II.

4.243 Membrane bound vesicles

Small spherical membrane bound vesicles are found associated with bundles of MTs (kMTs and nkMTs) in all three species studied. They can be seen, for example, in Figs. 20, 21, 31, 36, 37 and 40, but are best seen under high magnification. In Fig. 41a several vesicles of different sizes (40 - 80 nm) can be seen adjacent to both kMTs and nkMTs at metaphase II in *Iris*. Note the concentration of vesicles into regions containing MTs. Figure 41b illustrates a nkMT bundle in prometaphase I of *Iris* which contains several vesicles of different size. Of particular interest is the adjacent golgi body (= dictyosome) and its associated small membrane bound vesicles. These vesicles are similar in size, shape and electron density to the MT associated vesicles in Figs. 41a and b just described, and it is likely that MT associated vesicles are derived from golgi bodies. Small golgi bodies are commonly scattered throughout the cytoplasm and even within the spindle region (as in Fig. 41b) at all the stages of meiosis studied.

Vesicles are found close to MTs in the prometaphase cell of *Tradescantia* in Figs. 31a and b. No other meiotic stages were examined in *Tradescantia* in this study, although similar vesicles can be seen close to kMTs in metaphase II of *Tradescantia* sp. in Wilson (1968, Fig. 5). These are again similar to vesicles produced by golgi bodies in these cells.

Figure 41c illustrates a metaphase I chromosome and kMTs of *Allium*. Several golgi bodies are present and they are apparently producing vesicles similar to the golgi vesicles of *Iris* and *Tradescantia*. Golgi-type vesicles can be found close to the kMTs (small arrows, Fig. 41c), but larger vesicles approximately 150 nm in diameter (large arrows) are more predominant (see also Fig. 40a). These large vesicles are more electron dense than the MT associated vesicles previously described, and they therefore may not be derived from golgi bodies. The large vesicles are abundant at the pole and are scattered around the edge of the spindle, as well as being associated with spindle MTs (Fig. 40a). At prometaphase I, aggregations of these vesicles, and of ER, mark the probable positions of the future poles (Fig. 38b).

4.244 Summary

Invaginations and evaginations of the inner and outer membranes of the nuclear envelope were visible in interphase and in early prometaphase II. All membrane profiles examined within the spindle area of prometaphase II could be traced via invaginations to the inner nm. At least part of the membrane around the edge of the future spindle at early prometaphase II is continuous with evaginations of the outer nm. At early/mid-prometaphase II the spindle area is filled with irregularly shaped profiles of ER and at

41a

41b

41c

later stages ER is aligned along the interpolar axis. At metaphase and anaphase II most ER is found as large accumulations at both spindle poles. Endoplasmic reticulum within the spindle is usually aligned alongside MT bundles.

Membrane distributions in the first division of meiosis have not yet been examined in detail, but preliminary observations suggest that ER in these spindles is less abundant than in meiosis II. The manner of ER formation in early prometaphase I may be different from that in prometaphase II.

The amount of membrane in the spindle region doubles from interphase to mid-prometaphase II. The amount decreases at metaphase II, then increases slightly at anaphase II. Low values were recorded for telophase II.

Small (40 - 80 nm) spherical membrane bound vesicles were always found associated with MT bundles. These were probably derived from golgi bodies in *Iris* and *Tradescantia*. In *Allium* golgi body derived vesicles and vesicles of unknown origin were described.

4.25 The nucleolus

A nucleolus is visible in the right nucleus of the interphase cell in Fig. 33a, and one is still present in Fig. 34a although the nm has broken. In the upper spindle in Fig. 35a, a nucleolus is still present even though the nm has completely broken down. These observations can be related to the living cell studies of prometaphase II where chromosome movements did not apparently begin until the nucleolus had completely disappeared (section 4.111).

Chapter 5.

DISCUSSION

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5.3 CONCLUSION

5.1 LIVING CELL STUDIES

5.1.1 Anaphase chromosome velocity

Table 8 presents a summary of anaphase chromosome velocities in PMCs of a number of species. Data from several animal meiotic cells and plant mitotic cells are also given for comparison. A much more comprehensive summary of anaphase velocities in many different mitotic and meiotic cells is given by Carlson (1977, Table 1). The velocities from my own data cited in Table 8 were measured from the graphs over the period of constant velocity at early to mid-anaphase I or II (here called the mid-anaphase velocity). This was considered a more realistic representation of chromosome movement than the "mean velocity" given by Carlson (1977), which he defines as the slope of the line intersecting kinetochores at the beginning and end of a graph of anaphase. Chromosome velocity decreases considerably towards the end of anaphase, and this decline would contribute greatly to a low "mean" velocity despite a relatively high mid-anaphase velocity. Moreover, the period of constant (or near constant) velocity usually accounts for approximately 80% of the movement, and is thus the best basis for comparisons. For this reason the additional data in Table 8 are mid-anaphase velocities as defined above, and were recalculated by myself from the graphs given in the original articles.

The available data on mid-anaphase I and II velocities in PMCs are limited, which precludes detailed discussion. However, some tentative conclusions can be made.

1. Mid-anaphase I and II velocities in the four species of PMC examined are all of the same order ($0.25 - 1.40 \mu\text{m}/\text{min}$). In contrast, the anaphase I velocities of two species of grasshopper (*Melanoplus* and *Chortophaga*) vary considerably from each other ($0.65 - 0.87 \mu\text{m}/\text{min}$ versus $3.0 \mu\text{m}/\text{min}$). Of course, the absence of such variability between different plant species may simply reflect the lack of available data, but it might also indicate that anaphase velocity is more uniform in plant meiosis.
2. Carlson (1977) notes that mitotic animal cells tend to have higher anaphase velocities than meiotic cells of the same species. This correlation cannot be directly extended to plant cells due to the lack of data, but, from Table 8, it can be seen that mid-anaphase velocities of plant cells in mitosis are generally higher and rather more variable than those in PMCs in meiosis ($0.6 \mu\text{m}/\text{min}$ and $2.3 \mu\text{m}/\text{min}$ in endosperm mitosis of *Haemanthus* and *Tilia* respectively). This conclusion also applies when mitotic and meiotic anaphases are compared in cells of plants from the same

Table 8. Anaphase Velocities

Species	Anaphase Mitosis ($\mu\text{m}/\text{min}$)	Anaphase ($\mu\text{m}/\text{min}$) *	Anaphase II ($\mu\text{m}/\text{min}$) *	Reference
<i>Iris spuria</i>		0.55 (0.45 - 0.60)	0.42 (0.28 - 0.48)	own data section 4.1
<i>Allium triquetrum</i>		0.55 (0.25 - 1.40)	0.40	own data section 4.1
<i>Tradescantia flumenensis</i>		0.35 (0.3 - 0.35)		own data section 4.1
<i>T. paludosa</i>		approx. 0.2 - 0.5	approx. 0.3	Lambert (1978; and pers. comm. 1979)
<i>T. paludosa</i>		approx. 0.4	approx. 0.5	Garot <i>et al.</i> (1968)
<i>Melanoplus</i> sp.		0.65 - 0.87		Nicklas (1965)
<i>Chortophaga</i> sp.		3.0		Carlson (1977)
<i>Haemanthus katherinae</i>	0.6			Bajer and Molè-Bajer (1954)
<i>Iris aphylla</i>	1.0			Bajer and Molè-Bajer (1954)
<i>Pisum</i> sp.	2.0			Bajer and Molè-Bajer (1954)
<i>T. virginiana</i>	1.2			Barber (1939)
<i>Tilia americana</i>	2.3			Fuseler (1975a)

* range given in brackets where available.

genus. Thus, *Iris aphylla* endosperm chromosomes move about twice as fast as the chromosomes of *Iris stylosa* PMCs. Similarly, mid-anaphase velocity in staminal hair mitosis in *T. virginiana* is more than twice as fast as that in *T. flumenensis* and *T. paludosa* PMCs.

