Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Chromosome Distribution: Experiments on Cell Hybrids and In vitro [and Discussion]

R. B. Nicklas and K. Jones

Phil. Trans. R. Soc. Lond. B 1977 **277**, 267-276
doi: 10.1098/rstb.1977.0017

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: **http://rstb.royalsocietypublishing.org/subscriptions**

This journal is © 1977 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

Phil. Trans. R. Soc. Lond. B. **277**, 267–276 (1977) [267]
Printed in Great Britain

Chromosome distribution: experiments on cell hybrids and *in vitro*

By R. B. Nicklas
Department of Zoology, Duke University, Durham, North Carolina 27706, U.S.A.

[Plates 1–4]

Östergren (1951) provided a simple explanation for both chromosome distribution in mitosis and chromosome segregation in meiosis, and more recently a molecular extension of his hypothesis has been possible. This report focuses on experimental tests of these ideas. Micromanipulation experiments on cell hybrids containing both meiotic and mitotic spindles demonstrate that differences in meiotic and mitotic chromosome behaviour are determined by something intrinsic to the chromosome: meiotic chromosomes transferred to a mitotic spindle (or vice versa) behave just as they normally would. The molecular explanation postulates polarized growth or binding of microtubules at kinetochores. This has just been tested *in vitro* by McGill & Brinkley (1975) and by Telzer, Moses & Rosenbaum (1975), and their results are reviewed. In addition, a novel method for *in vitro* studies is described – mechanical demembranation of cells which is compatible with quite normal chromosome movement in anaphase. After addition of microtubule subunits to a demembranated prophase cell, chromosome orientation and movement toward an aster was observed for the first time *in vitro*.

It is concluded that important aspects of chromosome distribution are probably understood at both the cellular and molecular levels, but final tests are still required. The outlook is hopeful indeed because the gaps in our knowledge are well known – the necessity of observations on prophase is a recurrent theme – and the means of filling the gaps are in hand.

(At the discussion meeting itself, part of this work was presented as a ciné film.)

The mechanisms of equitable chromosome distribution in mitosis and meiosis have recently been extensively reviewed (Luykx 1970; Nicklas 1971, 1974). Here I will consider only the initial interactions of chromosomes with the spindle just after the breakdown of the nuclear envelope. However, these interactions determine not only the appropriate distribution of most chromosomes in both mitosis and meiosis but also the reduction of chromosome number in meiosis. The emphasis is on *in vivo* micromanipulation experiments in cell hybrids only briefly reported earlier, and on recent *in vitro* studies of isolated chromosomes and spindle fibre subunits. The discussion is limited to orthodox chromosome distribution mechanisms in organisms with localized kinetochores.

Cellular aspects of chromosome distribution

Background

Chromosome distribution depends upon the association of each chromosome with the spindle via chromosomal spindle fibres – microtubules – which run from a particular site or sites on each chromosome, the kinetochore or centromere, toward one pole. This 'orientation' of a particular chromosome to a given pole determines the pole to which that chromosome will move in the ensuing anaphase and therefore determines which daughter cell will receive that chromosome. The usual, equal distribution of chromosomes depends upon orientation of paired

The Royal Society is collaborating with JSTOR to digitize, preserve, and extend access to *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences.* STOR www.jstor.org

Downloaded from rstb.royalsocietypublishing.org

half-bivalents in meiosis, or of sister chromatids in mitosis, toward opposite poles: 'bipolar orientation'. In consequence, each daughter cell receives one representative of each chromosome.

Bipolar orientation is achieved flawlessly by most bivalents in meiosis at their first encounter with the developing spindle (Bauer, Dietz & Röbbelen 1961). How? First, suppose that any one half-bivalent tends to orient toward the pole its kinetochores more nearly face; for instance, if the kinetochoric end of a half-bivalent points straight toward one pole, then association with that pole via chromosomal spindle fibres will almost invariably follow (Östergren 1951). This proposition has passed a direct test by micromanipulation (Nicklas 1967). Second, suppose that when the kinetochores of one half-bivalent face one pole, those of the partner half-bivalent must face the opposite pole simply because bivalents are so constructed. This structural constraint has long been recognized (see, for example, figure 1*a*). Thus since each half-bivalent preferentially orients to the pole its kinetochores more nearly face, and bivalent structure constrains the kinetochores of partner half-bivalents to face in opposite directions, the flawless initial orientation of most bivalents is assured.

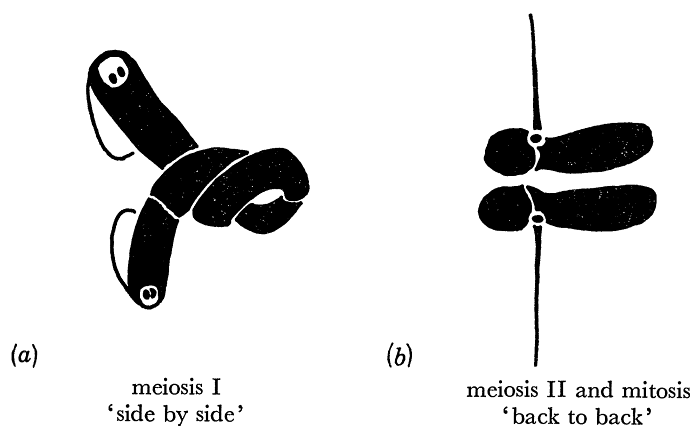


FIGURE 1. The arrangement of sister kinetochores in the first meiotic division contrasted with that in the second meiotic division or mitosis, as seen in *Amphiuma* spermatocytes. (After Schrader 1936; drawing by Dr D. Wise.)

The proposed explanation is readily extended to mitosis and to the difference in chromosome distribution between mitosis and meiosis (Östergren 1951), by using observations on kinetochore arrangement first made by Schrader (1936). Preferential orientation to the pole a given kinetochore or pair of kinetochores most nearly faces is assumed to operate in mitosis as in meiosis. The only difference is illustrated in figure 1: individual sister chromatids in mitosis have their kinetochores arranged back to back while in the first meiotic division they lie side by side. In mitosis, therefore, sister kinetochores tend to orient to opposite poles and one chromatid is distributed to each pole in anaphase. In contrast, at the first meiotic division, the reduction in chromosome number comes about because the closely appressed sister kinetochores orient to the same pole and the two chromatids are later distributed together to that pole. Later, in the second meiotic division, sister kinetochores lie back to back as in mitosis and hence the two chromatids are distributed to opposite poles. This explanation is straightforward and not in conflict with present knowledge, but present knowledge is incomplete because we lack a direct experimental demonstration of preferential orientation in mitosis, due to technical difficulties (Nicklas 1971, p. 268; Roos 1976). The essential features of kinetochore position in mitosis and meiosis are now established (older literature: Nicklas 1971, p. 267; mitotic prophase: Roos 1973; Heneen 1975). However, definitive evidence on kinetochore number and structure

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

in late meiotic prophase is lacking. For ease of exposition, meiotic prophase kinetochores will be assumed to be double as in mitosis and to differ only in their arrangement (figure 1). This is very likely if not certainly so, and anyway it is not essential for the present explanation of chromosome distribution. Observations on bivalents in prophase are especially important (see below) and careful electron microscopic investigations are required (Roos 1975). Parenthetically, compelling electron microscopic evidence for doubleness of sister kinetochores in meiotic *prometaphase* has been presented by Müller (1972).

Meiotic × mitotic cell hybrids

Östergren's (1951) simple explanation for the reduction of chromosome number in meiosis based on altered kinetochore arrangement was challenged by Lima-de-Faria (1958), who proposed instead that general physiological differences between meiotic and mitotic cells were responsible. Obviously, developmental changes in meiotic versus mitotic cells must be the ultimate cause of all observed differences, including the difference in kinetochore arrangement. At issue here, however, is the *immediate* cause: factors intrinsic to the chromosomes themselves (Östergren) or to the whole cell (Lima-de-Faria). The issue could be decided directly by transferring a meiotic chromosome to a cell in mitosis and vice versa. I attempted this and did not succeed, but the next best experiment was possible – the transfer of either a bivalent or a mitotic chromosome from one spindle to another in a meiotic × mitotic cell hybrid. This work was briefly reported earlier without photographic documentation (Nicklas 1971).

Grasshopper spermatocytes in the first and second meiotic divisions can sometimes be fused by micromanipulation to produce one cell containing two spindles – one spindle with bivalents, the other with unpaired chromosomes. In each spindle, the kinetochores display their usual orientation (i.e. as in figures 1*a* and 1*b* respectively). Fusion was achieved (occasionally!) by using a microneedle to bring a small area of the membrane of two cells into contact and then vigorously massaging one cell's membrane against the other's. Examples of fused cells are shown in figures 2 and 3, plate 1. First, division proceeded normally in hybrid meiosis I and II cells. Not only were hybrid cells as viable as adjacent unfused controls, but the chromosomes on each spindle were distributed as in the respective control cells. These facts by themselves argue against an immediate effect of postulated physiological differences. Second, as illustrated in figure 2, a bivalent, detached by micromanipulation from the division I spindle and placed near the division II spindle, oriented on the 'heterologous' spindle as it normally would on the division I spindle. Hence in anaphase the bivalent divided normally – two chromatids moved together to each pole – while on the same spindle all the other chromosomes divided in *their* normal fashion – a single chromatid moved to each pole. Five additional experiments of this type have been performed with identical results. Third, the reciprocal experiment has been done once, and is illustrated in figure 3. The second meiotic division chromosome oriented and divided as it normally would, though surrounded by bivalents behaving differently. Significantly, the manipulated chromosome or bivalent oriented according to its own specifications even when, as in both examples illustrated, it was placed at an angle to the heterologous spindle which should favour the contrary orientation.

In one cell fusion experiment, three prophase or *very* early prometaphase bivalents were transferred to a second division spindle; all three oriented and divided as bivalents usually do. More such experiments are needed. In fact, only observations on bivalents or mitotic chromosomes from prophase nuclei are definitive because we must be positive that the peculiarities of

Downloaded from rstb.royalsocietypublishing.org

chromosome behaviour are determined by intrinsic chromosome properties that exist before chromosome/spindle interaction. After prophase, any properties intrinsic to the chromosomes could be a result, not a cause, of a previous specific orientation on the spindle. Experiments with prophase chromosomes are not merely a formal necessity because we already know one circumstance in which interaction with the spindle seems to modify chromosome organization. Sister kinetochores sometimes orient to opposite poles in the first meiotic division (for review and a brief speculation on the causes, see Nicklas 1971, p. 270). Obviously, the usual spindle fibre forces might then separate the originally closely appressed sister kinetochores (Östergren 1951, Figure 132 and associated text) and preliminary electron microscopic studies (Müller 1972; Wagenaar & Bray 1973) are consistent with this possibility. For the same reason, *observation at prophase* is a criterion for definitive morphological studies of kinetochore number and disposition mentioned above, as well as for the molecular experiments described below.

The present cell fusion experiments therefore are not conclusive but they strongly suggest that a property intrinsic to the chromosome (kinetochore position, surely) is the immediate cause of the essential consequence of meiosis – the reduction in chromosome number. Some ancillary observations on fused spermatocytes are worthy of note. The first two are illustrated in the cell in figure 3, plate 1, and are found in all cells which were fused 1 h or longer before anaphase began. First, anaphase begins synchronously in both spindles of such cells, a well-known feature of most spindles sharing a common cytoplasm (for especially interesting examples, see Barber 1942). In the present case, synchrony arises because the spindle normally

Description of plate 1

Figures 2 and 3. Living cell-hybrids of first and second meiotic division spermatocytes from the grasshopper *Dissosteira carolina*. The time in minutes is given on each print. The pattern of chromosome distribution is especially clear in these cells because in the light microscope all the chromosomes have invisibly short second arms. Hence in anaphase, all V-shaped chromosomes (e.g. figure 2, the open arrowheads, 5 min print) in fact are two chromatids segregating together, while a rod shape characterizes single chromatids being distributed as in mitosis (e.g. figure 2, the arrow on the 0 and 5 min prints). Naturally, this distinction is not always possible from the single focal level shown in the photographs. For cell culture, recording and micromanipulation methods, see Nicklas & Staehly, 1967. The bar (figure 2, 7 min, and figure 3, 47 min print) equals 10 μm.

Figure 2 (above). Behaviour of a meiotic bivalent transferred from a first division to a second division spindle. The filled arrowheads on the middle print, lower row, identify the poles of the spindles; the second division spindle lies above the first, is perpendicular to it, and is slightly tilted vertically so that the chromosomes moving to the lower pole are out of focus on the other prints. Anaphase began only a few minutes after fusion and is already in progress in the second division spindle in the first print shown (0 min). Just before this, a bivalent had been detached by micromanipulation from the first division spindle and placed adjacent to the second division spindle; the kinetochoric ends of this bivalent are identified by the open arrowheads on the 0, 5, and two 17 min prints. The bivalent oriented (0–5 min prints) on the second division spindle just as it normally would in the first meiotic division and so the partner half-bivalents segregated to opposite poles (5–17 min prints). Meanwhile, the other chromosomes on the same spindle are distributed as single chromatids (arrow, 0 and 5 min prints).

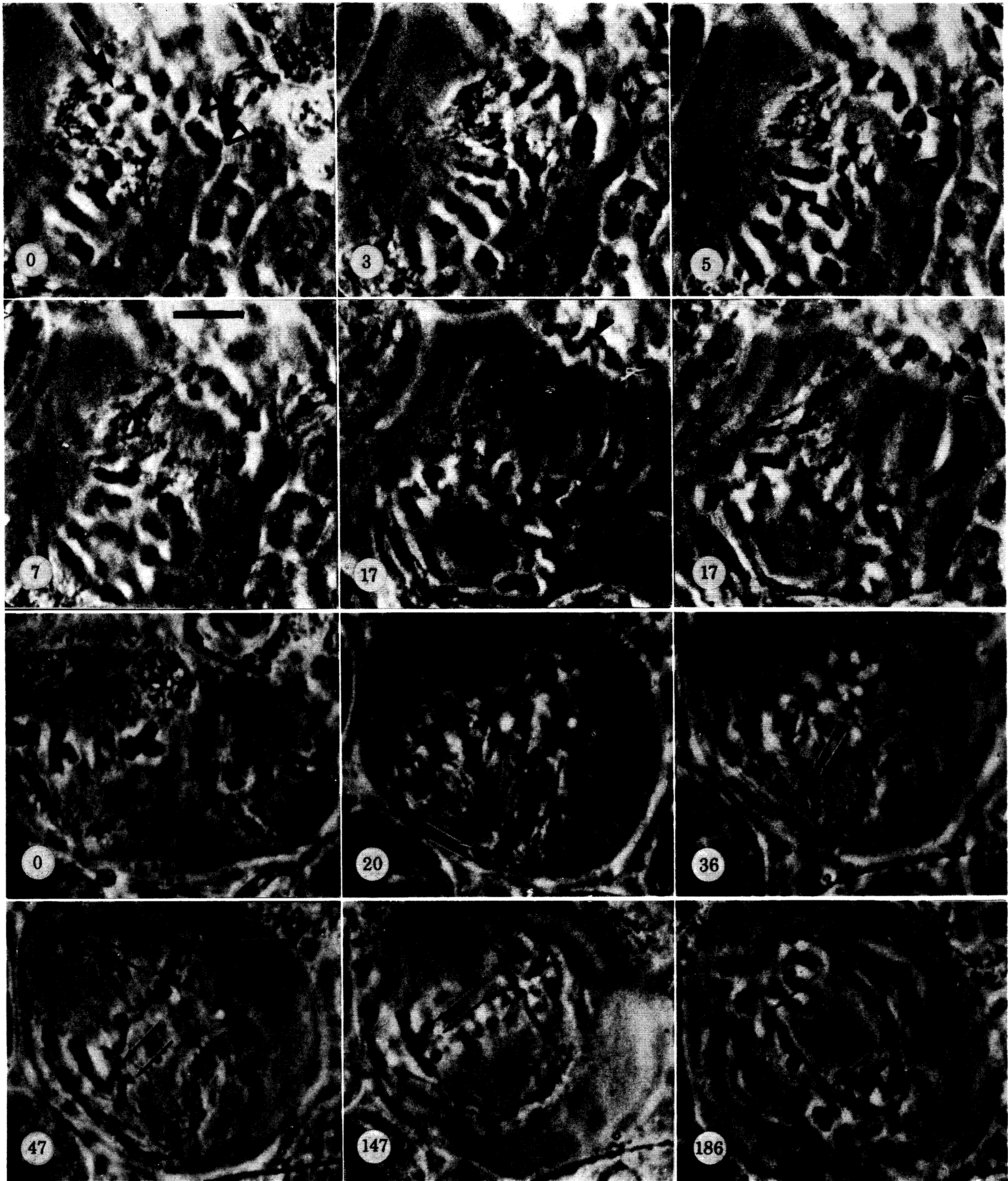
Figure 3 (below). The reciprocal experiment: a second meiotic division chromosome transferred to a first division spindle. The first, 0 min, print shows the two spindles lying side by side at a slight angle to one another with the division II spindle on the right. The arrows on every print thereafter identify the kinetochoric end of both chromatids of a chromosome detached from the division II spindle and placed near the division I spindle. Eventually the two spindles merged (see below), but before this happened the two chromatids of the transferred chromosome oriented toward opposite poles of the division I spindle (36 min print). The chromosome then moved to the equator (36–147 min prints) and divided in anaphase – the movement of one chromatid toward each pole is clear despite the non-specific stickiness that prevented final separation. Note that the two originally separate spindles (0 min print) moved together and eventually merged, establishing the common spindle axis clearly evident in anaphase (186 min). Thus at 0 min the distance from the left edge of the division I spindle to the right edge of the division II spindle was approximately 43 μm, while the width of the unified spindle in metaphase (147 min) was approximately 25 μm.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



FIGURES 2 AND 3. For description see opposite.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

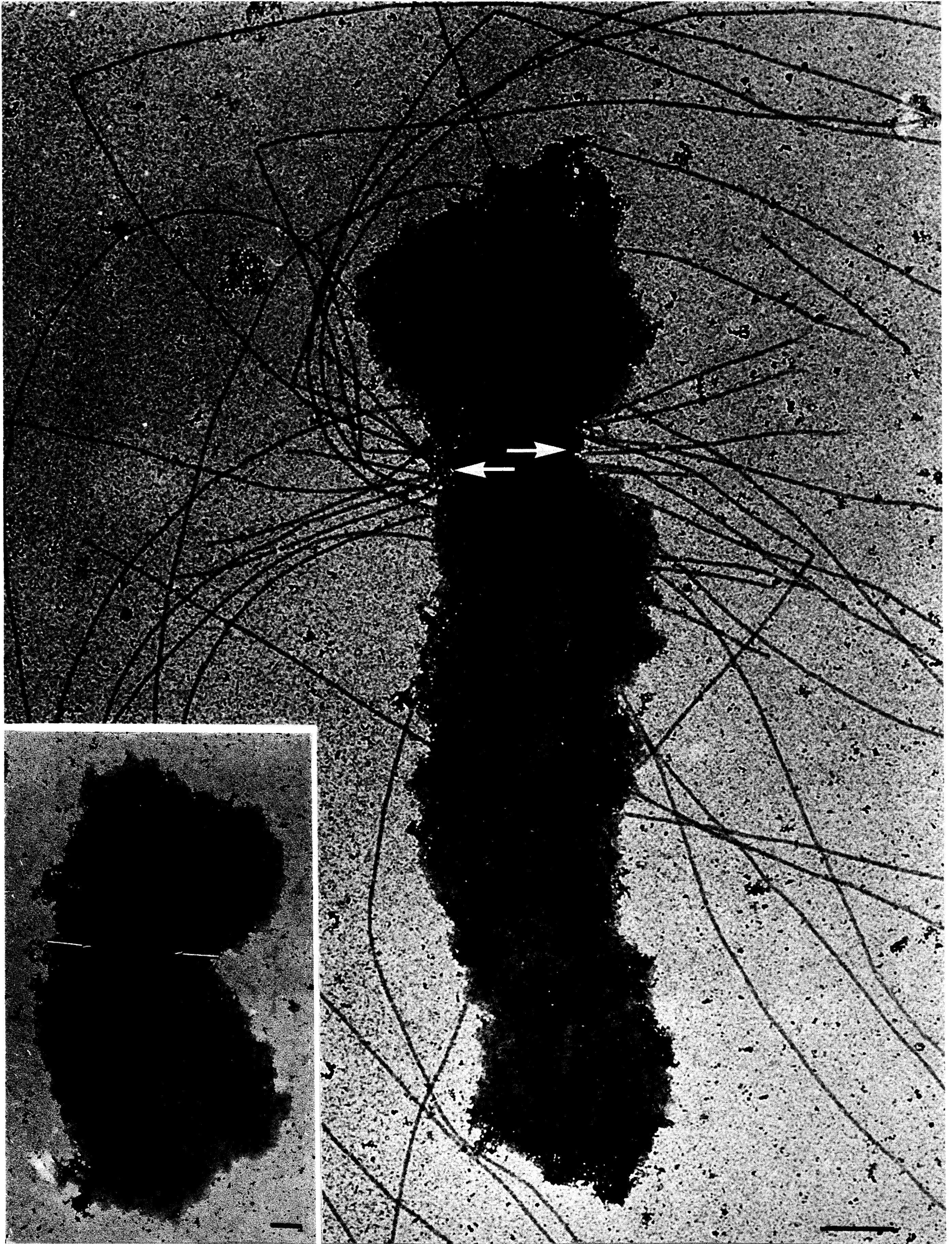


FIGURE 4. For description see opposite.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

having the shorter prometaphase–metaphase duration (division II) does not begin anaphase on schedule, but 'waits' for the spindle having the longer duration (division I). The record delay to date is more than five times the normal prometaphase–metaphase duration for division II (~1 h; the comparable value for division I is 3.5–6 h – data from unfused control cells). Second, the two spindles slowly come to lie parallel with one another, move together, and eventually meld so intimately that the larger first division spindle forms a saddle-shaped mantle about the second division spindle. This is particularly striking evidence for the tendency of microtubules, initially at various angles to one another, spontaneously to form a parallel array (review: Nicklas 1971, pp. 233–234). Third, the transfer of a chromosome or bivalent from one spindle to another is followed by orientation on the second spindle. This is incontrovertible proof that genuine chromosome detachment from the spindle can be induced by micromanipulation and that detachment can be followed by the spontaneous formation of unquestionably new spindle fibre attachments – in this instance to a totally separate spindle (this implication of the experiment was pointed out to me by Dr M. Y. Menzel of Florida State University; for earlier evidence on the point, see Nicklas 1967; 1971, p. 261).

Molecular aspects of chromosome distribution

Microtubule nucleation or binding in vitro

So far, our doubts and areas of ignorance have been stressed but I hope it is clear nevertheless that the cellular fundamentals of chromosome distribution are largely understood. What about a molecular explanation? More precisely, what is the molecular basis of preferential kinetochore orientation to the pole a given kinetochore more nearly faces? Proposal (McIntosh, Hepler & Van Wie 1969; Henderson, Nicklas & Koch 1970): the kinetochore is a polarized site for the nucleation of microtubule assembly or for the binding of already assembled microtubules. In mitosis, for instance, kinetochore polarity relative to the chromosome axis promotes the assembly or binding of microtubules which extend away from the chromosome in opposite directions from the two chromatids. These microtubules either already are, or tend to come into, parallel register with microtubules in the rest of the spindle by swinging through the smaller possible arc; this commonly produces the immediate orientation of chromatids to opposite poles.