3. Chromosome velocities in the second division of meiosis in animal cells tend to be faster than in the first meiotic division in animal cells (Carlson, 1977), but this is not confirmed so far by the data for PMCs. Indeed, in most of the PMCs studied to date, chromosome velocities in the second meiotic division are slower than in the first.

4. Anaphase velocity is positively correlated with distance travelled in animal cells (Carlson, 1977) but there is no such correlation in PMCs. In fact, *Allium* anaphase I velocities varied considerably (0.25 - 1.4 $\mu\text{m}/\text{min}$), yet the distance travelled in each case was approximately the same.

The wide range of velocities may reflect some sort of artifact in the culture of *Allium* PMCs, but this is unlikely for the following reasons.

- a. There was no relationship between anaphase I chromosome velocity and time in culture before anaphase onset (Table 2, section 4.112). For example, cell 7-1 was cultured for four and a half hours before anaphase I began, yet anaphase I velocities in this cell (0.60 - 0.85 $\mu\text{m}/\text{min}$) were in the centre of the observed range (0.25 - 1.40 $\mu\text{m}/\text{min}$, Table 2).
- b. There was no relationship between culture temperature and chromosome velocity, since temperatures were kept within a narrow range (23-25°C).
- c. Other culture conditions such as medium concentration and slide preparation time were kept constant and are therefore unlikely to contribute to variations in anaphase I velocity.

The wide range of anaphase I velocities in *Allium* PMCs compared with those in other species (Table 8) may simply reflect a greater tendency to variability in this species, although the variability may also be due to a lack of enough data in the other species.

Similar conclusions to a, b and c above can also be made with respect to *Iris* PMCs (section 4.111).

5.12 Anaphase chromosome group bowing

Chromosomes bowed away from the pole during late anaphase I in the *Allium* PMC illustrated in Fig. 11, and this was found to be due to an approximately 38% difference in poleward velocity between chromosomes at marginal versus central positions on the metaphase I plate (section 4.112). This observation is in contrast to that of Fuseler (1975a), who found that chromosome bowing away from the pole in *Tilia americana* endosperm cells was caused by all chromosomes moving polewards at the same velocity. As a result in *Tilia*, laterally positioned chromosomes had further to travel and therefore lagged behind central chromosomes.

However, bowing away from the pole was not a general feature of anaphase I in *Allium*, and in one case bowing towards the pole was observed. Again, differences in velocities were found to account for this; and, in particular, marginal chromosomes moved about 45% faster than those on the spindle axis (Table 2, section 4.112). Nicklas (1965) points out that this condition is the general one in *Melanoplus* spermatocyte meiosis, and he notes a 25% greater velocity for lateral chromosomes. In the remainder of the *Allium* cells studied in anaphase I, no differences in chromosome velocities were observed within the half-spindle, but differences in average mid-anaphase velocities of 0.1 - 0.2 $\mu\text{m}/\text{min}$ between sister half-spindles were common.

A possible explanation for these variable chromosome velocities, both within and between half-spindles, may lie simply in economy of anaphase movement: there may be no relationship between position on the spindle and chromosome velocity. *Allium* bivalents are large and variable in size in relation to the spindle (see Fig. 11), and thus in those cells where chromosomes bow towards (or away from) the poles, bowing may occur because it is the most economical method of movement. Similarly, economy of movement would explain the lack of variation in those cells which have, happily, an economical arrangement of bivalents at the equator.

Similar variations in chromosome velocities can be observed in the photographs of anaphase I in *Tradescantia paludosa* given by Garot, Lebrun-Peremans, Moreau and Gillies (1968) and Lambert (1978; and pers. comm.) (my observations). As in *Allium*, *T. paludosa* bivalents are large and variable in size in relation to the spindle. In both of these reports, variation in chromosome velocity does not appear to be related to position on the metaphase plate, and thus economy of movement may explain variations in chromosome velocities in these cells also.

5.13 Spindle elongation

Anaphase movement can usually be resolved into two components, namely chromosome-to-pole movement and spindle elongation. During spindle elongation the chromosome sets are moved apart by expanding the distance between the two poles. The relative contributions and timing of the two components vary from cell type to cell type, even within the same organism (Mazia, 1961). In some cases the two can be distinct, as, for example, in the second division of meiosis in the aphid *Tamalia*, where poleward chromosome movement occurs before spindle elongation (Ris, 1943). This case is unusual: usually the two components of anaphase movement overlap somewhat. Another unusual case is described by Fuseler (1975a) in *Tilia americana* endosperm, where spindle elongation precedes chromosome-to-pole movement.

Spindle elongation is often described as being negligible or absent in plant cells as, for example, in *Luzula* and "many other higher plants" (Östergren, 1949, p. 416). Another commonly cited example is from the study of staminal hair cell mitosis in *Tradescantia* sp. by Barber (1939), but an analysis of anaphase chromosome movement is not given. Carlson (1977) holds the view that there is no evidence of poleward movement without spindle elongation in any cell; and states that an increase in spindle length of almost one third has been reported in *Tradescantia* staminal hair cells by Bělař (1929). However, Ris (1943, p. 175) writes that in "*Tradescantia* staminal hair cells, as in other somatic plant cells, there is no elongation of the spindle and cell (c.f. Bělař's photographs, 1929). The situation is confused. My own (unpublished) observations of living staminal hair cells revealed that the spindle poles are difficult to distinguish and thus estimates of spindle length changes are likely to be inaccurate.

There is no spindle elongation during anaphase I in *Allium triquetrum*. This can be stated unequivocally, because both LM (Fig. 11) and EM (Fig. 40a) observations at metaphase I show that the spindle poles are situated at the edge of the cell, immediately adjacent to the cell membrane. Pollen mother cells do not change their shape or size during anaphase I, because there is a thick, rigid, callose wall around the cell. Thus spindle elongation cannot occur during anaphase I. No elongation is seen in the projected films of anaphase I and the graphical plots of pole positions are straight lines. These results are the first unequivocal demonstration of anaphase movement without polar elongation in living plant cells.

No spindle elongation was observed during anaphase II of *Iris* PMCs (Fig. 10), and the graphical plots of pole positions were straight lines, as for *Allium* anaphase I above.

Spindle elongation could be detected in the projected film of anaphase II in *Allium* and the graph shows an increase in spindle length of approximately 40% (Fig. 15). At anaphase I in *Iris*, there was no apparent change in spindle length when the film was projected at normal speed, but the graph shows a small increase (15%) in late anaphase I. The spindle pole cannot be determined in *Tradescantia* due to an accumulation of cytoplasmic granules (mostly starch grains; Fig. 31) over the polar regions; thus the contribution, if any, of spindle elongation to anaphase I movement cannot be determined.