As noted in the preceding sections, we already have some evidence that spindle microtubules tend to form a parallel array, hence the critical issue is whether polarized binding or nucleation occurs. Happily, two independent groups have just shown that direct *in vitro* tests are possible and have already obtained important results (McGill & Brinkley 1975; Telzer *et al.* 1975). These experiments were made possible by microtubule assembly *in vitro*, first achieved by Weisenberg (1972; recent studies are described by several authors in Goldman, Pollard & Rosenbaum 1976). In outline, a fraction from brain containing microtubule subunits – 'tubulin' – is mixed with isolated chromosomes (Telzer *et al.* 1975), or with whole cells in the presence of detergent to produce cell lysis (McGill & Brinkley 1975), under conditions permitting

---

Description of plate 2

Figure 4. Kinetochores and microtubule assembly *in vitro*. Main figure: an isolated chromosome from a HeLa (human) cell exposed to a chick brain tubulin preparation under conditions permitting microtubule assembly. Numerous microtubules extend from each kinetochore (arrows). Inset: the control – an isolated chromosome not exposed to tubulin. No microtubules are visible at the kinetochores. Both bars equal 0.5 μm. (From Telzer *et al.* 1975, with permission.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

microtubule assembly. Typical results are illustrated from the work of Telzer *et al.* in figure 4, plate 2. The chromosomes were isolated from cells in metaphase, but after isolation no microtubules were visible at the kinetochores (inset, figure 4). Six minutes after tubulin addition, however, numerous microtubules were found at the kinetochores (figure 4). In control preparations of the tubulin preparation incubated 6 min in the absence of added chromosomes no microtubules were found 'an indication that spontaneous microtubule assembly had not occurred' (Telzer *et al.* 1975). This suggests that the kinetochores of these chromosomes can nucleate microtubule assembly *in vitro* (Telzer *et al.* 1975). But just as important for the present discussion, the nucleation is *polarized:* the microtubules associated with the right-hand kinetochore (figure 4) extend predominantly to the right, those of the left-hand kinetochore to the left, before curving away in various directions (presumably due to mechanical factors present *in vitro* – cf. Telzer *et al.* (1975) on the distortion of kinetochore position). McGill & Brinkley (1975) obtained similar results but microtubules were observed at more diverse angles to the kinetochore, as they emphasize. Even so, it is plain that a polarity sufficient to account for considerable preference in orientation is present because the solid angle enclosing the microtubules associated with each kinetochore is less than 180°, always on the outward face of each chromatid. Neither McGill & Brinkley (1975) nor Telzer *et al.* (1975) discuss these questions of polarity and controlled chromosome distribution – the responsibility is mine.

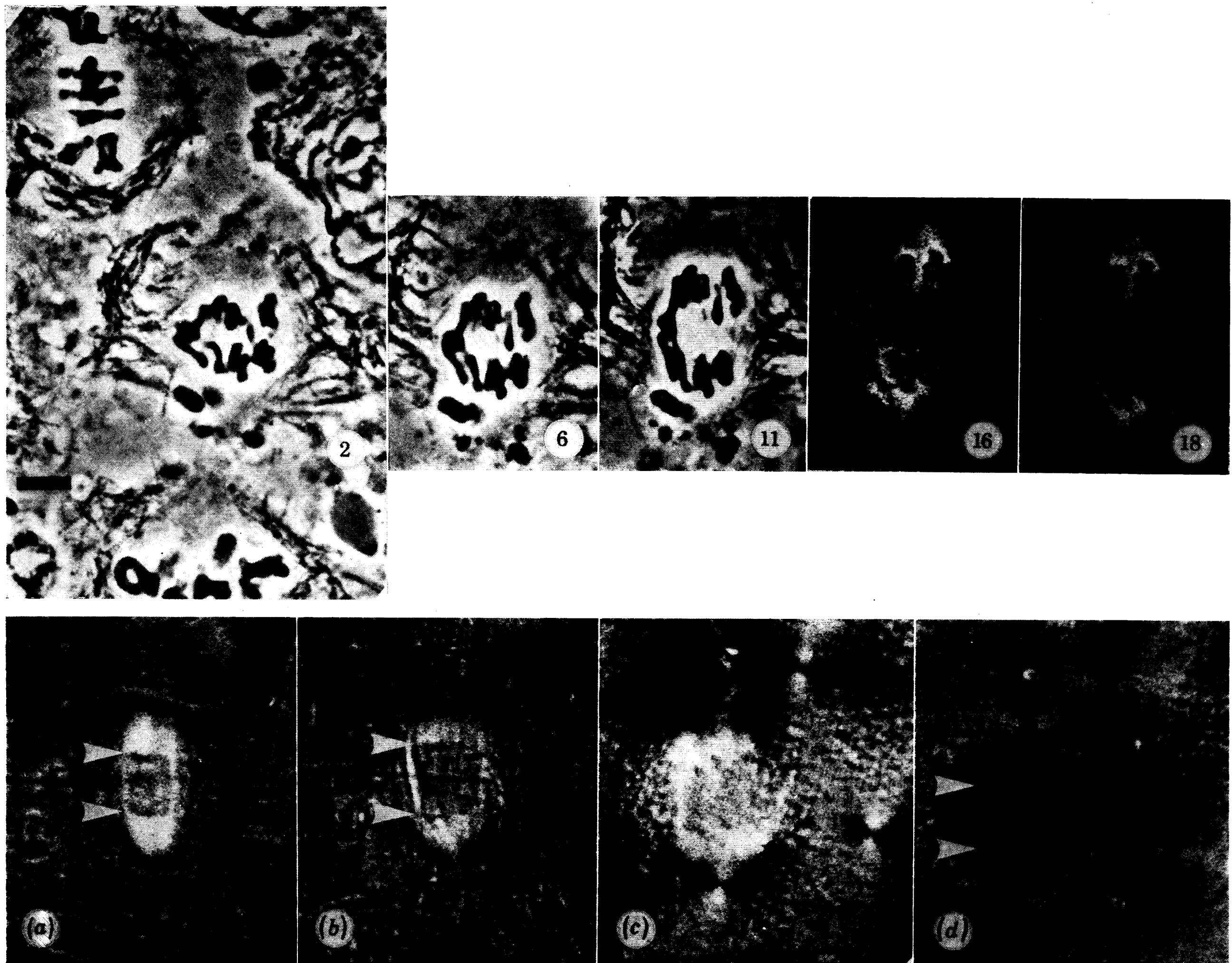
We have recently done similar experiments with meiotic bivalents, and obtained evidence for polarized microtubule nucleation or binding. Moreover, the bivalents differed as expected from mitotic chromosomes – from the pair of adjacent sister kinetochores of one half-bivalent, a single group of microtubules extended away from the chromosome with very little divergence in angle, while the microtubules from the kinetochores of the partner half-bivalent extended in the opposite direction (R. B. Nicklas, D. F. Kubai & H. P. Erickson, unpublished). The results will not be considered further because the experiments share two defects with the studies of Telzer *et al.* (1975). Both defects arise from the use of chromosomes from cells in metaphase. First, although the isolated chromosomes are free of detectable microtubule fragments (inset, figure 4), the kinetochores could well contain materials capable of nucleating microtubules which normal late prophase chromosomes do not possess. Second, only observations on prophase chromosomes can yield definitive information on the polarity of microtubule nucleation, for reasons given above. These problems were largely circumvented by McGill & Brinkley (1975) through the study of chromosomes in cells where spindle formation was suppressed by Colcemid. However, the evidence for kinetochore microtubule nucleation is not so compelling in the McGill & Brinkley (1975) study because microtubule assembly also occurred in the absence of chromosomes, providing a source of microtubules that could have bound to, rather than have been nucleated from, the kinetochores (some observations suggest true nucleation, however: McGill & Brinkley 1975, p. 197).

So some reservations are necessary but they do not diminish the achievements of the pioneers. They have given us not only highly suggestive observations, but also most of the methods required for conclusive experiments: prophase bivalents and mitotic chromosomes, reacting with tubulin preparations under conditions permitting either tubulin nucleation or microtubule binding but not both.

Orientation in vitro

Recently I have begun experiments using a novel method for preparing demembranated cell models. A few years ago I noticed that in some of our preparations of living spermatocytes,

Downloaded from rstb.royalsocietypublishing.org



Figures 5 and 6. Features of cells demembranated by a new method. A mechanical shock (see text) was delivered to cells in a well filled with halocarbon oil and viewed on an inverted microscope (Nicklas & Staehly 1967). The bar (figure 5, 2 min print) equals 10 μm and applies to both figures.

Figure 5 (above). Anaphase in a demembranated but otherwise untreated grasshopper (*Arphia xanthoptera*) spermatocyte. The time after demembranation is given in minutes on each print. Anaphase in the central spindle in the 2 min print is illustrated by phase contrast (2–11 min prints) and by polarization microscopy (16 and 18 min prints). The total increase in chromosome separation was 11 μm in 16 min, for an average speed of 0.7 μm/min (about two-thirds of that seen in control, undemembranated cells). By 16–18 min, spindle birefringence had decreased to about half the normal value.

Figure 6 (below). The consequences of adding tubulin to demembranated cricket (*Acheta domestica*) spermatocytes as seen by polarization microscopy. The arrowheads show the position of the chromosomes in anaphase spindles.

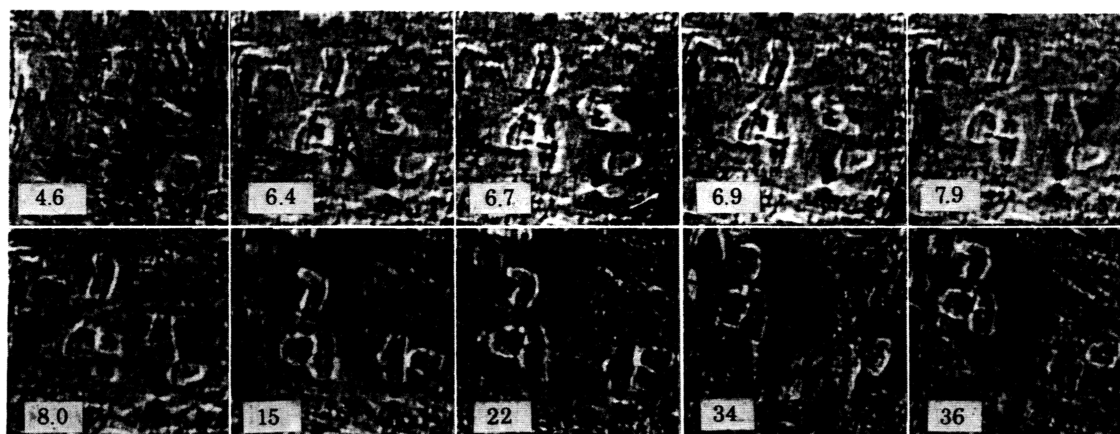
A control cell is shown (*a*) 0.5 min before demembranation and (*b*) 3 min after demembranation (*c*, *d*) Another preparation, showing an anaphase spindle and two asters (to the right) after tubulin addition in photographs made at opposite compensator settings. Note the striking enhancement of spindle and aster birefringence as compared with the control. The tubulin preparation was added 6 min after demembranation; the photographs were taken 5 min (*c*) and 18 min (*d*) after tubulin addition. The tubulin was purified from hog brain by one standard assembly/disassembly cycle, and was added to the demembranated cells at a concentration of 7 mg protein/ml in standard assembly medium (Erickson 1974). The tubulin was a gift of Dr H. P. Erickson.