5.14 Duration of meiosis

Table 9 presents a summary of the duration of meiosis as studied *in vitro*. Several examples of mitotic stages in plant and animal cells are also given for comparison. It is immediately obvious that there is considerable variation among the different plant species: anaphase I - tetrads took 5 hrs in *T. paludosa* and 15 hrs in *Iris*, yet the durations of anaphase I and II and prometaphase II in both species are approximately the same. This observation apparently contradicts that of Bennett (1977, p. 219) who states that "... changes in the duration of meiosis (from species to species*) in higher plants usually result from proportional alterations of all the stages ... rather than large changes in one or just a few". The most striking differences are in the metaphase II and telophase II - tetrad stages, and these might be explained by culture artifact. Pollen mother cells are particularly sensitive to culture conditions and if they are exposed to anisotonic conditions they quickly die (section 3.22; see also Shimakura, 1934). Slight changes in the tonicity of the culture medium, however, may not cause death, but can arrest cells at metaphase I, for example (Shimakura, 1934). Shimakura found that he could induce such arrested cells to enter anaphase by slightly increasing the tonicity of the culture medium around the cells. Lengthy delays at metaphase II in *Iris* might therefore be due to slightly anisotonic medium. Other observations support this view as follows. The optimum sucrose concentrations for culture increases during meiosis (p. 41; Shimakura, 1934) and chromosomes tend to lose contrast after about 5 hrs in culture. Therefore, the time taken to form tetrad cells almost certainly depends on time in culture. One cell (cell 10-1, Fig. 7 and Table 1b), initiated in culture at metaphase I, took 5 hrs from telophase II - tetrads, while another cell (cell 19-3 in Table 1b), initiated at metaphase II, took only 1 hr from telophase II to tetrad cells. Culture conditions may considerably affect the duration *in vitro* of some meiotic phases, especially stages where chromosome movement is minimal, i.e. metaphase I and II and post telophase I and II. As was noted earlier, culture conditions had little effect on anaphase chromosome velocities. It may be that the onset of anaphase can be relatively easily delayed, but once anaphase movement is initiated, chromosomes move at "normal" velocities.

* my insertion

Table 9a. Duration of Meiosis.

Species	Late Prophase I	Prometaphase I	Metaphase I	Anaphase I	Telophase I to Prophase II	Prometaphase II	Metaphase II	Anaphase II	Telophase II to tetrads	Total time Anaphase I to tetrads	References
<i>Iris spuria</i>	>4 hr	30 min	≈4 hr	40 min	4hr30min	30 min	4 hr	30 min	5hr(1hr)	15 hr 30 min	own data, Fig. 7
<i>Allium triquetrum</i>			≈4 hr	15-25min				15 min			own data, Figs. 11,12
<i>T. paludosa</i>			≈4 hr	≈30 min	2hr40min	≈30 min	<20 min	≈30 min	40 min	5 hr 10 min	Lambert (pers. comm.)
<i>T. paludosa</i>				25 min	40min			20 min	70 min		Garot <i>et al.</i> (1968)
<i>T. paludosa</i> (from cultured anthers)	17 hr	2 hr 30 min		36 min	5hr54min	3 hr	6min	1 hr	20 hr	≈11 hr	Steinitz (1944)
<i>T. paludosa</i> (from cultured anthers)			2-3 hr			3-5 hr				≈5 hr	Taylor (1950)
<i>T. flumenensis</i>				30 min							own data, Fig. 13
<i>T. virginiana</i> cell a			40 min	50±10min	3 hr 35 min	1 hr	20±10min	2 hr		7 hr 45 min	Shimakura (1934)
<i>T. virginiana</i> cell b			>1hr 25min	1hr 15min	8hr20min	1 hr 30 min	6 hr 45 min			17 hr 50 min	Shimakura (1934)
<i>Frittilaria</i> sp.			>25 min	30 min	>45min		15-20 min				Fujii and Yasui (1954)
<i>Chortophaga</i> sp.	45 min		2hr 40min	1hr 30min	2hr30min	25 min	1hr 30min	1 hr 45 min			Pis (1949)

Table 9b. Duration of Mitosis.

Species	Prophase	Prometaphase	Metaphase	Anaphase	Telophase	References
<i>Iris aphylla</i>	1 hr	12 min	10-20 min	17 min	40 min	Bajer and Molè-Bajer (1954)
<i>T. paludosa</i> (haploid mitosis)	10 hr	22 min	30 min	29 min	14 min	Beatty and Beatty (1953)
<i>Haemanthus</i> <i>katherinae</i>		≈30 min	≈40 min	25-45min	≈3 hr	Bajer and Molè-Bajer (1954)
<i>Pisum sativum</i>	40 min	← 20 min →	← 12 min →	12 min	70 min	Bajer and Molè-Bajer (1954)
<i>Tilia americana</i>		40 min	12 min	12 min	15 min	Bajer and Molè-Bajer (1954)
Mouse spleen	20-35min	← 6-15 min →	← 12 min →	8-14 min	9-26 min	Fuseler (1975a)
Yoshida sarcoma	14 min	← 31 min →	← 4 min →	4 min	21 min	Mazia (1961) Mazia (1961)

In vitro studies provide a direct check on the duration of meiosis as calculated by more classical means, such as half-anther culture (Steinitz, 1944; Taylor, 1950; Bennett, 1977). At present, comparative data are available only for *T. paludosa* (Table 9a). Lambert's observations compare favourably with the times estimated by Taylor (1950), but Steinitz's (1944) times are longer and this may reflect the lack of temperature control in her experiments. The concordance of Lambert's and Taylor's results suggests that if culture conditions are optimum, PMCs can be cultured through most of the meiotic stages in times comparable to those *in vivo*.

The remaining data of Table 9b illustrate that the duration of mitosis is less than that for the first division of meiosis (compare *T. paludosa* haploid mitosis and the first division of meiosis, and *Iris aphylla* endosperm mitosis with *I. spuria* meiosis I. Other data from classical studies are reviewed by Bennett (1977).

5.2 ELECTRON MICROSCOPY

5.21 Kinetochores structure

The kinetochore is defined as the special region(s) on a chromosome to which spindle fibres (kMTs) are attached (Fuge, 1977; Clapham and Östergren, 1978; reviews). Kinetochores are usually only visible in the EM, but they can sometimes be seen in suitable LM preparations (Roos, 1975; Clapham, 1978). The structure usually described in the LM is the centromere, which comprises the whole of the primary construction, namely the kinetochore and the chromatin of the "commissural region" (Clapham and Östergren, 1978). This centromeric or commissural chromatin is indistinguishable from "ordinary" chromatin in electron micrographs.