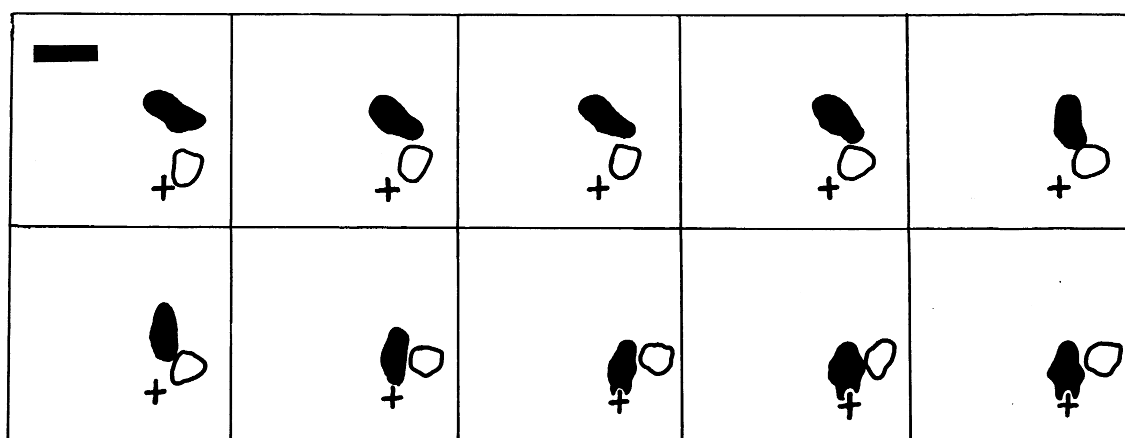
(*Facing p.* 272)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org





FIGURES 7 AND 8. Chromosome orientation *in vitro*. Chromosome behaviour can be followed most readily by reference to figure 8, using figure 7 for verification and whenever birefringence is in question. The bar (figure 8, upper left) equals 10 μm and applies to both figures.

FIGURE 7 (above). A slide containing a grasshopper (*Arphia xanthoptera*) spermatocyte in diakinesis was placed at 7 °C, the cells were demembranated as in figures 5 and 6, tubulin was added 10 min later, and 3 min after that the slide was returned to 22 °C. Bivalents from the cell in diakinesis are shown by polarization microscopy; the time in minutes after the return to 22 °C is indicated on each print. An aster is indicated by A on the 6.4 and 15 min prints. One bivalent oriented to that aster; the kinetochoric end involved is indicated by an arrow on the 4.6, 6.7, 7.9, 8.0, 22, and 36 min prints. The bivalent first showed a twitching movement toward (4.6–6.4 min) and then away from (6.4–6.7 min) the aster; the twitch is just visible in the prints but is very striking in the movie record. The bivalent then gradually swung toward the aster (6.7–8.0 min prints) and then moved straight toward the centre of the aster (8.0–36 min prints), bypassing a bivalent on the right. The bivalent moved initially at a velocity of 1.9–3.6 μm/min (4.6–8 min) and later at 0.28 μm/min (8–22 min). Birefringence developed between two bivalents identified by paired arrows on the 22 and 34 min prints. The 4.6 and 34 min photographs were made at the compensator setting opposite that used for all the others. The tubulin, a gift of Dr H. P. Erickson, was prepared and used as described for figure 6 except that two assembly/disassembly purification cycles were carried out and the concentration was approximately 3 mg protein/ml.

FIGURE 8 (below). A tracing of figure 7 showing the orientating bivalent (in black) as it moved toward the aster (+), passing a stationary bivalent (in outline), which later moved or drifted *away* from the pole. Drawn by Dr D. Wise.

Downloaded from rstb.royalsocietypublishing.org

BIOLOGICAL SCIENCES THE ROYAL SOCIETY PHILOSOPHICAL TRANSACTIONS OF

all the cells had lost their plasma membrane – nuclei and chromosomes floated free in the medium. The cause was soon traced to a mechanical shock delivered to the slide and the cells by my clumsy release of the spring-loaded metal 'finger' that holds the slide on the stage of the microscope. Equally accidentally I recently observed that anaphase can continue quite normally after such 'mechanical demembranation', in those areas of the preparation which contain very little fluid between the cells before demembranation. An example is shown in figure 5, plate 3. The first ('2 min') print shows portions of three spindles sharing a common cytoplasm. The total loss of plasma membranes is confirmed (1) by micromanipulation – it is easy to transfer mitochondria or chromosomes from one spindle to another, and (2) by the accessibility of the spindle to macromolecules (see below). Typical chromosome-to-pole movement is evident in figure 5 as well as normal chromosome and mitochondrial morphology as seen by phase contrast microscopy and the persistance of some spindle birefringence as seen by polarization microscopy. The only noteworthy differences from the intact cell are diminished chromosome speed and spindle birefringence, and very little or no spindle elongation. The result illustrated can be obtained without fail in selected cells. In the most favourable circumstances, not only does an anaphase already in progress continue, but, remarkably, anaphase is initiated in metaphase spindles up to 30 min after demembranation. Evidently chromosome movement is so nearly normal because wherever extracellular fluids are sparse before demembranation, the cell's interior situation is hardly altered afterwards: its cytoplasm contacts on one side the inert glass coverslip, on the other the equally inert halocarbon oil, and laterally only the cytoplasm from other cells.

Independently, Inoué and Fuseler have discovered a different method for producing demembranated cells, which does not involve mechanical shock: in *Haemanthus* endosperm preparations exposed to fluorocarbon oil, the cell surface materials spontaneously disperse. These membrane-less endosperm cells are very sensitive to mechanical disturbance, but not only do they continue anaphase and enter anaphase from metaphase, but also they show normal birefringence changes and the formation of a complete phragmoplast in late anaphase (S. Inoué, personal communication).

Both mechanical demembranation and the one previous method producing equally normal chromosome movement (Cande *et al.* 1974; McIntosh *et al.* 1975) yield a cell model nearly as complicated as the living cell but with one essential difference: the ionic and macromolecular environment of the spindle can be altered at will. Here this is accomplished by lowering a micropipette through the layer of halocarbon oil covering the demembranated cells and expelling a controlled volume of the desired medium wherever desired. An example of tubulin addition is illustrated in figure 6, plate 3. The striking enhancement of spindle and aster birefringence (figure 6*c*, *d*) due to assembly of heterologous tubulin has been observed in numerous previous investigations (Cande *et al.* 1974; Inoué *et al.* 1974; Rebhun *et al.* 1974; Snyder & McIntosh 1975). The chief differences between the two cell models for chromosome movement are: (1) mechanical demembranation, unlike detergent-induced cell lysis (Cande *et al.* 1974; McIntosh, Cande & Snyder 1975), permits continued chromosome movement after unequivocal, total loss of the cell membrane (probably via fragmentation and dispersal), but (2) only the detergent methods permit repeated, total replacement of one bathing medium by another.

Chromosome orientation has been produced *in vitro* for the first time by the addition of exogenous tubulin after mechanical demembranation (figures 7 and 8, plate 4). Unlike

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

detergent-induced cell lysis, mechanical demembranation usually causes the loss of the nuclear envelope as well as the cell membrane. Therefore, the behaviour of chromosomes or bivalents can be studied before normal spindle formation. The bivalents shown in figures 7 and 8 were in a cell demembranated in late prophase (diakinesis), when the spindle was represented only by small asters. After demembranation and tubulin addition, the bivalents were scattered and a single aster was seen, slightly larger than the asters observed *in vivo* at this stage (A, 6.4 and 15 min prints, figure 7). One bivalent (figure 8) unmistakably oriented to the aster: one end swung around until it faced directly toward the aster, and orientation was followed by continued chromosome movement to within 1 μm of the centre of the aster (for details, see figure 7 and legend). The bivalent moved a total of 11 μm at velocities within the usual range for related movements *in vivo*. The movement toward a pole with one kinetochoric-end foremost, and at the velocities observed, are diagnostic consequences of true chromosome orientation (see, for example, Bauer *et al.* 1961; Nicklas 1967). The absence of non-specific displacement is verified by the bivalent just to the right (figure 8) which remained stationary as the oriented bivalent moved past.

The birefringence observed here is due, mainly at least, to aligned microtubules. Birefringence developed between the kinetochoric ends of two bivalents that happened to be facing one another – see figure 7, 22 and 34 min prints, the small, paired arrows on the left. This provides some evidence for polarized microtubule nucleation or binding at the kinetochores of *prophase* bivalents, but final proof must await the electron microscopic studies now in progress.

We have high hopes for the experimental exploitation of orientation *in vitro*. The goals include the study *in vitro* not only of microtubule nucleation but also the later events in chromosome orientation and eventually the reconstitution of the whole spindle from its elements. An experiment we are especially anxious to try is the transfer by micromanipulation of a single bivalent or chromosome from a first to a second meiotic division spindle and vice versa. First, however, additional studies on the necessary preconditions for orientation *in vitro* are required because at present success is very rare.

Conclusions and prospects

The cellular and molecular basis of one aspect of chromosome distribution is now probably known, although conclusive proof is missing. The key elements are preferential kinetochore orientation based upon polarized microtubule nucleation plus differing kinetochore arrangements in mitotic and meiotic chromosomes. Together, it is argued, these two elements determine in part the equitable chromosome distribution in both mitosis and meiosis and determine *in toto* the fundamental difference between mitosis and meiosis – parity of chromosome number in parental and daughter cells in mitosis versus reduction of chromosome number in meiosis. And in the most straightforward, simplest manner imaginable.

From here it is no great leap to identify the kinetochore with a DNA sequence adapted to bind proteins which in turn mediate polarized chromosome attachment to the division apparatus (Nicklas 1971, pp. 279–284). Recent evidence strongly suggests that formally identical sequences exist in the chromosomes of prokaryotes (review: Liebowitz & Schaechter 1975): a functional, and possibly an evolutionary, continuum extending across one to two billion years of divergence. The difference between pro- and eukaryotes is equally clear on this formulation – for 'division apparatus' read 'cell membrane' in the prokaryotes and 'spindle' in the eukaryotes (review: Kubai 1975).

Downloaded from rstb.royalsocietypublishing.org

Now what? A first step must be final tests of the hypothesis of kinetochore orientation. The necessary information has been identified – observations in prophase on kinetochore arrangement and microtubule nucleation or binding properties, and the required methods apparently are now available. Then we must understand the later events in orientation: the integration of a chromosome with its associated microtubules with the rest of the spindle. Also, the molecular biology of reorientation must be understood, a subject not touched on here simply because we know essentially nothing about it. Finally, our ignorance about chromosome condensation poses a challenge here as in so many other areas in cell biology. No clearer or more important consequence of controlled chromosome condensation exists than the change in kinetochore position during the brief interkinesis between the two meiotic divisions.

I am grateful to Barbara Layton and Carolyn Staehly for expert technical assistance and to Dr Dwayne Wise for skilful drawing. To Dr Shinya Inoué of the University of Pennsylvania go heartfelt thanks for exceptional generosity in lending us the rectified polarization optics used in some of these studies. Our studies were supported in part by research grant GM-13745 from the Division of General Medical Sciences, United States Public Health Service.

References (Nicklas)

Barber, H. N. 1942 The pollen-grain division in the Orchidaceae. *J. Genet.* **43**, 97–103.

Bauer, H., Dietz, R. & Röbbelen, C. 1961 Die Spermatocytenteilungen der Tipuliden. III. Das Bewegungsverhalten der Chromosomen in Translokationsheterozygoten von *Tipula oleracea*. *Chromosoma* **12**, 116–189.

Cande, W. Z., Snyder, J., Smith, D., Summers, K. & McIntosh, J. R. 1974 A functional mitotic spindle prepared from mammalian cells in culture. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1559–1563.

Erickson, H. P. 1974 Assembly of microtubules from preformed, ring-shaped protofilaments and 6-S tubulin. *J. Supramolec. Struct.* **2**, 393–411.

Goldman, R., Pollard, T. & Rosenbaum, J. (eds.) 1976 *Cell motility*. Cold Spring Harbor, Long Island, New York: Cold Spring Harbor Laboratory Press.

Henderson, S. A., Nicklas, R. B. & Koch, C. A. 1970 Temperature-induced orientation instability during meiosis: an experimental analysis. *J. Cell Sci.* **6**, 323–350.

Heneen, W. K. 1975 Ultrastructure of the prophase kinetochore in cultured cells of rat-kangaroo (*Potorous tridactylis*). *Hereditas* **79**, 209–219.

Inoué, S., Borisy, G. G. & Kiehart, D. P. 1974 Growth and lability of *Chaetopterus* oocyte mitotic spindles isolated in the presence of porcine brain tubulin. *J. Cell Biol.* **62**, 175–184.

Kubai, D. F. 1975 The evolution of the mitotic spindle. *Int. Rev. Cytol.* **43**, 167–227.

Liebowitz, P. J. & Schaechter, M. 1975 The attachment of the bacterial chromosome to the cell membrane. *Int. Rev. Cytol.* **41**, 1–28.

Lima-de-Faria, A. 1958 Recent advances in the study of the kinetochore. *Int. Rev. Cytol.* **7**, 123–157.

Luykx, P. 1970 *Cellular mechanisms of chromosome distribution* (*Int. Rev. Cytol.*, Suppl. 3). New York: Academic Press.

McGill, M. & Brinkley, B. R. 1975 Human chromosomes and centrioles as nucleating sites for the in vitro assembly of microtubules from bovine brain tubulin. *J. Cell Biol.* **67**, 189–199.

McIntosh, J. R., Cande, W. Z. & Snyder, J. A. 1975 Structure and physiology of the mammalian mitotic spindle. In *Molecules and cell movement* (eds. S. Inoué & R. E. Stephens), pp. 31–76. New York: Raven Press.

McIntosh, J. R., Hepler, P. K. & Van Wie, D. G. 1969 Model for mitosis. *Nature, Lond.* **224**, 659–663.

Müller, W. 1972 Elektronenmikropische Untersuchungen zum Formwechsel der Kinetochoren während der Spermatocytenteilungen von *Pales ferruginea* (Nematocera). *Chromosoma* **38**, 139–172.

Nicklas, R. B. 1967 Chromosome micromanipulation. II. Induced reorientation and the experimental control of segregation in meiosis. *Chromosoma* **21**, 17–50.

Nicklas, R. B. 1971 Mitosis. In *Advances in cell biology* (eds. D. M. Prescott, L. Goldstein & E. McConkey), vol. 2, pp. 225–297. New York: Appleton-Century-Crofts.

Nicklas, R. B. 1974 Chromosome segregation mechanisms. *Genetics* **78**, 205–213.

Nicklas, R. B. & Staehly, C. A. 1967 Chromosome micromanipulation. I. The mechanics of chromosome attachment to the spindle. *Chromosoma* **21**, 1–16.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

Östergren, G. 1951 The mechanism of co-orientation in bivalents and multivalents. *Hereditas* **37**, 85–156.
Rebhun, L. I., Rosenbaum, J., Lefebvre, P. & Smith, G. 1974 Reversible restoration of the birefringence of cold-treated, isolated mitotic apparatus of surf clam eggs with chick brain tubulin. *Nature, Lond.* **249**, 113–115.
Roos, U.-P. 1973 Light and electron microscopy of rat kangaroo cells in mitosis. II. Kinetochore structure and function. *Chromosoma* **41**, 195–220.
Roos, U.-P. 1975 Are centromeric dots kinetochores? *Nature, Lond.* **254**, 463.
Roos, U.-P. 1976 Light and electron microscopy of rat kangaroo cells in mitosis. III. Patterns of chromosome behavior during prometaphase. *Chromosoma* **54**, 363–385.
Schrader, F. 1936 The kinetochore or spindle fibre locus in *Amphiuma tridactylum*. *Biol. Bull. mar. biol. Lab., Woods Hole* **70**, 484–498.
Snyder, J. A. & McIntosh, J. R. 1975 Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. *J. Cell Biol.* **67**, 744–760.
Telzer, B. R., Moses, M. J. & Rosenbaum, J. L. 1975 Assembly of microtubules onto kinetochores of isolated mitotic chromosomes of HeLa cells. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4023–4027.
Wagenaar, E. B. & Bray, D. F. 1973 The ultrastructure of kinetochores of unpaired chromosomes in a wheat hybrid. *Can. J. Genet. Cytol.* **15**, 801–806.
Weisenberg, R. C. 1972 Microtubule formation in vitro in solutions containing low calcium concentrations. *Science, N.Y.* **177**, 1104–1105.

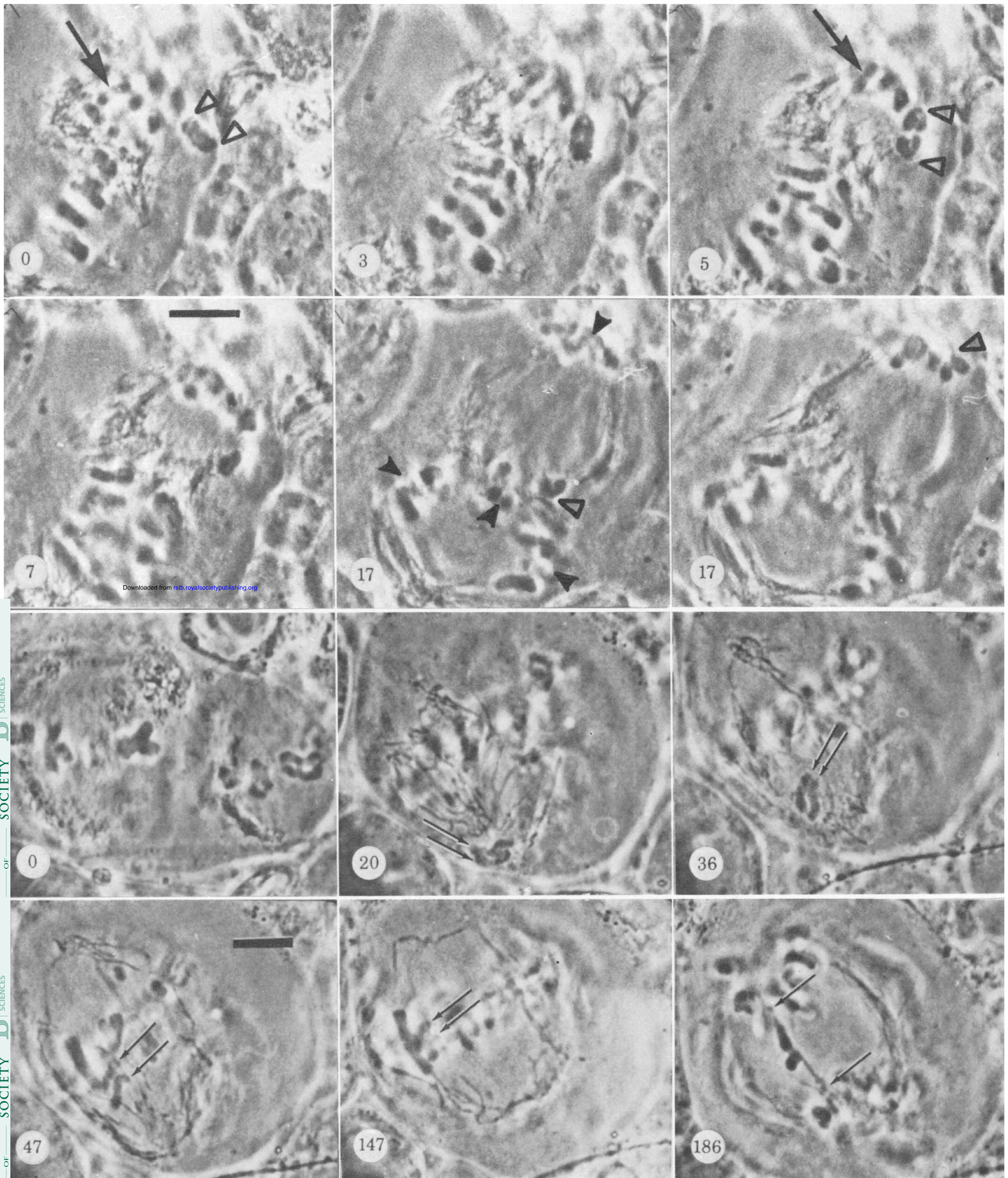
Discussion

K. Jones (*Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey*). In your paper you were showing how you were able to move a meiotic bivalent into the mitotic environment of the second meiotic division. We might remember, however, that in hybrids and other plants such as uneven polyploids it is common to find both paired associations and univalents. The latter can be observed to behave in different ways, some, for example, can undergo a virtual mitotic division following the first metaphase while others segregate as half bivalents. We therefore see a centromere behaving quite differently in the same cell. And of course we can observe that this behaviour can be under strict genetic control as in the case of *Rosa canina* for example. I have also observed in inbred lines of *Lolium italicum* that when the genetic control of meiosis is disturbed very rarely a rod bivalent can show auto-orientation of both centromeres and the bivalent then lies with its axis at right angles to that of the spindle. It is clear therefore that univalent chromosomes can behave in several ways and for several reasons at meiosis.

In earlier days there was the belief that in general the centromere of the mitotic chromosome was exposed in a way quite different from the meitoic because of coiling differences which leave the centromere fully exposed on both sides in mitosis. Furthermore the occurrence of chiasmata at meiosis could be a means of preventing the rotation of a paired chromosome in this division. Perhaps you would care to comment on this?

B. Nicklas. Yes, the orientation of univalents certainly is variable, possibly because the frequent reorientation of univalents places them repeatedly at hazard (Nicklas 1971, pp. 270 and 272). Two examples of the much rarer mitosis-like orientation of a bivalent or half-bivalent in meiosis have been described from living cells, where the consequences of such mal-orientation can be directly observed (Nicklas 1967, Figs. 10 and 11).

Concerning a possible difference in kinetochore exposure in mitosis versus meiosis, my feeling is that only electron microscopic observations on mitotic and meiotic prophase chromosomes in the same material can be decisive. On the basis of present information, however, I do not expect that a striking difference will be found. Chiasmata may play some role in determining kinetochore position, but many univalent chromosomes lacking chiasmata nevertheless orient just as do bivalents with chiasmata (e.g., the X-chromosome of numerous orthopterans).



Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

FIGURES 2 AND 3. For description see opposite.