The kinetochore of higher plants is a ball shaped mass of diffuse material having a more electron dense core; and the ball is embedded in a cup shaped depression in the centromeric chromatin (Bajer, 1968; Bajer and Molè-Bajer, 1971, 1972; Wilson, 1968; Braselton and Bowen, 1971; Wagenaar and Bray, 1973; this study, Figs. 20 and 41a). In contrast, the kinetochore of mammalian cells and many other eukaryotes is more differentiated and typically consists of "trilaminar, roughly circular plaques" (Roos, 1973b). The outer and innermost layers are electron dense and the middle layer is electron light (e.g. Jokelainen, 1967; Comings and Okada, 1971; see Alov and Lyubskii, 1977; Fuge, 1977; Heath, 1980 for reviews); and kMTs are attached to the outer layer.

The newt kinetochore appears to have a somewhat intermediate structure in mitotic cells (Molè-Bajer, Bajer and Owczarzak, 1975; Rieder, 1979a). It has a ball and cup structure, but part of the ball is differentiated into layers.

Rieder (1979b) reports that all three layers of the mammalian kinetochore contain DNA and the inner layer also contains ribonucleo-protein (RNP). However, kinetochores of colchicine treated cells have only a single RNP containing structure, which corresponds to an enlarged inner layer of the kinetochore. Braselton (1975) and Rieder (1979a) also describe RNP staining in the ball shaped kinetochores of *Allium cepa* and newt, respectively. Other reports describe RNP or RNA in centrioles or the pericentriolar material (e.g. Peterson and Berns, 1978, 1980 review), and Heidemann, Sander and Kirschner (1977) present evidence that suggests that the presence of RNA in centriolar basal bodies is required for the nucleation of MTs. Rieder (1979b) suggests that RNA may be a universal component of MT organizing centres (MTOCs, Pickett-Heaps, 1974) and thus it could be argued that the innermost layer of the trilaminar kinetochore is equivalent to the ball shaped kinetochore of higher plants. Fuge (1977, p. 5), however, states that the outer layer of the trilaminar kinetochore is the equivalent structure. The question is unresolved at present.

5.22 Kinetochore orientation

The almost flawless arrangement of chromosomes on the spindle ensures that there is equal distribution of chromosomes to daughter cells. Thus, homologous half-bivalents are drawn to opposite poles during the first division of meiosis, while sister chromatids are drawn to opposite poles during the second division of meiosis and during mitosis. The difference in distribution pattern is brought about by a difference in chromosome orientation. Sister kinetochores are oriented to the same pole in the first division of meiosis (syntely), and to opposite poles in the second division and in mitosis (amphitely) (Bauer, Dietz and Röbbelen, 1961). In both cases the preferred orientation ensures the bipolarity of the bivalent or the chromosome, respectively, on the spindle.

Östergren (1951) suggested that the reason for the orientation difference was due to a difference in kinetochore arrangement. In his view, sister kinetochores are arranged side-by-side in the first division of meiosis; they therefore face the same direction and tend to orient to the same pole. In the second division of meiosis the sister kinetochores are back-to-back, separated by chromatin; they point in opposite directions and tend to orient to opposite poles. Lima-de-Faria (1958) proposed instead that the type of orientation depends on the general physiological condition in the spindle. He writes "... there is no fundamental difference in structure between the kinetochores at mitosis and at the first division of meiosis" (Lima-de-Faria, 1958, p. 148).

A relevant example is given by Wagenaar and Bray (1973). Univalents regularly formed in the first division of meiosis of a wheat hybrid, and at prometaphase I most univalents were close to one or ^{the} other pole. In EM sections of these univalents, the two sister kinetochores from each sister chromatid were adjacent to each other and both faced the nearest pole. Both kinetochores were connected via MTs to that pole, i.e. they were syn-oriented. At later stages of prometaphase and metaphase I, other univalents from midway between the pole and the equator were amphio-oriented, i.e. the sister kinetochores were connected to opposite poles, although the sister kinetochore oriented to the closer pole had more MTs attached to it than did its sister kinetochore. Sister kinetochores of all metaphase I univalents at the equator were all amphio-oriented and both had approximately equal numbers of KMTs. Wagenaar and Bray (1973) report that the sister kinetochores of some of the equatorial univalents were arranged back-to-back. They suggest that amphitelicly oriented univalents at the equator change from a side-by-side to a back-to-back arrangement. This suggests that an orientation change causes a change in the kinetochore arrangement (the converse of Östergren's theory). However, it appears that Wagenaar and Bray may have misinterpreted their results (see Fig. 42a and b, below).

Fig. 42. Tracings of sister kinetochores in metaphase I univalents taken from Wagenaar and Bray (1973; Figs. 4 and 6).

If the chromosome in Fig. 42a had been sectioned in a longitudinal plane perpendicular to the page (i.e. in the plane X-Y), instead of in the plane of the page, the sister kinetochores would appear to be separated by a small amount of chromatin. This is exactly what is seen in Fig. 42b (Wagenaar and Bray, 1973, Fig. 6), so it would appear that the two kinetochores are still side-by-side. Parenthetically, these observations emphasise the necessity for 3-D analyses in studies of kinetochore structure.

According to Lima-de-Faria (1958), physiological changes account for the differences in kinetochore orientation. On his theory, amphitelically oriented univalents would not be expected to occur in the same spindle as normal syntelically oriented bivalents or syntelically oriented univalents. Nicklas (1977) further examined this argument by transferring a metaphase I bivalent to a metaphase II spindle in a grasshopper spermatocyte cell hybrid. If Lima-de-Faria is correct, the transferred chromosome should orient in a manner typical of the recipient spindle. However, the bivalent syn-oriented and segregated during anaphase as if it were in an anaphase I spindle. Similarly, a metaphase II chromosome was transferred to a metaphase I spindle and it amphi-oriented and segregated as normal. Nicklas's results support Östergren's hypothesis but, as he points out, metaphase chromosomes were used, i.e. chromosomes which had already been in contact with the spindle. This contact might predispose the chromosome to a particular orientation. Nicklas suggests that the use of prophase chromosomes would have been more definitive.

Electron microscopy of kinetochores in other studies also support the views of Nicklas (1977) and Östergren (1951). In mitotic cells, for example, sister kinetochores are arranged back-to-back (Lambert and Bajer, 1977) even during prophase (Roos, 1973b; Heneen, 1975). The kinetochore region of metaphase I bivalents often appears to contain a single kinetochore (Wagenaar and Bray, 1973; Braselton and Bowen, 1975; Nicklas, 1977; Harris, A., 1980, pers. comm.) but Nicklas (1977) proposes that this is actually composed of two "closely appressed" sister kinetochores, which are syn-oriented.

In favourable material such as *Iris* and *Allium*, the kinetochore region in the first division of meiosis is clearly double. The two adjacent sister kinetochores can be distinguished from prometaphase I to anaphase I in LS and TS (Figs. 20-25). Distinct sister kinetochores have not been observed previously in metaphase I bivalents of PMCs, but Müller (1972) and Fuge (1980) have briefly described double kinetochores at prometaphase I and anaphase I respectively in crane fly spermatocytes. Moens (1979) also reports double kinetochores at metaphase I in locust spermatocytes, but photographic documentation is not given.