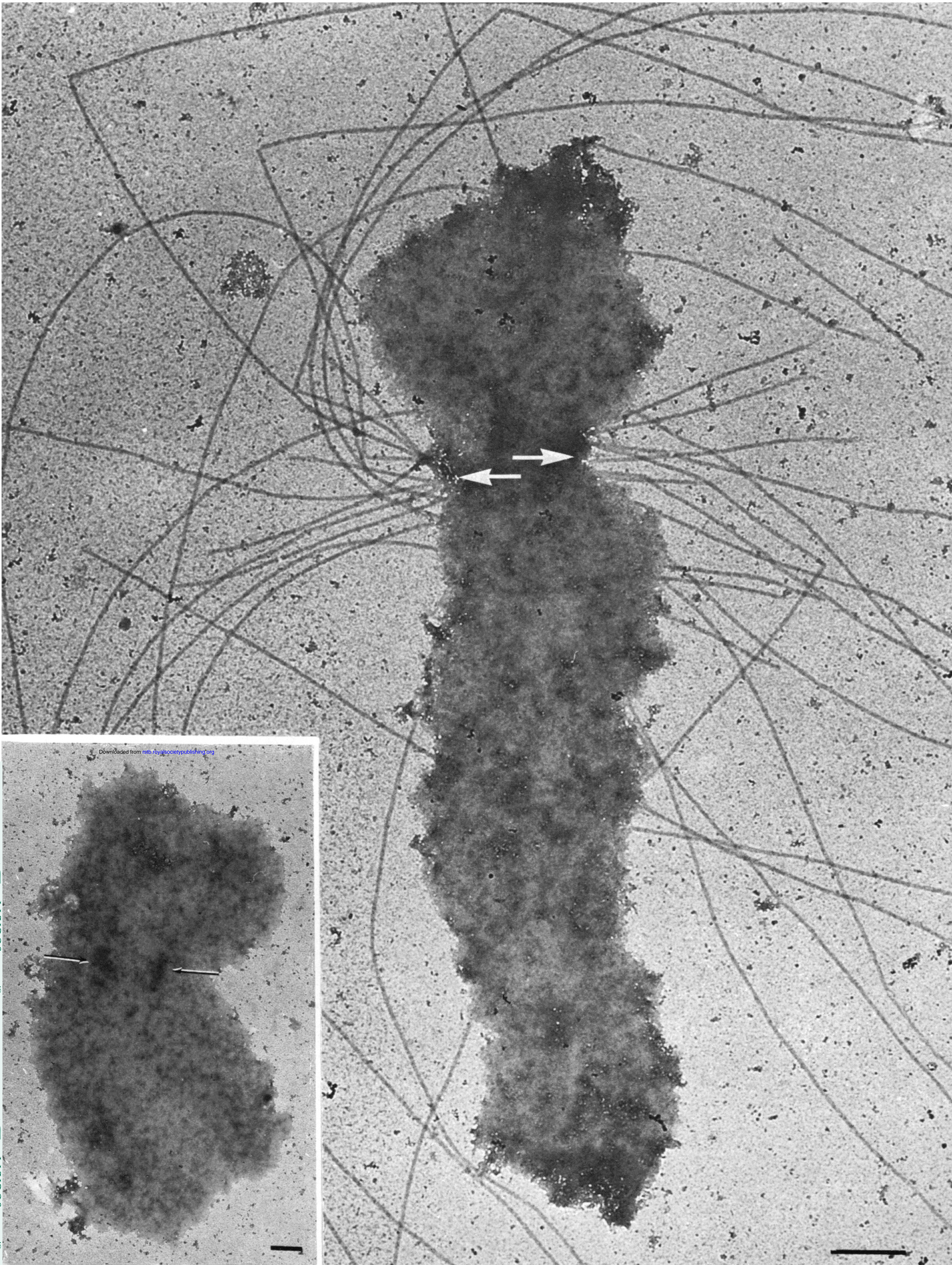
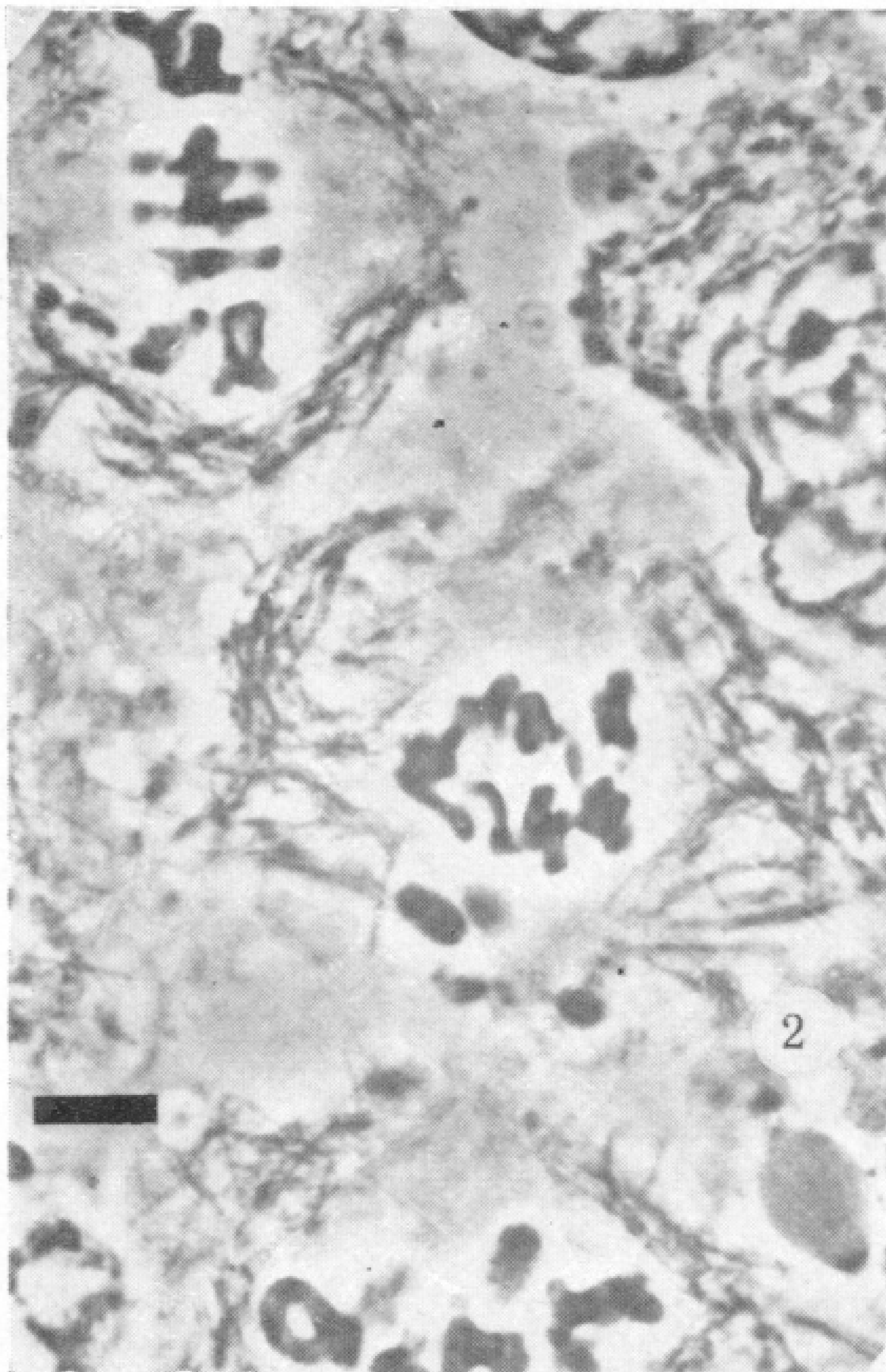


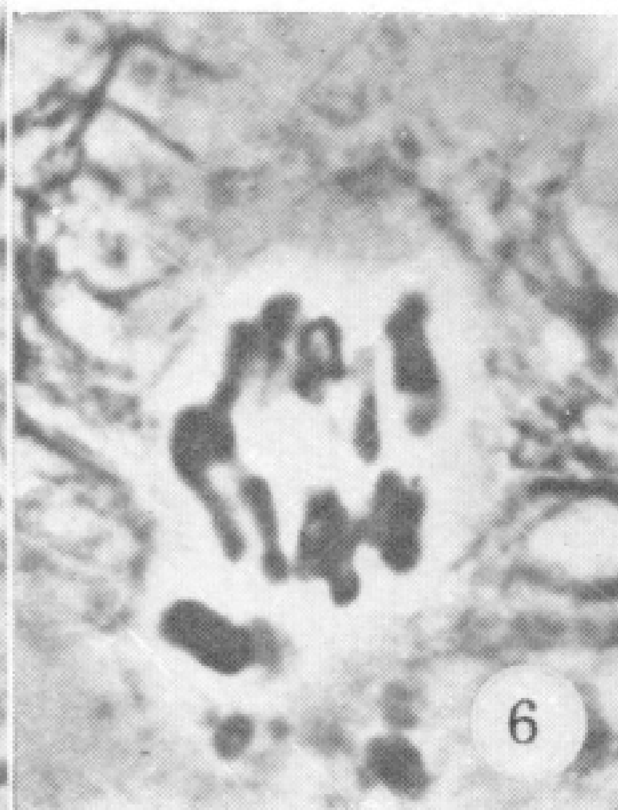
FIGURE 4. For description see opposite.

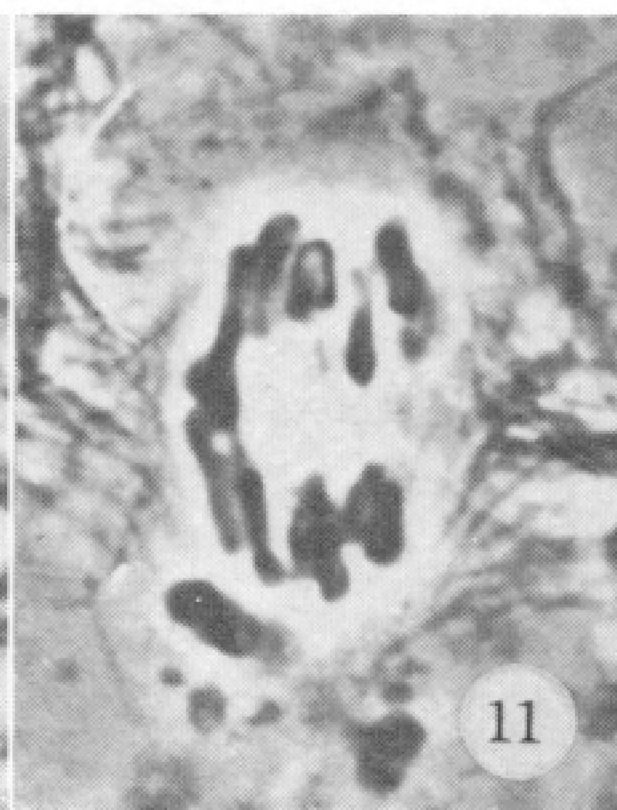
Downloaded from rstb.royalsocietypublishing.org

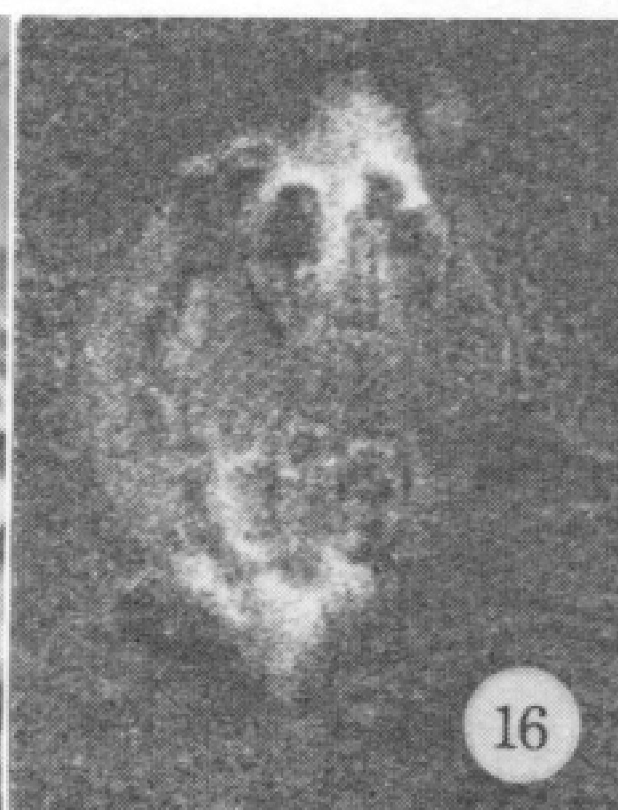
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

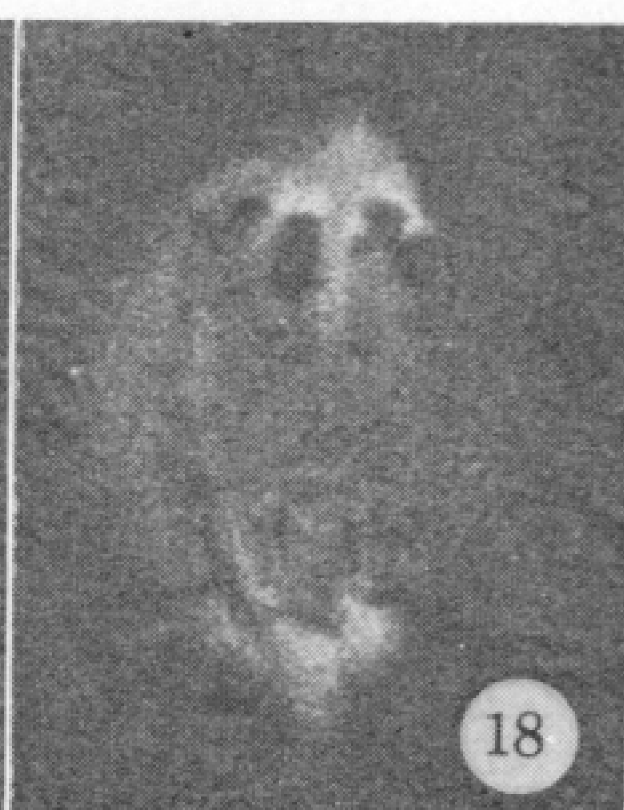
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