Östergren's (1951) hypothesis states that sister kinetochores which are arranged side-by-side will tend to orient to the same pole. This does not mean, however, that they necessarily do so, and in fact, univalents with side-by-side kinetochores regularly amphiorient, as noted earlier (Wagenaar and Bray, 1973). Sister kinetochores of bivalents can also orient amphitelically, although this is rare (some recent examples are: Parker, Ainsworth and Taylor, 1978, in *Hypochoeris* autosomes in meiocytes

containing large numbers of B chromosomes; and Goldstein, 1980, in meiotic mutants of *Drosophila*). In *Iris*, separate sister KMT bundles can sometimes be distinguished for several microns polewards from the kinetochore region (Fig. 27a), and thus, not only are the kinetochore regions structurally double, the sister kinetochores are acting as separate functioning units, although in this case they are acting together.

Sister kinetochores in the second division of meiosis in *Iris* are back-to-back (Figs. 27c, 34b, 35b, 36), as suggested also by Östergren (1951); and are clearly separated by the thickness of two chromatids. The ultra-structure of the kinetochore at meiosis II in higher eukaryotes is very poorly recorded and I know of only two reports dealing with this issue: Fuge (1973), TS of a metaphase II spindle of *Pales*; and Wilson (1968), LS of *Tradescantia* sp. metaphase II chromosomes (his Figs. 1-3). Both studies support the kinetochore arrangement shown here.

The sister kinetochore arrangement has changed from side-by-side at late anaphase I to back-to-back by prometaphase II. When, precisely, and how might this occur? Two possibilities are as follows.

1. The two kinetochores could be rearranged sometime during interphase or prophase II.
2. The two kinetochores could be still side-by-side at the end of prophase II, and then be pulled apart by the action of mitotic forces during early prometaphase II.

The implications of these alternatives to the first movements on the spindle of prometaphase II chromosomes should be examined. If the sister kinetochores are back-to-back at the beginning of prometaphase II, amphitelic orientation should commonly occur. This is because MTs associated with each sister kinetochore tend to extend in opposite directions perpendicular to the chromosome axis (see the *in vitro* experiments of Snyder and McIntosh, 1975; Telser, Moses and Rosenbaum, 1975; McGill and Brinkley, 1975; Gould and Borisy, 1978; Pepper and Brinkley, 1979; Summers and Kirschner, 1979; and Bergen, Kuriyama and Borisy, 1980). If one kinetochore faces and thus orients to one pole, its sister will be facing and will orient to the opposite pole; immediate bipolar orientation will result and most chromosomes will move to the equator. If the kinetochores are still side-by-side, then syntelic orientation (as in meiosis I) should commonly occur, since both kinetochores still face the same direction. This would result in unstable unipolar orientation and initial movement polewards. Re-orientation to the more stable bipolar orientation would eventually follow and the chromosomes would move to the equator.

The first movement of all chromosomes at early prometaphase II in living PMCs of *Iris* was towards the equator (section 4.111). Each chromosome moved individually to the centre of the spindle, and thus the movements are considered to be different from those of the "contraction stage" in *Haemaphysalis* (Bajer and Molè-Bajer, 1972). These contraction stage movements appear to be due to the invasion of the disrupted nucleus by the developing spindle and apparently are not caused by direct interaction of the spindle with kinetochores. The pattern of movement in *Iris* suggests that bipolarity is attained immediately in most chromosomes and, therefore, the sister kinetochores were likely to be back-to-back at the beginning of prometaphase II (alternative 1 above). The possibility of monotelic orientation (the orientation of one kinetochore to one pole while the other kinetochore is unoriented, Roos, 1976) can be excluded, because initial monotelic orientation would usually result in movement polewards. This would be so regardless of the kinetochore arrangement.

Electron microscope observations on *Iris* PMCs also support alternative 1. Thus, sister kinetochores are back-to-back at the earliest stages of prometaphase II (Fig. 34b). A low number of very short ($< 1.5 \mu\text{m}$) MTs are associated with most kinetochores at this stage, but it is doubtful whether such short MTs could generate or transmit sufficient force to draw the kinetochores apart, as required in alternative 2 above. In any case, it is unlikely that prometaphase movement had begun in this spindle, since a large, well developed nucleolus is still present: living cell studies (section 4.111) have suggested that chromosome movements do not begin until the nucleolus has broken down. This implies a delay of unknown duration after nm breakdown and before movement.

The sister kinetochore arrangement in *Iris* probably changes from side-by-side to back-to-back sometime during interphase/prophase II. The most likely period is during chromosome recondensation during early prophase II, and if this is so it may be a reason why a decondensation-recondensation cycle is usually necessary between meiotic divisions. In some organisms (as e.g. in *Trillium*; Rhoades, 1961), however, telophase I and interphase are omitted and the late anaphase I half-bivalents enter the second division of meiosis with little or no change in chromosome length or in coiling. Continuing chromosome movement in living cells of *Trillium* has not been documented, although Shimakura (1937) was able to maintain *Trillium kamtschaticum* PMCs in an arrested state at metaphase I. On the basis of the above discussion we might predict that sister kinetochores of early prometaphase II chromosomes would still be side-by-side, since there has been no opportunity for the kinetochores to be rearranged. The first movements of *Trillium* prometaphase II chromosomes should therefore be polewards.

5.23 Microtubule distribution

Serial section studies on MT distribution in the higher eukaryotes have concentrated on three main spindle types: mitosis in mammalian cells (Brinkley and Cartwright, 1971, 1975; McIntosh and Landis, 1971; McIntosh, Cande and Snyder, 1975a; McIntosh, Cande, Snyder and Vanderslice, 1975b; McIntosh, Cande, Lazarides, McDonald and Snyder, 1976; Roos, 1973a); crane fly spermatocytes (Forer and Brinkley, 1977; Fuge, 1972-1980; La Fountain, 1974, 1976); and plant mitosis in *Haemanthus* endosperm (Jensen and Bajer, 1973, 1974; Lambert and Bajer, 1975). This study adds an important cell type: meiosis in a higher plant.

Fuge (1977) suggests that for an understanding of spindle function in higher eukaryotes it is useful to search for morphological similarities in different species, since these similarities might point to important features for spindle function. Such similarities are rare, however. The major ones are as follows.

5.231 Non-kinetochore MTs and kinetochore MTs

Non-kinetochore MTs run alongside kMT bundles in all higher eukaryote spindles studied to date. In many (most ?) cases the nkMTs are parallel to kMTs. The arrangement of nkMTs into bundles in plant cells (*Iris*, Figs. 19, 27; and *Haemanthus*, Jensen and Bajer, 1973) makes the arrangement of nkMTs readily visible in LS, but careful examination of published photographs in other cell types (McIntosh and Landis, 1971, Fig. 2b, HeLa cells; Roos, 1973a, Fig. 16, PtK₁ cells; Fuge, 1974, Fig. 6, *Pales* spermatocytes) indicates that nkMTs pass alongside kMT bundles in these cells also. The observation that nkMTs connect the two half-spindles by spanning the interzone between sister kinetochores in *Iris* and in *Haemanthus* may also be a more widespread phenomenon, not only in the higher eukaryotes (see the published figures cited above and the brief description of continuous MTs between bivalents at metaphase I in *Lilium* by Braselton and Bowen, 1975), but also in some of the lower organisms such as in the micronuclear division in *Tetrahymena* (La Fountain and Davidson, 1979). Of course, spindle organization is much more variable in the lower organisms and similarities with higher organisms should be regarded with caution (Pickett-Heaps and Bajer, 1977; Heath, 1980).

The arrangement of nkMTs and kMTs described above immediately suggests some sort of sliding mechanism between the two classes of MTs which might directly cause chromosome movement (e.g. McIntosh, Hepler and Van Wie, 1969; McIntosh and Landis, 1971; Nicklas, 1975; Subirana, 1968). Sliding mechanisms require close interactions between nkMTs and kMTs, and at least in *Iris* (and probably also in mammalian cells, lit. cit.) this is

not observed. Only a small degree of interdigitation of MTs was observed along the margins of adjacent bundles in *Iris* at all stages studied. Also, in metaphase I of *Iris* MT interdigitation occurred only along the edge of one of the two sister kMT bundles. A much clearer association of the nkMTs with both sister kMT bundles might be expected if MT sliding caused movement. Sliding mechanisms also require cross bridges between MTs and these were not found to be a regular feature in meiotic spindles in this study (see section 4.231). If cross bridges were functional in chromosome movement then they should be regularly and clearly seen in spindles. In other systems where a functional relationship between MT cross bridges and intra-cellular movement has been suggested, cross bridges are routinely observed (e.g. ciliary beating, Warner 1976; axopodia feeding mechanisms, Tilney, 1971). Also, in *Iris* where there is apparent nkMT-kMT interaction (circled region, Fig. 26b), no cross bridges can be observed.

Another model for chromosome movement, "Zipping" (Bajer, 1973, 1977; Bajer and Molè-Bajer, 1975), also requires interdigitation of kMTs and nkMTs (section 1.423 for details). The lack of interdigitation described above in *Iris* and in other cells suggests that the zipper model cannot be applied to these cells. The model also fails on other grounds when applied to *Iris*. Thus kMT bundles in *Iris* do not increase in divergence during anaphase I or II, and maximum numbers of MTs are not found behind the advancing anaphase kinetochores, as predicted on the zipper hypothesis (see Figs. 27 and 32 and section 4.232). In *PtK₁* (Roos, 1973a) and in *Pales* (Fuge, 1980) the degree of splaying of kMTs during anaphase appears to be minimal. Anaphase MT distribution graphs for mammalian cells show maxima in the region of the kinetochores (McIntosh *et al.*, 1975a), but the depth of the kinetochore region in these cells precludes further determination of the position of the MT maxima as being in front of, or behind, the kinetochores.

Preliminary studies in *Iris* also suggest that there is no change in kMT number from metaphase I to anaphase I (Tables 5 and 6; section 4.232). No further information is available on changes in kMT number during anaphase other than in *Haemanthus* (Jensen and Bajer, 1973, 1974) where kMT numbers decrease during anaphase, as predicted by the zipper model.

Jensen and Bajer (1973) note that the connection of sister kinetochores to the same nkMT bundle might explain the in-concert responses of both sister half-bivalents after UV microbeam irradiation of the kinetochore region of only one half-bivalent (Forer, 1965, 1966; Bajer, 1972). Begg and Ellis (1979), however, have recently suggested that in-concert responses of sister half-bivalents (to micromanipulation) are directly related to the

age of the preparation, and thus the response might be abnormal. Possibly, in older preparations, closer interactions with nkMTs might develop. Jensen and Bajer also observed that adjacent kinetochores in *Haemanthus* can often be connected to the same, or a branch of the same, nkMT bundle (see also Fig. 27e) and this might explain the in-concert movement of adjacent kinetochores in living cells (Bajer and Molè-Bajer, 1956). Of course, MTs in these examples need not be directly involved in force production and may only have a supportive role (Forer, 1978a).

5.232 Microtubule and spindle elongation

Truly continuous nkMTs (MTs which individually span the entire pole-to-pole distance) are virtually non-existent in ^{the} higher eukaryotes ^{studied to date}. In *Iris*, most nkMTs are found in the middle region of the spindle since in the MT distribution graphs, MT numbers fall to low levels at the poles. At metaphase I, for example, only 6% of the total nkMTs could run from pole to pole. In fact, it is unlikely for a single MT to be that long (80-100 μm); the longest seen in this study was 5 μm (Fig. 41a), and most nkMTs in *Pales*, for example, are quite short: Fuge (1974) estimates a mean nkMT length of 3.0 μm in metaphase and 2.6 μm in anaphase.

Computer facilitated MT trackings in serial sections of mammalian cells (McIntosh *et al.*, 1975b) and of diatoms (McDonald, Pickett-Heaps, McIntosh and Tippit, 1977; McDonald, Edwards and McIntosh, 1979; McIntosh, McDonald and Edwards, 1978; McIntosh, McDonald, Edwards and Ross, 1979a; Pickett-Heaps and Tippit, 1978), and high voltage EM (HVEM) of mammalian cells (McIntosh, Siskin and Chu, 1979b) show that in these cells nkMTs consist of two groups of MTs emanating from each pole and overlapping at the equator. The zone of overlap is "sloppy" since the nkMTs are of variable lengths. Only one or two MTs stretch from pole-to-pole in the very small (5-6 μm) *Diatoma* spindle. These results have suggested that nkMT-nkMT sliding in the central spindle region contributes largely to spindle elongation at late anaphase (McIntosh *et al.*, 1979a). Spindle elongation is not a universal feature of mitosis (e.g., it is absent in anaphase I of *Allium* and in anaphase II of *Iris*, section 4.1; see also Mazia, 1961), but where it does occur the nkMT distribution is not always so easily related to nkMT sliding as in mammalian and diatom spindles. The nkMT central spindle in *Pales* is composed of an array of quite short MTs with no zone of overlap at the equator (Fuge, 1977), yet an overlap is required on any model of MT sliding. There is some spindle elongation (15%) in *Haemanthus*, but nkMTs are arranged in discrete bundles crossing the interzone at anaphase. Here, there is a minimum of MT numbers at the equator, instead of the maximum required for MT sliding (Jensen and Bajer, 1973).

There is negligible spindle elongation during anaphase II in *Iris*, yet the nkMT distribution is most similar to that of *Haemanthus*, where there is elongation. If the nkMT distribution in *Haemanthus* is directly related to spindle elongation the situation in *Iris* becomes anomalous. Of course, it might be argued that elongation has been secondarily lost in the evolution of the *Iris* spindle, but an equally plausible alternative might be that there is no direct relation between nkMT distribution and spindle elongation. Indeed, while the nkMT distribution in mammalian cells is consistent with spindle elongation via MT sliding, other interpretations, such as MT polymerization, are possible. Also the nkMT distributions found in elongating *Pales* and *Haemanthus* spindles are quite different from each other and from those of mammalian spindles. Clearly, any model loses its appeal once its universality is lost, but of course that does not mean that it is necessarily wrong (see Pickett-Heaps and Bajer, 1977). Another interpretation of nkMT distribution, however, might be that nkMTs provide a structural support for the spindle. This could be provided by a number of different but equally effective nkMT distributions. This interpretation implies the involvement of a different force producer, or at least a different method of force production in spindle elongation. Actin has been proposed as a mitotic force producer (see Introduction on p. 27 for review) and could be involved in spindle elongation as well as chromosome-to-pole movement. An alternative view could be that MT polymerization could provide the force for spindle elongation (Inoué and Ritter, 1978). Certainly, even in mammalian cells, some MT polymerization probably takes place since the spindle elongates by as much as eight times (McIntosh *et al.*, 1975b). On this view the actual distribution of nkMTs within the spindle would matter little, so long as polymerization at one or both ends, or even in the middle of the MT, were possible.

5.233 Kinetochore MT length changes

Most kMTs are shorter than the kinetochore-to-pole length, since graphs of MT distribution fall to low levels at the poles. A maximum of 10% of the kMTs might span the kinetochore-to-pole distance at metaphase I in *Iris*, although this percentage is probably somewhat lower since both nkMTs and kMTs would be present at the pole. The percentage of possible kMTs at the pole rises to a maximum of about 66% at mid anaphase I. This indicates a shift polewards of kMT protein (Fuge, 1973), either by translocation of whole MTs, or MT depolymerization and repolymerization. Similar conclusions can be drawn from MT distribution graphs in other organisms (Fuge, 1973 ; Jensen and Bajer, 1973; McIntosh *et al.*, 1975b). In

conjunction with this, kMTs must shorten or be depolymerized during anaphase, for otherwise kMTs would "pierce" the polar regions (Fuge, 1977). Several long kMTs were observed in metaphase spindles of *Allium* (Fig. 40), which were longer than the eventual kinetochore-to-pole distance at late anaphase. Jensen and Bajer (1973) also note that the average length of kMTs decreases during anaphase in *Haemanthus*. These observations most clearly support a theory of chromosome movement via MT polymerization and depolymerization, although similar changes in kMT distribution are also expected on other theories where MTs have a different (sliding, zipper or structural) role.

5.234 Lateral interactions between chromosomes and MTs

Fuge (1972, 1974, 1975) describes lateral filamentous connections between nkMTs, and regular longitudinal flanges on half-bivalents at late anaphase I, and on experimentally induced akinetochoric chromosomal fragments. Intimate associations of MTs and chromatin have not been reported in other spindle types, but some associations can be seen in *Iris* (Fig. 28b, 41a). These are most easily seen in regions where shrinkage has taken place during fixation. Shrinkage in large cells during fixation is probably unavoidable and, in general, produces wavy MTs (Bajer and Molè-Bajer, 1972). If a MT is held at several points during shrinkage via MT-chromatin bridges, break-

5.24 Kinetochore MT numbers

Kinetochore MT numbers were estimated by Moens (1979) for several species of grasshopper. He found a statistically significant difference in the number of kMTs in *Locusta* between metaphase I cells (12.5 kMTs per kinetochore; i.e. 25 per sister kinetochore pair) and cells in metaphase of mitosis (21 kMTs per kinetochore). This difference was observed even though the volumes occupied by the chromosomes in each spindle were approximately the same.

Moens (1979) noted distinct, functional, sister kinetochores in transverse sections of metaphase I cells and thus was able to exclude the obvious explanations for his results that only one of the two sister kinetochores is functional or that the kinetochore region in each half-bivalent is undivided. He concludes that "for unknown reasons, kinetochores at meiosis I initiate fewer MTs than at spermatogonial mitosis" (Moens, 1979, p. 561).

The estimates of kMT number per kinetochore in *Iris* PMCs (Table 5, section 4.23) were different for metaphase I and metaphase II (62.5 and 83 respectively). The trends are the same as those noted by Moens (1979) in that there are less kMTs per kinetochore at the stage when the kinetochore region is double versus when it is single. However, the difference was not statistically significant.

5.25 Membrane distribution

The presence of membrane in the spindle has been known since the studies of Porter and Machado (1960). The form and amount of membrane varies considerably with cell type, but it is probably a universal component of the mitotic apparatus of both plants (e.g. Pickett-Heaps and Northcote, 1966; Dietrich, 1968; Esau and Gill, 1969; Hepler, 1977, 1980) and animals (e.g. Cohen and Rebhun, 1970; and Harris, 1975). For a review of membrane in the spindle see Hepler (1977).

5.251 Origin and growth of spindle ER

Spindle ER in the second division of meiosis in *Iris* is derived largely from invaginations and evaginations of the inner and outer nms (section 4.241 and Figs. 33 and 34). It was also suggested that the amount of spindle ER approximately doubles from interphase to mid-prometaphase II (section 4.242).

The origin of spindle ER in the second meiotic division is envisaged as follows. During interphase and prophase II, membranes increase in size so that the area enclosed by invaginations and evaginations increases. After the breakdown of the nm at the beginning of prometaphase II, the

amount of membrane continues to increase until the entire spindle area is filled with irregular profiles of ER. A maximum amount of membrane is reached at mid-prometaphase II.

Several possibilities exist which could account for this increase in spindle ER as follows.

(a) Existing ER profiles might stretch so that their enclosed area increases. If this were so then one might expect the ER outline to become more circular in spindles with higher amounts of membrane, i.e. there would be a tendency towards a lower cross section area to volume ratio. The shape of ER profiles does not change from interphase through early to mid-prometaphase II (compare Figs. 33, 34 and 35), yet the relative area of membrane in these spindles (or future spindle regions) increased from 19.4 to 24.7 to 39.1% respectively (Table 7). Stretching of ER is therefore considered to be an unlikely explanation of the increase in amount of ER in these spindles.

(b) Membrane from the cytoplasm might invade the spindle region. However, most ER observed in the spindle region at the beginning of prometaphase II was continuous with the nm and therefore not of cytoplasmic origin. There was also an increase in the amount of membrane in this spindle from that in interphase (Table 7), and this increase at least cannot be accounted for by invasion of cytoplasmic ER. Invasion of cytoplasmic ER into the spindle, however, might still account for the ER increase in early to mid-prometaphase II.

(c) Existing membrane might grow. This is the best interpretation of the observation that ER area increases without change in ER shape, and it must account for the increase in ER from interphase to early prometaphase II. It is likely, although not proven, that membrane growth also accounts for later increases in amount of ER in the spindle.

The fragmentation of the nm into components of spindle ER has been documented previously (Esau and Gill, 1969; Roos, 1973a; Hepler, 1977; and Lambert, 1980), but to my knowledge the extensive invaginations and evaginations described here have not been previously reported.

The origin of spindle ER in the first division of meiosis is not clear since data from only one prometaphase I cell was available. The amount of membrane in this spindle (Fig. 38a) is less than in prometaphase II and is almost exclusively confined to regions containing MTs. These observations suggest an origin of first division spindle ER different from that in the second division of meiosis, but further study is required.

5.252 Changes in spindle ER distribution

The ER distribution clearly changes during meiosis II. Randomly arranged cisternae of ER become aligned along the interpolar axis during mid-prometaphase II. By metaphase II, ER in the spindle is aligned alongside kMTs, is abundant and unaligned at the poles, and is no longer abundant at the spindle periphery. These observations suggest ER is moved in the spindle. This might occur as follows.

The randomly oriented ER cisternae at early-prometaphase II are passively incorporated into pole-to-pole alignment by the developing spindle fibres (which consist at least of MTs) as follows. During later stages of prometaphase and metaphase II, MT bundles occupy more and more space in the spindle and ER becomes confined to the long narrow regions between MT bundles. During this latter stage of alignment, ER cisternae actively move or are moved polewards, where they accumulate and lose their aligned nature. Some cisternae may ~~breakdown~~ at the pole or be incorporated into the cytoplasm. Membrane at the equator at anaphase II will no longer be constrained by large bundles of MTs and will assume an unaligned structure.

The following observations support the above hypothesis.

- a. ER is unaligned at early-prometaphase II yet short MTs, which are aligned along the interpolar axis, are present (Fig. 34).
- b. At later stages of prometaphase II, more MTs are present and the ER is more aligned (Figs. 35a and b).
- c. The polar accumulations of ER are larger at metaphase II than at prometaphase II (compare Figs. 35 and 36).
- d. The amount of membrane in the spindle at metaphase and anaphase II is less than at mid-prometaphase II (Table 7).

Translocation of remnants of the nm to the pole has been suggested for both animal cells (e.g. Roth, Wilson and Chakraborty, 1966) and plant cells (Esau and Gill, 1969; Hepler, 1980; Lambert, 1980). The alignment of ER cisternae alongside MTs has been reported for animal cells (Harris, P., 1975; and unpublished work in this laboratory by Mr A. Harris on cricket spermatocytes) as well as plant cells (Hepler, 1977; review). Polar aggregations of membrane are common in spindles of plant cells (Hepler, 1977; review).

On the above hypothesis, the movement of membrane would comprise two parts: (i) passive alignment and (ii) active transport. Passive alignment is due to ER being confined to increasingly narrower regions between MT bundles during prometaphase II, while active transport to the poles requires the action of a force on the ER cisternae. The mechanisms of this force is not known but it could be similar to that causing poleward movement of

granules in the spindle, as reviewed by Nicklas (1971). Lambert (1980) reports that small nm remnants are surrounded by MTs and are actively transported polewards during prometaphase I in spore mother cells of the moss *Mnium*. However, she does not suggest any possible mechanism for this transport.

The sequence of events proposed above to explain changes in membrane distribution in the second division of meiosis are consistent with the results in *Iris* and with other published data, but other interpretations are possible. For example, selective breakdown of some cisternae between the chromosomes and the poles, and concurrent growth of other cisternae at the pole could explain both the redistribution and the change in amount of membrane from mid-prometaphase II to metaphase II. But this interpretation does not explain the observation that ER becomes aligned on the spindle, and it would also require a high degree of control of ER metabolism in the spindle. While such metabolic controls may well exist in the spindle, there is no evidence, at present, that they do. The hypothesis proposed here, however, requires only membrane growth throughout the spindle, and the presence of forces in the spindle.

5.253 Membrane bound vesicles

In *Iris* and *Tradescantia*, the small vesicles found associated with MT bundles appear to be derived from golgi bodies (Fig. 41; section 4.243). Golgi bodies are commonly found throughout the spindle and they are often considered part of the cellular membrane system (the "endomembrane system" Morr and Mollenhauer, 1976). It may be, therefore, that the golgi derived vesicles have a similar function to that of ER (see section 5.254 below).

Two types of membrane bound vesicles were described in *Allium* spindles. The smaller ones were similar to those described above for *Iris* and *Tradescantia* and appear also to be derived from golgi bodies (Fig. 41c). The origin of the larger vesicles (See e.g. Fig. 41c) is not known.

5.254 Membrane function

Several possible roles for ER in the spindle have been proposed (Hepler, 1977).

a. Chance. Membrane might simply be trapped in the spindle during spindle formation and have no function. While this may be true, it is unlikely, due to the apparent ubiquity of membranes in higher eukaryotic spindles (Harris, 1975).

- b. Structural support. Hanzely and Schjeide (1973) have suggested that ER provides a structural support or an anchor against which the spindle might push or pull. If membrane is shifted polewards during the second meiotic division in *Iris*, as suggested above, it is unlikely for membrane to also have a structural capacity. Also, membranes are easily deformed (see e.g. the micromanipulation experiments of Nicklas and Staehly, 1967) and would be unlikely to provide support to the spindle.
- c. Tubulin transport. Cisternae of ER might transport tubulin subunits into the spindle and thus take part in MT assembly (Burgess and Northcote, 1968). This might explain the close association of ER and MTs seen in this study (and others, lit. cit.) and is consistent with current theories of ER function (Morre and Mollenhauer, 1976).
- d. Control of calcium. Hepler (1977, 1980) draws a parallel between ER in the spindle and the sarcoplasmic reticulum (SR) of muscle. Hepler (1980) suggests that ER "regulates the level of free Ca^{++} in the spindle and thus controls either the assembly and/or activation of the motile machinery". There is good evidence for this view since, as reviewed in the introduction, the total quantity of calcium in the spindle is too high to be compatible with the presence of MTs (Forer, Gupta and Hall, 1980), and therefore Ca^{++} is probably sequestered in the spindle. This is because in general an increase in the concentration of Ca^{++} in a cell will cause the MTs to depolymerize, and in dividing cells this usually causes retardation of mitosis (see section 1.24). The results of Silver, Cole and Cande (1980) and Wick and Hepler (1980) (see section 1.24) support the view that ER sequesters Ca^{++} from the spindle matrix.

5.3 CONCLUSION

These studies provide a groundwork in technique for: the LM study of living PMCs; the EM examination of thin sections of cells at known developmental stages and at known planes of section; and for the correlative living cell and EM of one and the same cell or chromosome). Three-D reconstructions of kinetochore structure and MT distribution from serial LS's, and MT distribution graphs from MT counts of TS's, are also possible. The way is now open for future studies.

A particularly interesting problem concerns the relationship between kinetochores and the rest of the bivalent at the first division of meiosis. In the *Allium* metaphase I bivalent in Fig. 14b (see also LM insert), the two sister kinetochore pairs are at opposite ends of the diamond shaped bivalent. The two chiasmata are apparently at the equator (at the other two corners of the diamond). Sister chromatids in each half-bivalent should therefore be one above the other in this plane of section (see e.g. Moens,

1979, Fig. 1d). Why, therefore, can both sister kinetochores be seen at the upper end of the bivalent in this one section? Should not the sister kinetochores also be one above the other, one for each chromatid?

Possibly my interpretation of the number of chiasmata in the bivalent, and its identification as a pair of metacentric chromosomes, are in error. But this possibility only serves to emphasise the need for detailed study of chiasmata and centromere position in the bivalent in the LM; and for subsequent EM study of the same bivalent. Such a study would require 3-D reconstruction not only of the kinetochores but also of the whole bivalent.

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Appendix 1. Graph of anaphase I movement, cell 16-10, Iris spuria.

Appendix 2. Graph of anaphase II movement, cell 16-12, Iris spuria.

APPENDIX 1. IRIS ANAPHASE I, CELL 16-10 APPENDIX 2. IRIS ANAPHASE II, CELL 16-12

Appendix 3. Graph of anaphase I movement, Allium triquetrum, cell 8-3.

Appendix 4. Graph of anaphase II movement, Allium triquetrum, cell 5-3.

APPENDIX 3. ALLIUM ANAPHASE I, CELL 8-3 APPENDIX 4. ALLIUM ANAPHASE II, CELL 5-3