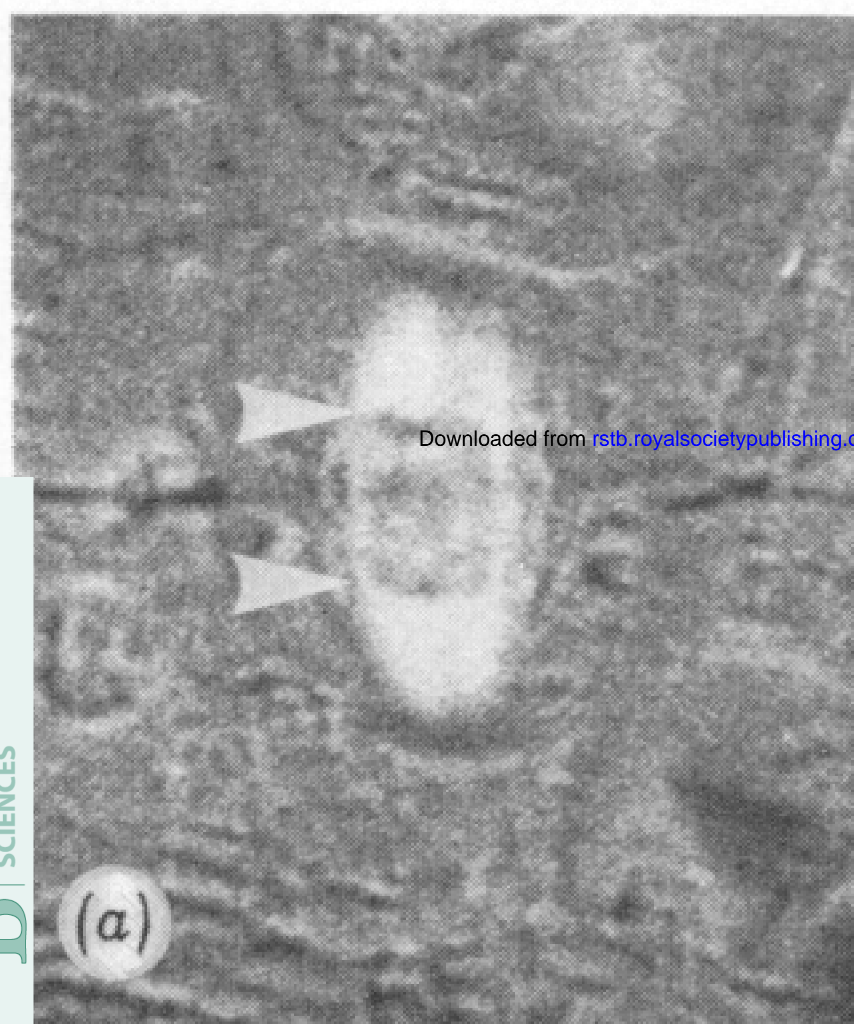




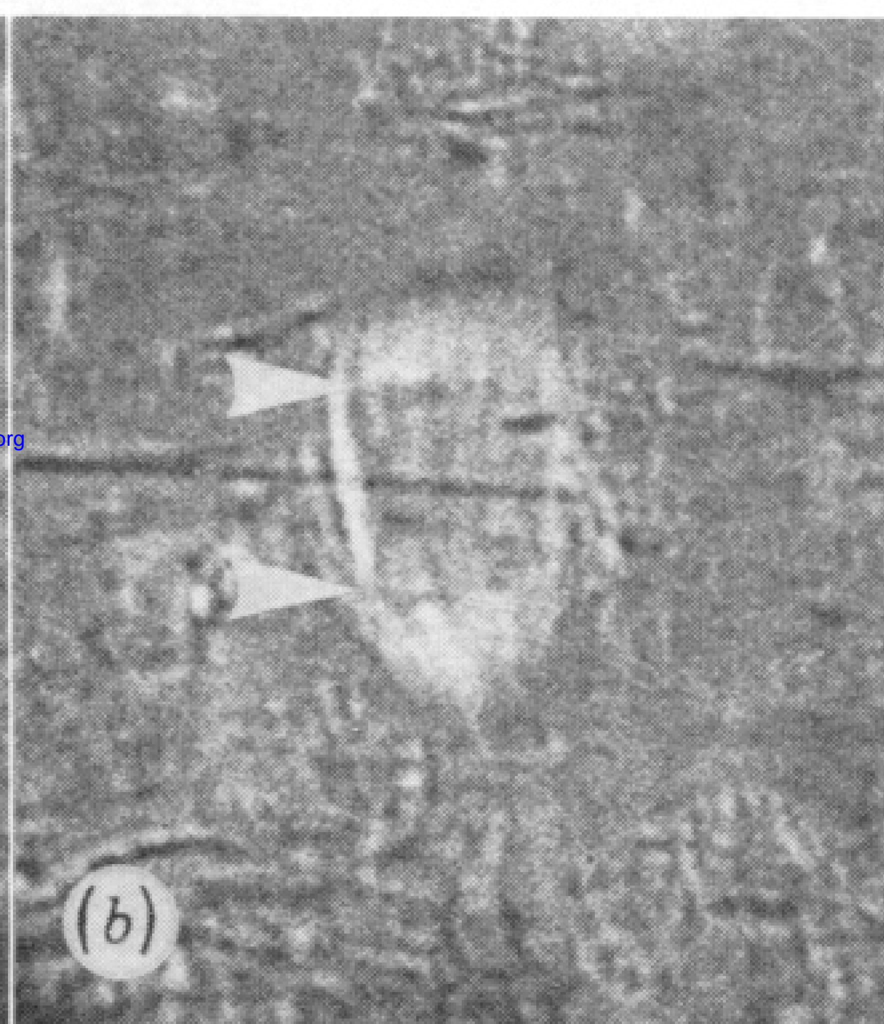


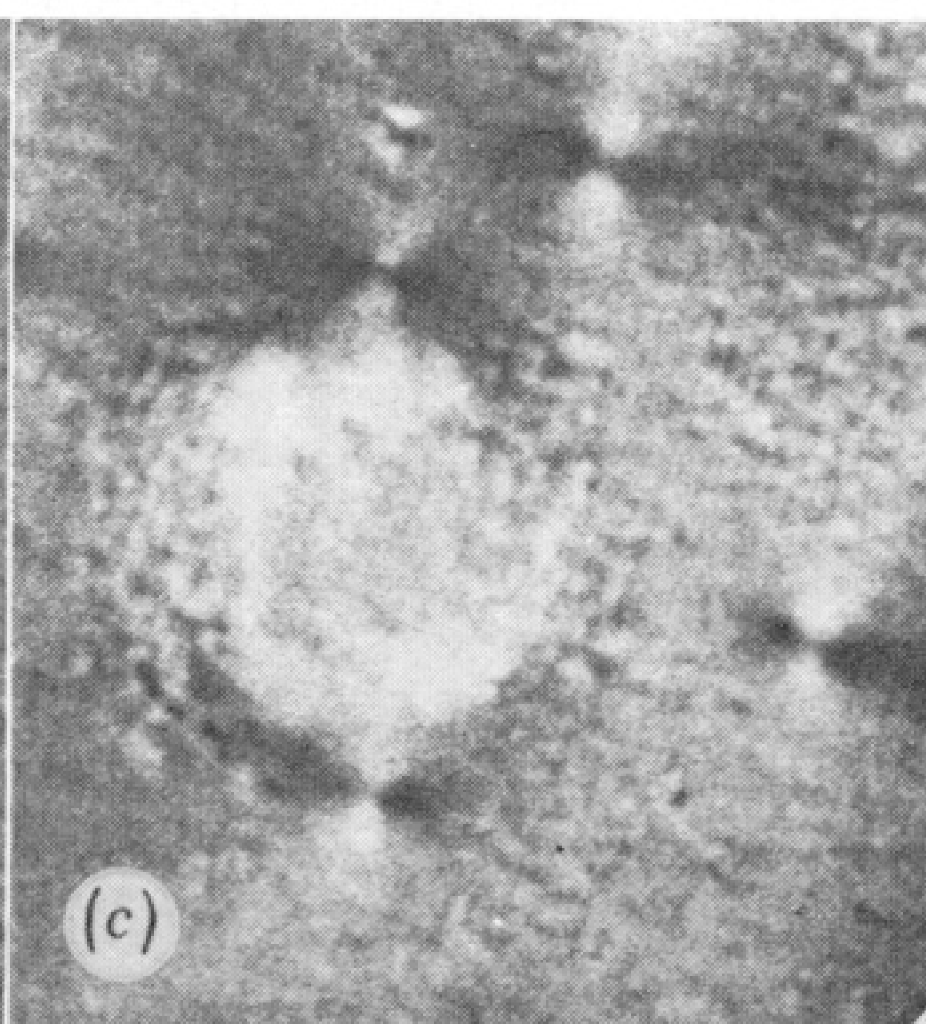


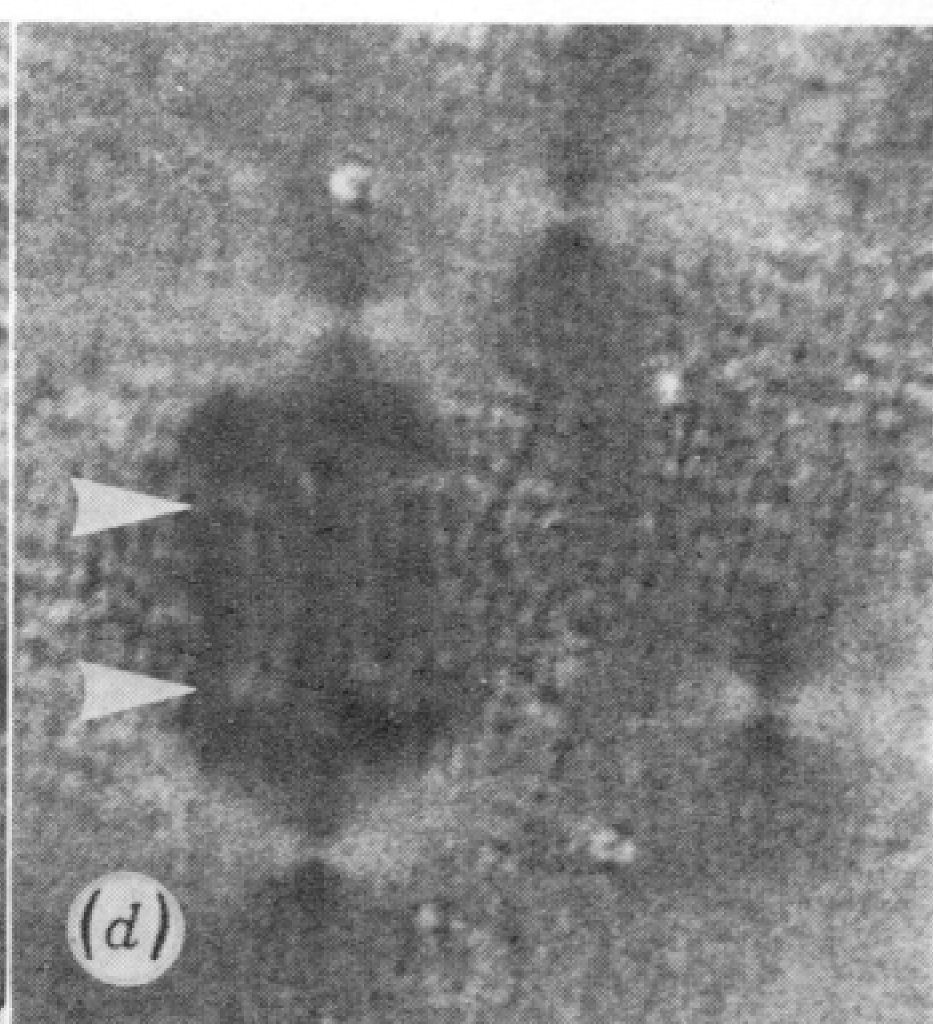




Downloaded from rstb.royalsocietypublishing.org







FIGURES 5 AND 6. Features of cells demembranated by a new method. A mechanical shock (see text) was delivered to cells in a well filled with halocarbon oil and viewed on an inverted microscope (Nicklas & Staehly 1967). The bar (figure 5, 2 min print) equals 10 μm and applies to both figures.

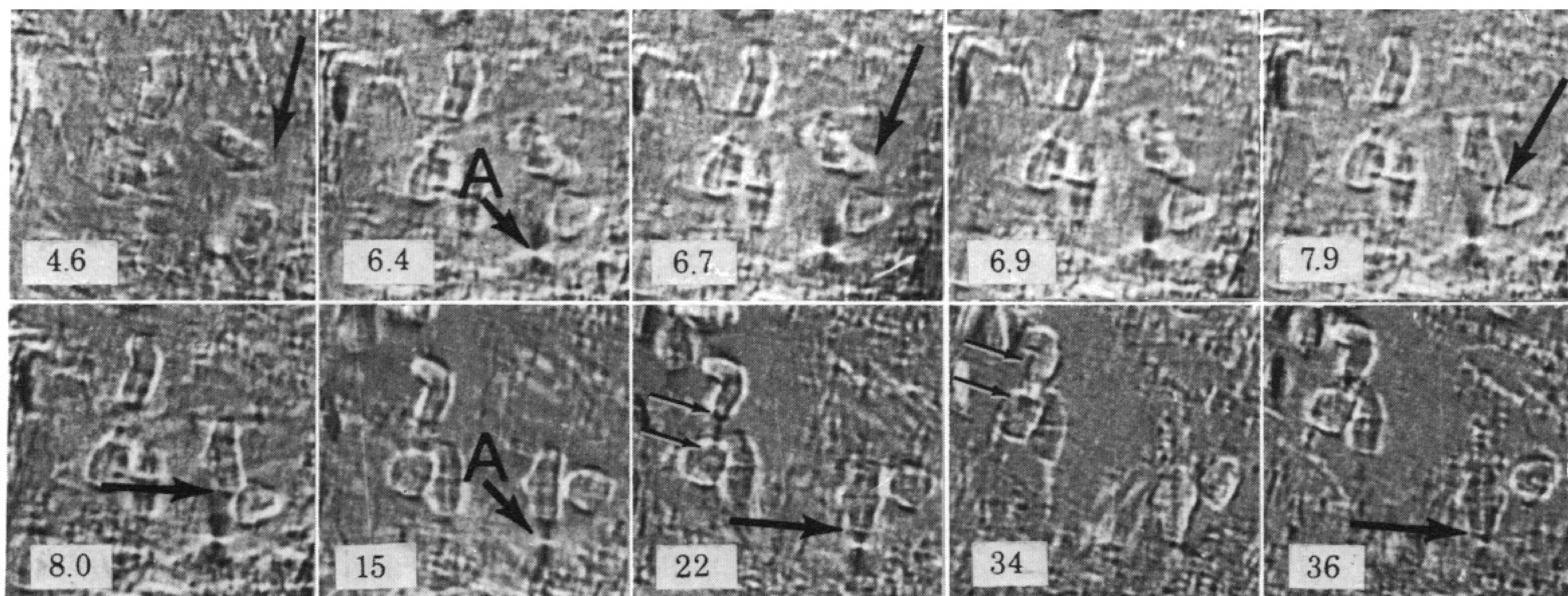
FIGURE 5 (above). Anaphase in a demembranated but otherwise untreated grasshopper (*Arphia xanthoptera*) spermatocyte. The time after demembranation is given in minutes on each print. Anaphase in the central spindle in the 2 min print is illustrated by phase contrast (2–11 min prints) and by polarization microscopy (16 and 18 min prints). The total increase in chromosome separation was 11 μm in 16 min, for an average speed of 0.7 μm/min (about two-thirds of that seen in control, undemembranated cells). By 16–18 min, spindle birefringence had decreased to about half the normal value.

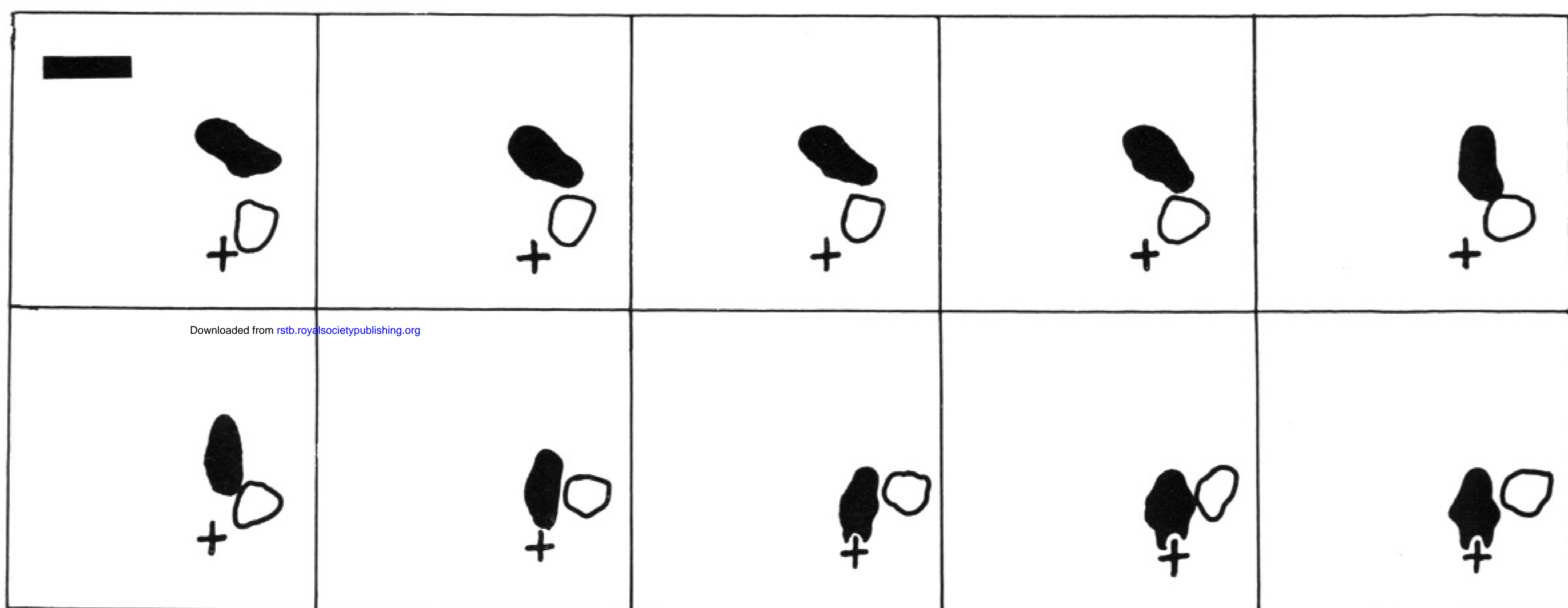
FIGURE 6 (below). The consequences of adding tubulin to demembranated cricket (*Acheta domestica*) spermatocytes as seen by polarization microscopy. The arrowheads show the position of the chromosomes in anaphase spindles.

A control cell is shown (*a*) 0.5 min before demembranation and (*b*) 3 min after demembranation (*c*, *d*) Another preparation, showing an anaphase spindle and two asters (to the right) after tubulin addition in photographs made at opposite compensator settings. Note the striking enhancement of spindle and aster birefringence as compared with the control. The tubulin preparation was added 6 min after demembranation; the photographs were taken 5 min (*c*) and 18 min (*d*) after tubulin addition. The tubulin was purified from hog brain by one standard assembly/disassembly cycle, and was added to the demembranated cells at a concentration of 7 mg protein/ml in standard assembly medium (Erickson 1974). The tubulin was a gift of Dr H. P. Erickson.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES





Downloaded from rstb.royalsocietypublishing.org

FIGURES 7 AND 8. Chromosome orientation *in vitro*. Chromosome behaviour can be followed most readily by reference to figure 8, using figure 7 for verification and whenever birefringence is in question. The bar (figure 8, upper left) equals 10 μm and applies to both figures.

FIGURE 7 (above). A slide containing a grasshopper (*Arphia xanthoptera*) spermatocyte in diakinesis was placed at 7 °C, the cells were demembranated as in figures 5 and 6, tubulin was added 10 min later, and 3 min after that the slide was returned to 22 °C. Bivalents from the cell in diakinesis are shown by polarization microscopy; the time in minutes after the return to 22 °C is indicated on each print. An aster is indicated by A on the 6.4 and 15 min prints. One bivalent oriented to that aster; the kinetochoric end involved is indicated by an arrow on the 4.6, 6.7, 7.9, 8.0, 22, and 36 min prints. The bivalent first showed a twitching movement toward (4.6–6.4 min) and then away from (6.4–6.7 min) the aster; the twitch is just visible in the prints but is very striking in the movie record. The bivalent then gradually swung toward the aster (6.7–8.0 min prints) and then moved straight toward the centre of the aster (8.0–36 min prints), bypassing a bivalent on the right. The bivalent moved initially at a velocity of 1.9–3.6 μm/min (4.6–8 min) and later at 0.28 μm/min (8–22 min). Birefringence developed between two bivalents identified by paired arrows on the 22 and 34 min prints. The 4.6 and 34 min photographs were made at the compensator setting opposite that used for all the others. The tubulin, a gift of Dr H. P. Erickson, was prepared and used as described for figure 6 except that two assembly/disassembly purification cycles were carried out and the concentration was approximately 3 mg protein/ml.

FIGURE 8 (below). A tracing of figure 7 showing the orientating bivalent (in black) as it moved toward the aster (+), passing a stationary bivalent (in outline), which later moved or drifted *away* from the pole. Drawn by Dr D. Wise.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES