Cell Motility by Labile Association of Molecules

The nature of mitotic spindle fibers and their role in chromosome movement

SHINYA INOUÉ and HIDEMI SATO

From the Laboratories of Biophysical Cytology, Department of Biology, University of Pennsylvania, Philadelphia

ABSTRACT This article summarizes our current views on the dynamic structure of the mitotic spindle and its relation to mitotic chromosome movements. The following statements are based on measurements of birefringence of spindle fibers in living cells, normally developing or experimentally modified by various physical and chemical agents, including high and low temperatures, antimitotic drugs, heavy water, and ultraviolet microbeam irradiation. Data were also obtained concomitantly with electron microscopy employing a new fixative and through measurements of isolated spindle protein. Spindle fibers in living cells are labile dynamic structures whose constituent filaments (microtubules) undergo cyclic breakdown and reformation. The dynamic state is maintained by an equilibrium between a pool of protein molecules and their linearly aggregated polymers, which constitute the microtubules or filaments. In living cells under physiological conditions, the association of the molecules into polymers is very weak (absolute value of $\Delta F_{25°C} < 1$ kcal), and the equilibrium is readily shifted to dissociation by low temperature or by high hydrostatic pressure. The equilibrium is shifted toward formation of polymer by increase in temperature (with a large increase in entropy: $\Delta S_{25°C} \simeq 100$ eu) or by the addition of heavy water. The spindle proteins tend to polymerize with orienting centers as their geometrical foci. The centrioles, kinetochores, and cell plate act as orienting centers successively during mitosis. Filaments are more concentrated adjacent to an orienting center and yield higher birefringence. Astral rays, continuous fibers, chromosomal fibers, and phragmoplast fibers are thus formed by successive reorganization of the same protein molecules. During late prophase and metaphase, polymerization takes place predominantly at the kinetochores; in metaphase and anaphase, depolymerization is prevalent near the spindle poles. When the concentration of spindle protein is high, fusiform bundles of polymer are precipitated out even in the absence of obvious orienting centers. The shift of equilibrium from free protein molecules to polymer increases the length and number of the spindle microtubules or filaments. Slow depolymerization of the

polymers, which can be brought about by low concentrations of colchicine or by gradual cooling, allows the filaments to shorten and perform work. The dynamic equilibrium controlled by orienting centers and other factors provides a plasusible mechanism by which chromosomes and other organelles, as well as the cell surface, are deformed or moved by temporarily organized arrays of microtubules or filaments.

In this paper, we shall attempt to relate the mitotic movement of chromosomes to the structure, physiology, and function of the mitotic spindle.

At each division of a eukaryotic cell a spindle is formed, and the chromosomes are oriented, aligned, and then separated regularly into two daughter cells. The spindle is disassembled when the task is done.

In a living cell, the fibrous elements of the mitotic spindle and its astral rays are weakly birefringent. The appearance and growth, and contraction and disappearance, of the fibers can therefore be observed in living cells with a sensitive polarizing microscope. The change in birefringence can also be measured with the polarizing microscope. Correlations can then be established between spindle fiber birefringence and alteration of fiber fine structure in cells altered physiologically or experimentally.

Many conditions and agents have been found in which spindle morphology and fiber birefringence are systematically and reversibly altered. From the various observations described or reviewed in this paper, we conclude that spindle fibers are composed of parallel arrays of thin filaments which are formed by a reversible association of globular protein molecules. The molecules in functioning spindle fibers are not stably aligned and cross-linked as in more stable fibers. Instead, spindle fibers are labile structures existing in a dynamic equilibrium with a large pool of unassociated molecules. The association of the molecules and formation of filaments are controlled by the activities of orienting centers and concentrations of active pool material.

We hypothesize that the contraction and elongation of the spindle fibers are responsible for regular mitotic movement of chromosomes. The spindle fibers, however, do not contract and elongate by folding and unfolding of polypeptide chains in the protein molecules. For example, the fiber birefringence remains unchanged during anaphase movement. Instead, the fibers are believed to elongate by increased addition and alignment of the molecules, which contribute to a pushing action. Conversely, they shorten and pull chromosomes as molecules are slowly removed from the filaments.

We view this hypothesis as providing a plausible mechanism, not only for mitotic movement of chromosomes, but also for movements of various other organelles and mechanical modulations of the cell surface.

In the following, we shall:

1. Review the visible changes of spindle fiber birefringence during normal mitosis
2. Discuss the mechanical integrity of the spindle fibers
3. Describe the dynamic nature of the spindle fibers
4. Discuss the dynamic equilibrium model
5. Describe the effect of heavy water on spindle fiber birefringence, protein content, and electron microscopic appearance
6. Provide evidence that the bulk of the spindle fiber protein comes from a preformed pool
7. Describe factors which control the formation and orientation of the fibers
8. Discuss the extension and contraction of spindle fibers
9. Discuss the nature of the spindle protein molecules
10. Relate spindle fiber birefringence to microtubules and filaments observed in fixed cells with the electron microscope
11. Discuss cell movements which appear to be brought about by labile association of molecules

1. VISIBLE CHANGES OF SPINDLE FIBER BIREFRINGENCE DURING MITOSIS

Spindle fibers and astral rays generally possess a positive birefringence whose magnitude is of the order of 1 mμ, or $^2/_{1000}$ of a wavelength of green light. With a sensitive polarizing microscope, they can be clearly visualized (Fig. 1) and their fine structural changes can be followed by interpreting the measured change in birefringence[1] (Bajer, 1961; Dietz, 1963; Forer, 1965, 1966; Inoué, 1953, 1964; Inoué and Bajer, 1961; Schmidt, 1937, 1939, 1941; Taylor, 1959; for a thorough discussion and general bibliography of mitosis, and for definition of terms, also see Schrader, 1953, and Mazia, 1961).

The change in spindle fiber birefringence during mitosis in a wide variety of animal and plant cells has been documented photographically (Inoué, 1964; also 1953). The same paper (Inoué, 1964) also describes a sequence of time-lapse motion pictures depicting the birefringence changes during mitosis and cytokinesis in several types of cells.

The major patterns of birefringence changes can be summarized as follows. In prophase before nuclear membrane breakdown, birefringent fibers are formed as astral rays in animal cells (Fig. 2 *A*) (Inoué and Dan, 1951; Forer, 1965; also see Cleveland, 1938, 1953, 1963), or as clear zone fibers in some plant cells (Fig. 3 *A*) (Inoué and Bajer, 1961; also see Bajer, 1957). In newt fibroblasts, linear growth of the birefringent central spindle has been measured

[1] In this paper, the term "birefringence" is used synonymously with retardation [i.e. $d(n_1 - n_2)$] and should not be interpreted to mean coefficient of birefringence $(n_1 - n_2)$, or retardation per unit specimen thickness.

by Taylor (1959). In the central spindle formed between the separating asters, and in the clear zone material, the birefringence fiber axis (slow axis) corresponds to the direction in which more continuous fibers, sheath fibers, and chromosomal fibers appear. The central (continuous) spindle fibers and clear zone fibers thus establish a fine structural foundation for subsequent orientation of other fibers (also see Wada, 1965; however, see Inoué and Dan, 1951, for anomalous situations regarding axis of streak material vs. aster and spindle, and for changes to negative birefringence under presumed compression).

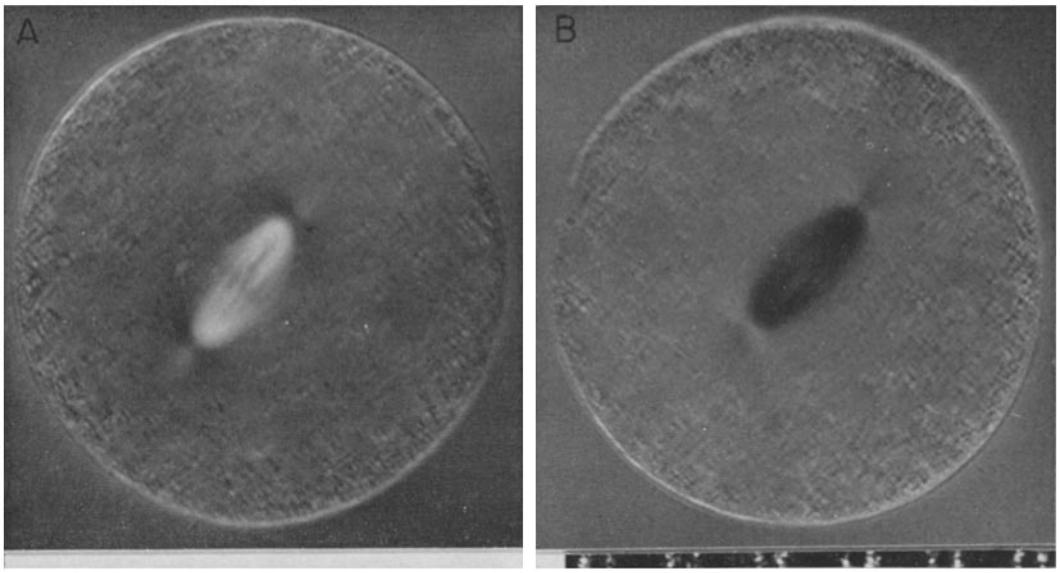


FIGURE 1. Mitotic spindle in arrested metaphase oocyte of *Pectinaria gouldi*. The over-all birefringence[1] of the spindle has been enhanced with 40% heavy water (D_2O)–sea water to approximately 3 mμ. Polarization microscopy. *A*, chromosomal fibers appear bright; *B*, compensator axis reversed, chromosomal fibers are dark. Scale interval, 10 μ.

In prometaphase following nuclear membrane breakdown (Fig. 3 *B*), more spindle material becomes oriented around the chromosomes to form sheath fibers in a crane fly, *Pales crocata* (Dietz, 1963; Went, 1966; also see Dietz, 1959, for special attributes of crane fly spindle poles), and in pollen mother cells of the Easter lily (Inoué, 1953, 1964). The birefringent sheath fibers represent a distinct accumulation of oriented material. In many cases, sheath fibers are not observed, and chromosomal fibers arise parallel to continuous fibers directly as in Fig. 3 *C*. In either case, the continuous or sheath fiber material appears to transform into chromosomal fibers. The birefringence of the former decreases as the birefringence of the latter increases (Fig. 3 *B* and *C*).

The birefringent chromosomal fibers are established parallel to the con-

tinuous fibers or sheath fibers of prometaphase. Their orientation in turn forecasts the direction of movement of the chromosomes (Figs. 2 *B–E* and 3 *B–E*). The chromosomal fibers are attached to the kinetochore of the chromosomes. In plant cells devoid of centrioles, the fiber birefringence is strongest adjacent to the kinetochore and weaker toward the poles (Fig. 3 *B–D*). Chromosomal spindle fibers with strong birefringence from kinetochore to poles are found in animal cells with active centrioles (Fig. 2 *B–D*).

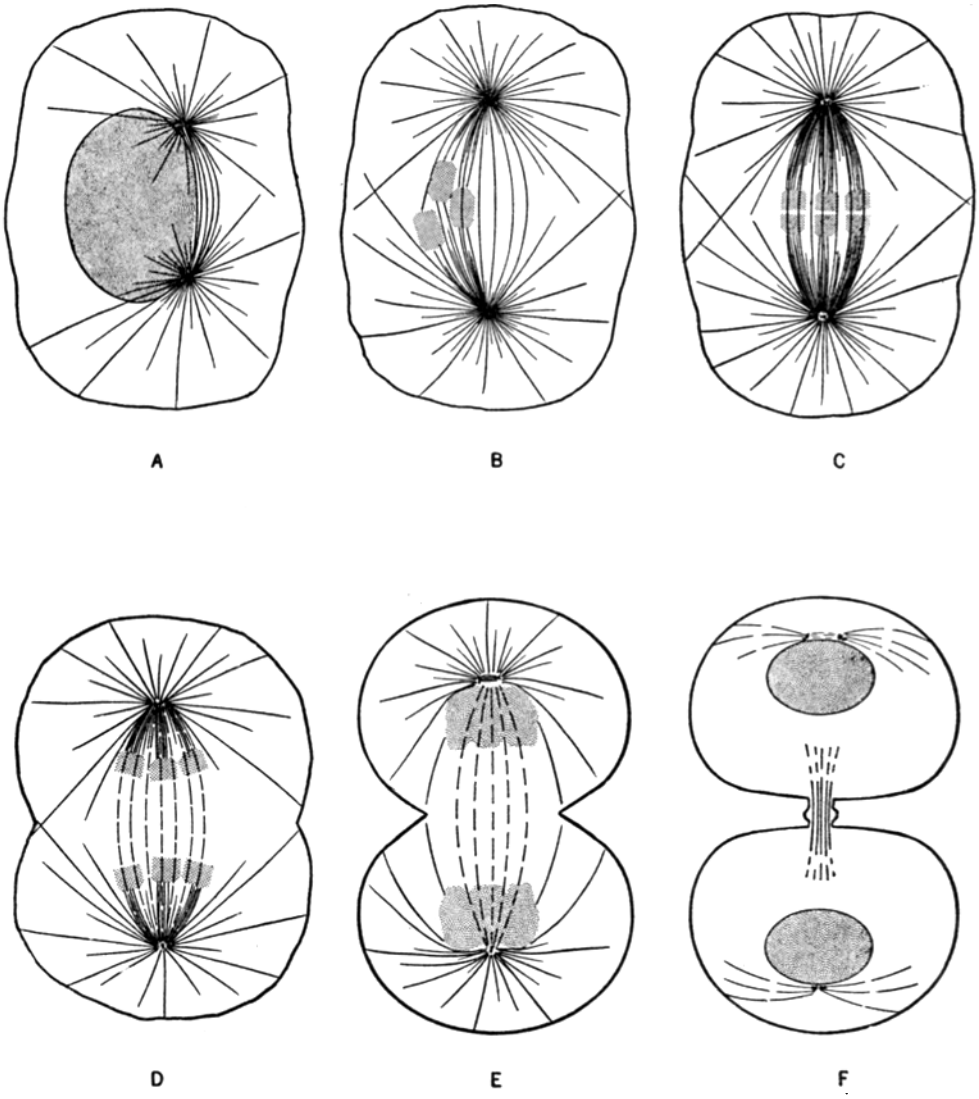
In prometaphase, chromosomal spindle fibers also appear to participate in the orientation of the chromosomes and their alignment on the metaphase plate. (See Östergren, 1951, for a proposed mechanism of metaphase equilibrium and orientation.)

During prometaphase, fluctuation of birefringence is observed in the chromosomal spindle fibers of a grasshopper spermatocyte, *Dissosteira carolina*. In a time-lapse motion picture, the fluctuation of birefringence resembles that of northern lights (Inoué, 1964). It is interpreted to reflect the fluctuation in the amount of molecules oriented in the chromosomal spindle fibers at this stage. Such changes might contribute to the achievement of exact alignment of chromosomes on to the metaphase plate as their equilibrium position (Östergren, 1951).

In anaphase, the chromosomes are led by the strongly birefringent chromosomal spindle fibers to the poles of the spindle. The birefringence adjacent to the kinetochore and in much of the chromosomal spindle fiber remains unchanged for the major part of anaphase movement (Figs. 2 *C–D* and 3 *C–E*). The chromosomal spindle fiber may or may not shorten during most of the anaphase, depending on the exact mode of chromosome separation relative to spindle elongation.

Continuous fibers whose birefringence is extremely low in anaphase regain birefringence during late anaphase and telophase and establish a phragmoplast in the case of a plant cell (Fig. 3 *E*). The phragmoplast fibers guide the accumulation of vesicles onto its midplane (Inoué, 1953; Bajer, 1965). The vesicles fuse and form the primary cell wall (Fig. 3 *F*). (For electron microscopy of this process, see Frey-Wyssling et al., 1964, and Porter and Machado, 1960). In animal cells, continuous spindle fibers also become more strongly birefringent, and presumably more stable in telophase, forming the core of the stem (spindle rest, stem *Körper*) connecting the separating cells (Fig. 2 *F*). In this region electron micrographs show an especially large number of microtubules (e.g. Robinson and Gonatas, 1964).

Thus, what appears to be a transition of birefringent material from one component of the spindle to another is seen throughout division. Much of this is presumably brought about by successive activation and inactivation of orienting centers (Inoué, 1964). At the same time, the orientation of the birefringent fibers of an earlier stage appears to contribute to the orientation



FIGURES 2 and 3. Schematic diagrams of mitosis. These figures were drawn specifically to illustrate change of birefringence distribution in various spindle regions. They are composites of observations on a variety of living cells undergoing normal mitosis. See references given in the text for photographic documentation.

Fig. 2 shows mitosis in spindles with centrioles, as commonly seen in animal cells.

of the subsequent fibers. (Also see Costello, 1961, for the role of centrioles in relating the axis of successive divisions.)

The successive fibers formed appear to co-orient and align chromosomes to the metaphase plate, pull them apart to the poles, and correlate cytokinesis with karyokinesis.

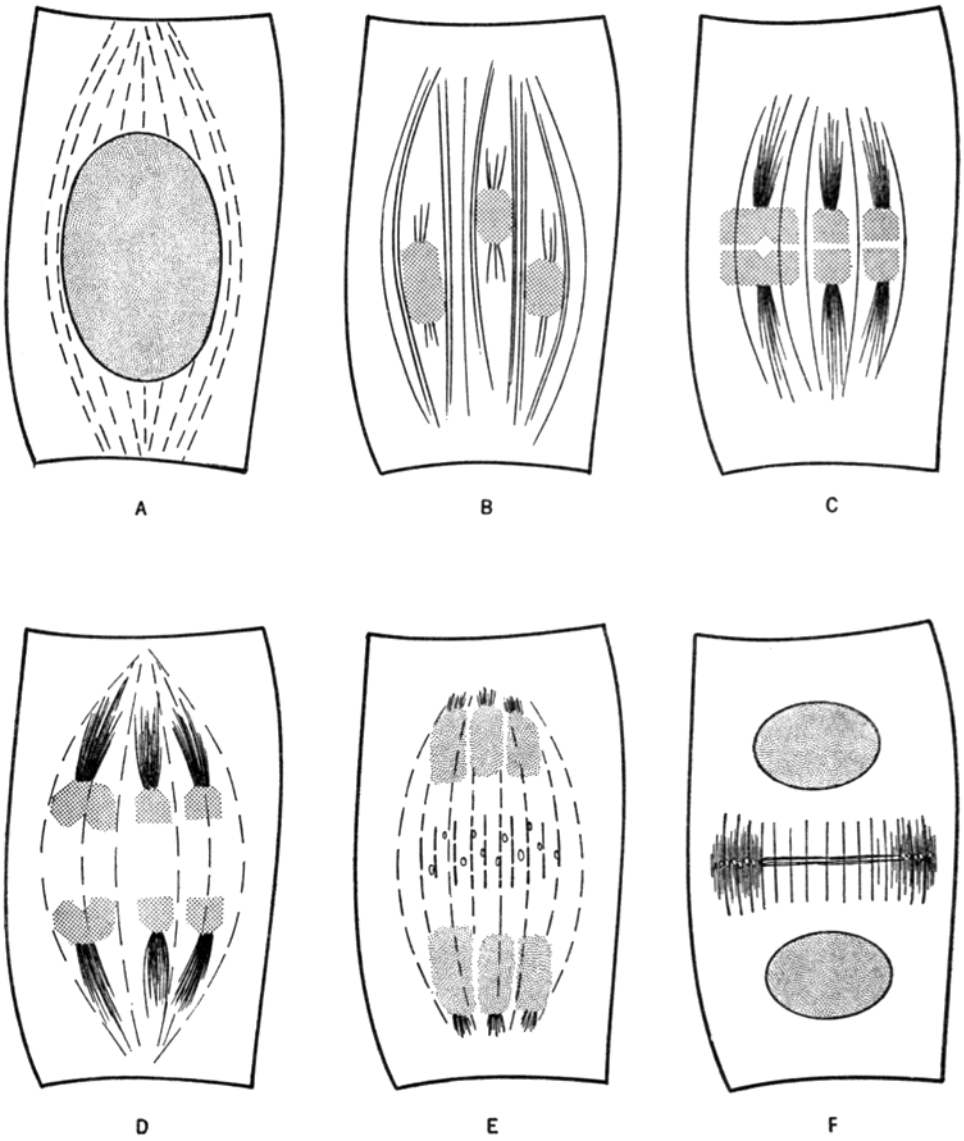


Fig. 3 shows mitosis in spindles without centrioles. Certain features shown in this illustration may appear in cells with the type of spindle shown in Fig. 2, as explained in the text.

2. MECHANICAL INTEGRITY OF SPINDLE FIBERS

Recently, the mechanical integrity of spindle fibers in prometaphase and anaphase movement was demonstrated directly by elegant micromanipulation experiments (Nicklas, 1965, 1967; also see earlier experiments of Carlson, 1952). Taking advantage of the Ellis micromanipulator (Ellis, 1962), Nicklas

managed to pull and stretch chromosomes which were attached by their kinetochores to chromosomal spindle fibers. He was also able to sever the chromosomal spindle fiber, turn the chromosome, and point its kinetochore to the opposite spindle pole. The chromosome then moved toward the new pole, presumably acquiring a new spindle fiber attachment to that pole. The chromosomal spindle fibers are thus capable of resisting extension when the same force deforms the chromosome considerably. The fiber maintains a mechanical integrity within limits, but a new fiber can be reformed rapidly if the old one is broken.

The mechanical integrity of the chromosomal spindle fibers and the resistance of the central spindle to compression were also seen in centrifugal experiments performed by Shimamura (1940) and by Conklin (1917).

3. DYNAMIC NATURE OF SPINDLE FIBERS

The reformation of the spindle fibers following direct mechanical disruption by micromanipulation (Nicklas, 1965, 1967; also see Chambers, 1924) was described above. The spindle fibers have also been shown to reform rapidly after other means of disruption. Their birefringence is abolished in a matter of seconds by treatment with low temperature in *Chaetopterus* oocytes (Inoué, 1952 *a*) and in *Lilium* pollen mother cells (Inoué, 1964). Upon return to normal temperature, they recover in the course of a few minutes, after which chromosome movement can continue (Inoué, 1964). Low temperature disintegration of the spindle can be repeated for as many as 10 times in the same cell. In *Chaetopterus* oocytes, *Lilium* pollen mother cells, *Halistaura* developing eggs, and *Dissosteira* spermatocytes, recovery from low temperature treatment is not affected by the duration of chilling, even up to several hours or longer (Inoué, 1952 *b*, and unpublished data).

Colchicine, Colcemid, and many other drugs eliminate the spindle birefringence (Inoué, 1952 *a*; Inoué, Sato, and Ascher, 1965; also see Gaulden and Carlson, 1951, and Molé-Bajer, 1958). The effects are reversible, and *Pectinaria* oocytes treated with 10^{-5} M griseofulvin may recover their birefringent spindles in as little as 5.5–11 min when the cells are washed with normal sea water (Malawista and Sato, 1966).

High hydrostatic pressure is known to destroy the spindle organization reversibly (Pease, 1946; Zimmerman and Marsland, 1964; also see Marsland, 1966, on synergic action of colchicine and pressure). UV microbeam irradiation of portions of spindle fibers introduces temporary lesions which recover rapidly (Campbell and Inoué, 1965; Forer, 1965, 1966; Inoué and Sato, 1964; Izutsu, 1961 *a*, *b*).

The natural fluctuation of birefringence in prometaphase has already been described. The apparent assembly of the spindle material, first into the astral ray or clear zone material, then into the continuous fiber and sheath fiber

material, from there to the chromosomal fiber, from chromosomal fiber to the continuous fiber, and, finally, to the phragmoplast fiber material, has also been described. We interpreted these changes to reflect the orderly assembly and disassembly of the same material, sequentially into different fibrous structures to perform different functions.

The various fibers of the spindle are thus extremely labile and their birefringence can be readily abolished. The spindle is also a dynamic structure, and, in most cases, the birefringence recovers rapidly when the external agent is removed. The birefringence can also be reduced or increased to another level by many agents and maintained at an equilibrium value.

4. THE DYNAMIC EQUILIBRIUM MODEL

In order to explain the dynamic nature of the spindle fiber and the response of the spindle birefringence and structure to various experimental alterations, Inoué (1959, 1960, 1964) earlier proposed a dynamic equilibrium model. In the model, spindle fibers are made up of oriented polymers which are in an equilibrium with dissociated molecules. The equilibrium is temperature-sensitive, the polymers dissociating at lower temperatures and the molecules associating to form oriented polymers at higher temperatures, up to a maximum.

Assuming that the birefringence measures the amount of oriented molecules, that the total amount of material which can be oriented is constant, and that the equilibrium between oriented and nonoriented material respresents a simple thermodynamic system, a van't Hoff plot was made of the equilibrium constant, log [(birefringence)/(maximum birefringence minus birefringence)] vs. $1/T$ (where T is absolute temperature). A straight line relationship was obtained which showed a very large *increase* in entropy (100 eu at 25°C) and a large heat of activation (29 kcal/mole) as the molecules polymerized. The standard free energy at 25°C was less than -1 kcal. Similar thermodynamic data have now been obtained, using *Pectinaria* oocytes with metaphase equilibrium spindles, in ordinary sea water and in sea water substituted with approximately 40% heavy water (Carolan et al., 1965, 1966).[2]

The high heat and the very high entropy increase associated with the formation of the polymers could be explained primarily by the loss in regularity of the water molecules, which is associated with the free protein molecules (see Kauzmann, 1957; Klotz, 1958, 1960; Robinson and Jencks, 1965; Scott and Berns, 1965; and Scheraga, 1967, for relevant discussions).

Higher temperatures are thought to dissociate bound water from the protein "subunit" molecules and allow them to interact closely (very likely by

[2] As of now, the thermodynamic data obtained comparing behavior of spindle birefringence in deuterated and normal sea water at different temperatures can be explained only by assuming an increased active pool size in D_2O.

hydrophobic interaction) and to associate. The low free energy reflects the weakness of the forces holding the "polymer" together.

The higher degree of alignment of material at higher temperature, as well as the thermodynamic parameters determined, resemble closely those obtained in the polymerization of tobacco mosaic virus (Lauffer et al., 1958) and in G- to F-actin transformation (Asakura et al., 1960; Grant, 1965; Oosawa et al., 1965).

Stevens and Lauffer (1965) have recently measured the buoyancy change of tobacco mosaic virus A-protein as it associated into virus-shaped rods. They concluded that in fact some 150 moles of water are dissociated from each 10^5 g of protein during their polymerization.

These simple model systems also respond to high hydrostatic pressure and to heavy water in a manner similar to the spindle in living cells (Ikkai and Ooi, 1966; Grant, 1965; Khalil et al., 1964).

5. EFFECT OF HEAVY WATER ON SPINDLE BIREFRINGENCE

In the dynamic equilibrium model, structured water is believed to dissociate from the protein molecules upon polymerization. Substitution of heavy water (D_2O) for normal water (H_2O) might then be expected to alter the equilibrium. Gross and Spindel (1960 *a*, *b*) and Marsland and Zimmerman (1965) have shown high concentrations of D_2O to "freeze" mitosis, or to overstabilize the gel structure of the spindle. Marsland and Hiramoto (1966) have shown the stiffness of the sea urchin egg to rise with D_2O.

According to Sidgwick (1950), heavy water molecules are held together more tightly than ordinary water molecules. Heavy water then may be expected to strip the protein molecules of ordered water and shift the equilibrium toward greater association of the molecules. This would enhance polymerization and birefringence of the spindle fibers (see Tomita et al., 1962, and Némethy and Scheraga, 1964, for other possible effects of heavy water on protein structure.)

This was, in fact, found to be so, as shown in Figs. 4–7, 9, and Table I, both in *Pectinaria* metaphase equilibrium oocyte and in dividing eggs of a sea urchin, *Lytechinus variegatus* (Inoué et al., 1963; Sato et al., 1966). When 45% D_2O was applied at the appropriate stage of mitosis, the spindle birefringence was found to increase at least 2-fold, and the volume occupied by the birefringent spindle, to increase as much as 10-fold at times. Fig. 7 illustrates the rapid change in the responsiveness of the spindle to D_2O (and temperature treatment) during mitosis in developing sea urchin eggs.

The action of heavy water is rapid, being 80% complete within 40 sec (see Tucker and Inoué, 1963, for determination of the rapid penetration rate of D_2O into sea urchin eggs). It is completely reversible, and the experiment can

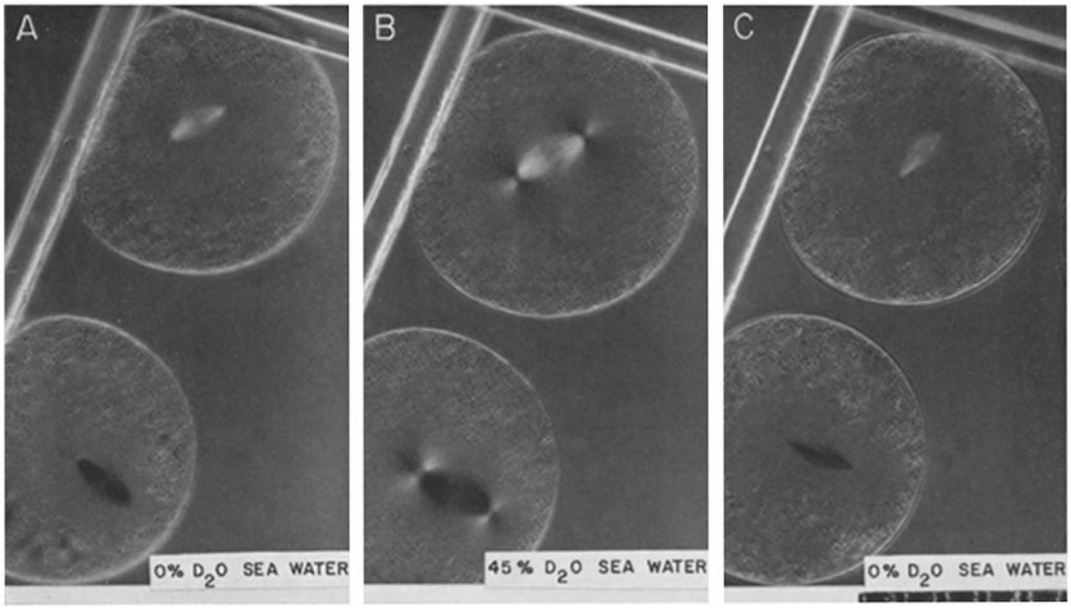


FIGURE 4. Reversible enhancement of spindle volume and birefringence by heavy water in arrested metaphase oocyte of *Pectinaria gouldi*. Cells supported by Butvar film and slightly compressed. Polarization microscopy. Scale interval, 10 μ. *A*, before D_2O–sea water perfusion; *B*, perfused for 2 min in 45% D_2O–sea water; *C*, approximately 3 min after return to normal sea water perfusion.

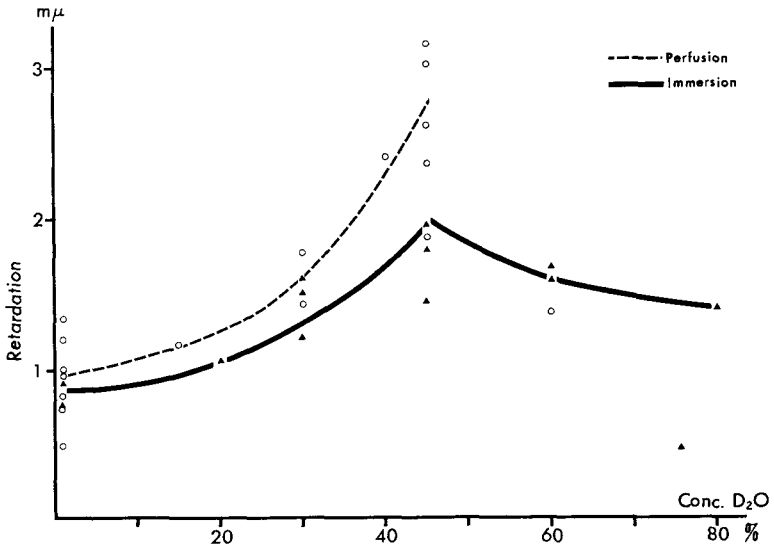


FIGURE 5. Effect of various concentrations of D_2O on the measured birefringence (retardation) of the mitotic spindle.

be repeated many times over on the same metaphase equilibrium cell. In many respects, it is similar to the effects of higher temperature and opposite to that of chilling.

Electron microscopy of spindles fixed with a new fixative, which prevents alteration of spindle birefringence during fixation (see section 10), showed the heavy water–treated spindle to have a larger bulk and more spindle filaments (Fig. 9 *B*) than the control (Fig. 9 *A*). The density of filaments was approximately equal to the control (Inoué, Kane, and Sato, unpublished data). Thus there is a parallel between birefringence observed in living cells under

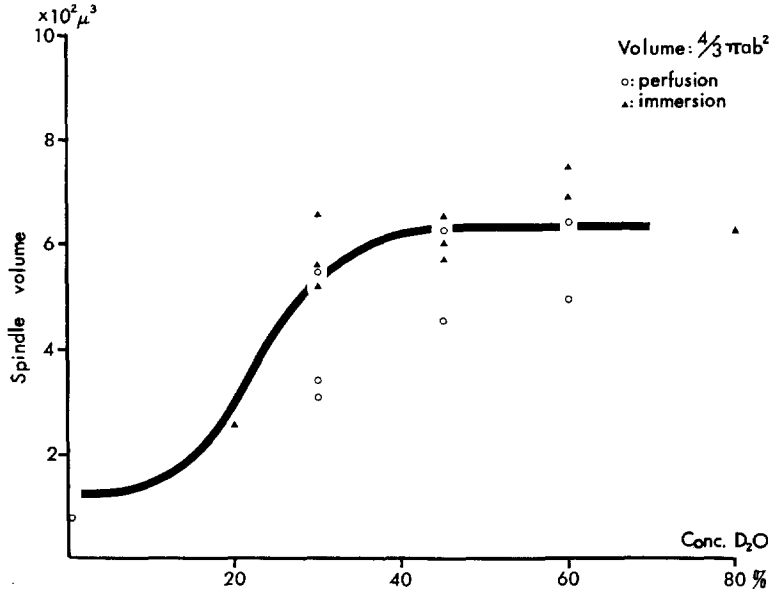


FIGURE 6. Spindle volume vs. D_2O concentration of *Pectinaria* oocyte. Temperature, 22° ± 1°C.

polarized light and the number of filaments (microtubules) observed with the electron microscope.

Similarly, a parallel increase is found in the quantity of the major (22S) protein extractable from an isolated spindle with and without heavy water treatment (Table I). In this connection, it should be pointed out that the total 22S protein extracted from each whole unfertilized egg treated with heavy water was no greater than that found in the untreated control eggs.

6. ASSEMBLY FROM POOL, NOT DE NOVO SYNTHESIS

Given the lability of the spindle fiber and its ability to recover rapidly from disruption and even to rapidly add more birefringent material, are the spindle fiber molecules synthesized rapidly each time they are required, or are they

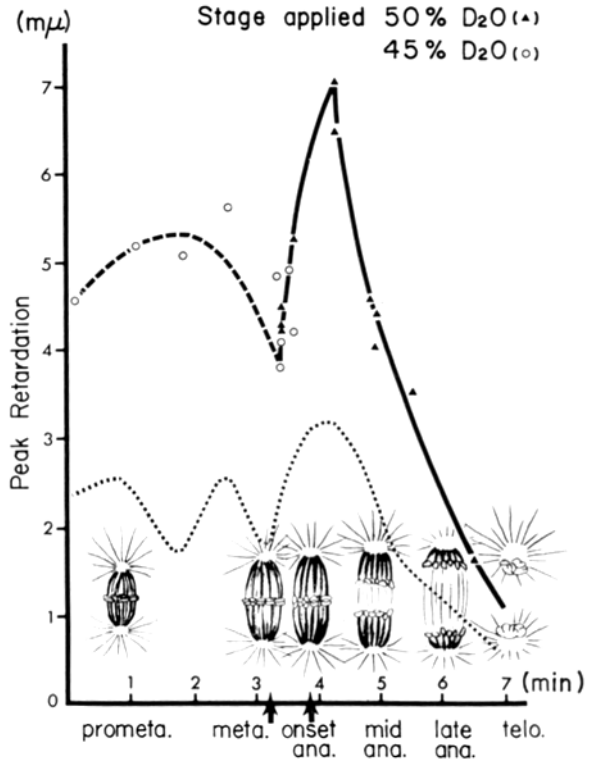


FIGURE 7. In developing eggs of sea urchin *Lytechinus variegatus* placed in D_2O–sea water, the spindle volume and birefringence increase, but with different sensitivities at different stages of mitosis. In 30–40% D_2O–sea water, mitosis proceeds. In 45–60% D_2O–sea water, mitosis is arrested unless the cell is exposed to D_2O during or after anaphase. At 50% D_2O, the retardation can be doubled and may reach a maximum of 7 mμ only if D_2O reaches the spindle at a particular 10 sec interval in early anaphase. (See Tucker and Inoué, 1963, for D_2O penetration rate.) The differential sensitivity of the spindle fiber birefringence to D_2O at different stages of mitosis closely parallels its sensitivity to chilling (Inoué, unpublished data). Heavy broken line and heavy black line in this figure indicate measured peak retardation achieved by spindle fiber with 45 and 50% D_2O–sea water at different stages of mitosis. Thin broken line indicates control.

TABLE I

ANALYTICAL ULTRACENTRIFUGATION DATA

Material as in Fig. 11 (Sato et al., 1966).

Concentration of D_2O in sea water	22S protein per spindle	Ratio*
%	*mg* × 10^{-7}	
0	2.4–5.1‡	2.6–10.0
40	9.0–35.0	

* Ratios of 22S protein per spindle in D_2O vs. H_2O sea water eggs, calculated for each experiment.

‡ Average = 3.5.

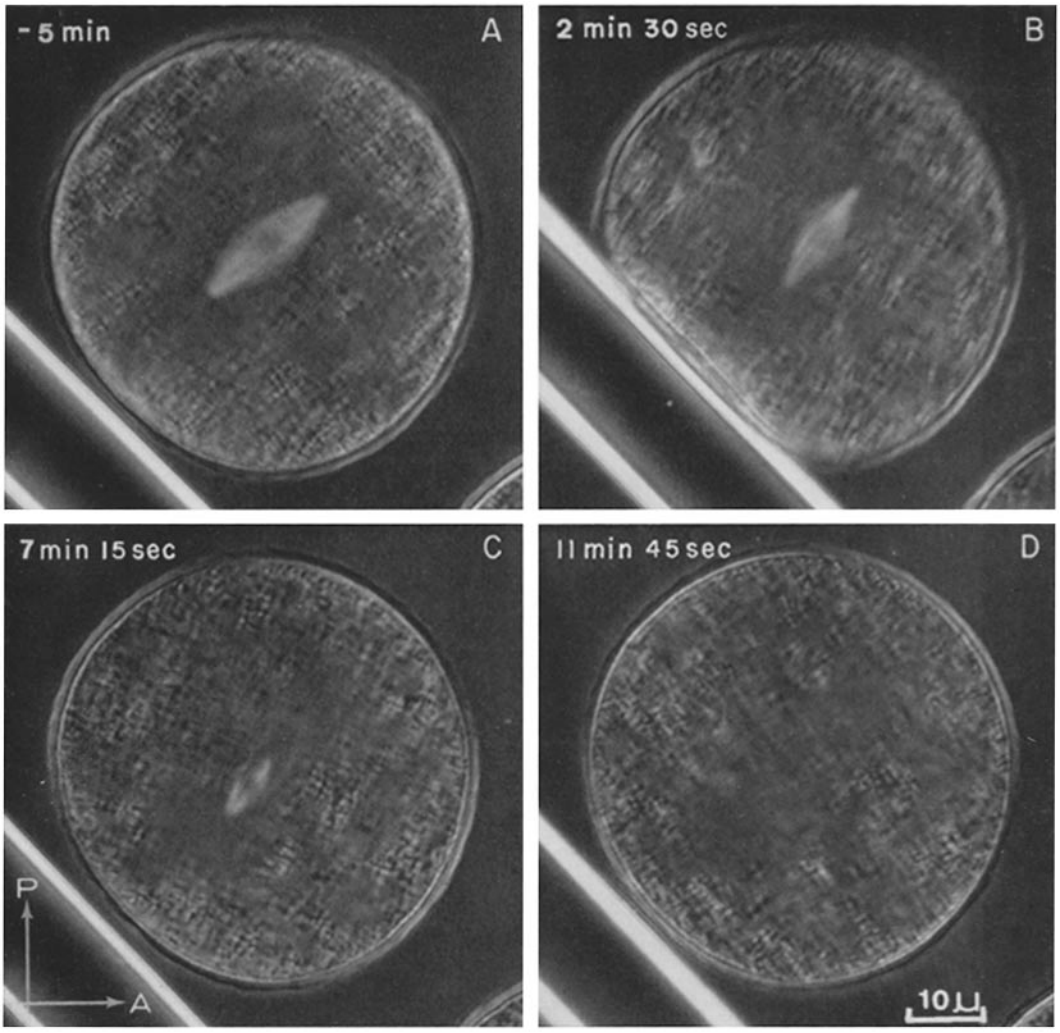


FIGURE 8. Effect of 10^{-5} M Colcemid-sea water perfusion on the arrested metaphase spindle of *Pectinaria* oocyte. The eggs, without compression, were placed on a slide glass coated with a thin layer of Butvar film, to which the eggs adhere. They were surrounded by glass wool, which further prevents the eggs from flowing away during rapid perfusion. *A*, 5 min before the perfusion; *B*, 2 min 30 sec after the Colcemid perfusion was started; *C*, 7 min 15 sec in Colcemid, the miniature spindle is still visible; *D*, after 11 min 45 sec, the spindle has disappeared completely. $P\uparrow$, vibrating direction of polarized light; $A\rightarrow$, direction of analyzer; applies to all figures in polarized light.

available from a ready pool? Colchicine and analogues reversibly abolish the spindle birefringence as reported earlier (section 3). As shown in Fig. 8, the metaphase spindle of *Pectinaria* oocyte shrinks and loses its birefringence in the course of approximately 10 min with 10^{-5} M Colcemid in sea water.

As described in the case of *Chaetopterus* oocytes (Inoué, 1952 *a*), when low concentrations of colchicine or of Colcemid are applied, the chromosomal spindle fibers contract and pull the chromosomes to the cell surface to which they are closest. Fig. 9 *D* shows an electron micrograph of a spindle in the process of dissolution in Colcemid. The cell was fixed by the method described in section 10. Remnants of short fragments of spindle filaments could still be seen around the chromosome, and the chromosomes moved closer to the cell surface during contraction of the spindle (compare with control spindle, Fig. 9 *A*). Fig. 9 *C* shows a similar situation in a cell treated with cold. When cells treated with Colcemid are washed with normal sea water, their spindle recovers in the course of 45–60 min. The recovery is not shortened or prolonged by washing with heavy water.

If this recovery requires protein synthesis, recovery would be expected to be delayed or prevented by the addition of inhibitors of protein synthesis. In fact, addition of actinomycin D, puromycin, or chloramphenicol did not hinder recovery of the spindle to completion. Sato and Inoué (manuscript in preparation) describe the effects of these agents on regular mitosis and cleavage. For some unknown reason, puromycin and actinomycin D accelerated the recovery, as shown in Fig. 10 (Inoué et al., 1965). There is no suggestion, then, that protein synthesis is required during recovery of spindle filaments or microtubules.

As described earlier, heavy water increases the birefringence retardation and the volume of the birefringent spindle material (Figs. 4–7). It also increased both of these parameters in isolated spindles, provided the cells were treated with heavy water while still intact (Fig. 11). The amount of the major spindle (22S) protein in the isolated spindle increases approximately proportionately with the amount of birefringent material, as shown in Table I, while the total 22S protein per cell remains unchanged (Sato et al., 1966).

The rapid rise of birefringence and volume of the spindle in D_2O–sea water, and the parallel increase in the spindle protein content without increase in the same protein in the whole cell, suggests that a sizable pool of protein must preexist in the cell. Kane (1967) has, in fact, found a large quantity of the major spindle protein already present in the unfertilized sea urchin egg. The amount of 22S protein in the unfertilized whole egg is some 20 times greater than that extracted from the first mitotic metaphase spindle.

These observations fit our working hypothesis that there exists in the cell a large quantity of pool material which can be associated to form the filaments and fibers of the mitotic spindle.

7. CONTROL OF ORIENTATION

From observations of birefringence in the living spindle, and changes induced in spindle birefringence by ultraviolet microbeam irradiation (Campbell and Inoué, 1965; Forer, 1965, 1966; Inoué and Sato, quoted in Inoué, 1964),

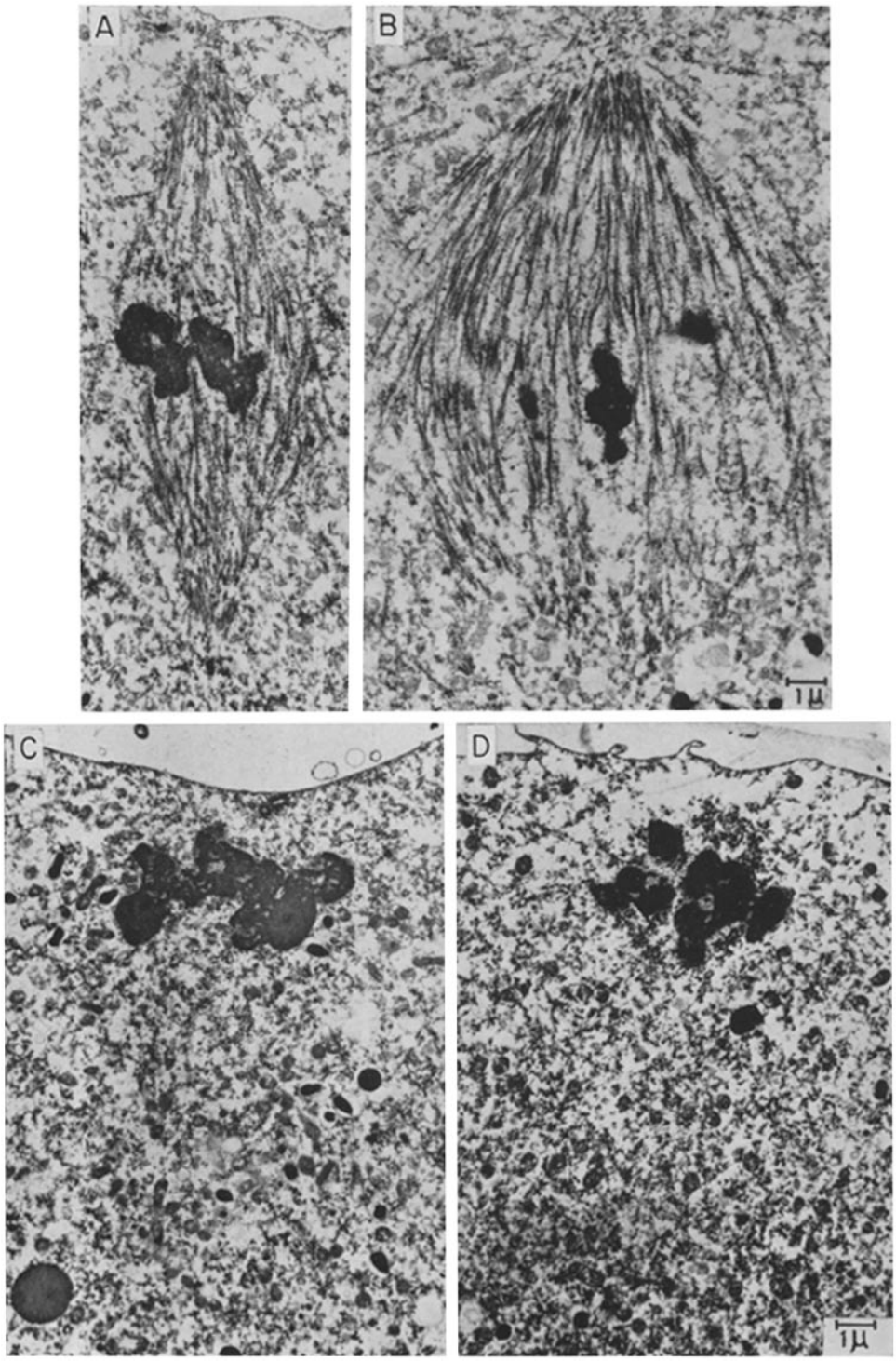


FIGURE 9

we believe that the spindle material can be assembled and oriented by three general mechanisms: (*a*) the activity of orienting centers, such as centrioles, kinetochores, and cell plate in the phragmoplast; (*b*) condensation of high concentration of monomer material into, for example, clear zone and sheath

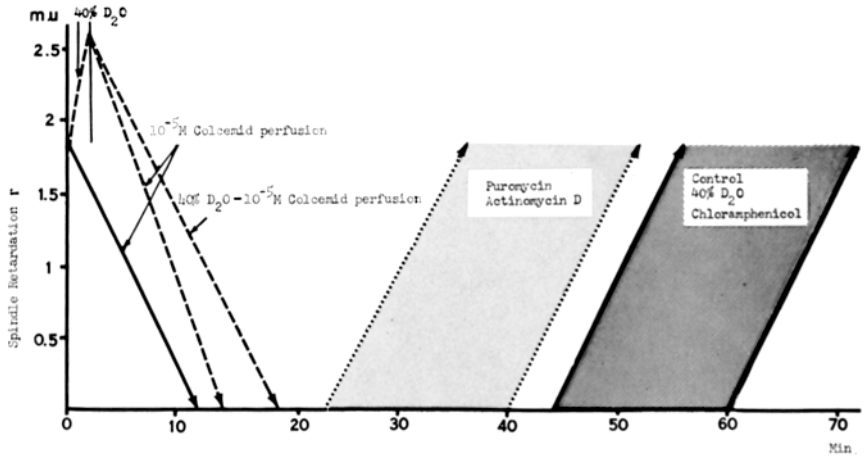


FIGURE 10. Summary of *Pectinaria* spindle behavior in 10^{-5} M Colcemid. Reduction of birefringence and spindle length are both delayed, but proceed at their same rates if 40% D_2O is mixed with Colcemid. Recovery upon washing, which was commenced 2 min after spindle birefringence became undetectable, is not accelerated by D_2O but is increased by the presence of puromycin or actinomycin D. No delay or inhibition of recovery is observed by the addition of these two antimetabolites or by chloramphenicol (Inoué et al., 1965).

fibers as seen in *Haemanthus*, *Lilium*, and *Pales* (also see Molé-Bajer, 1953 *a*,*b*); and (*c*) alignment parallel to other filaments or fibers already existing. Mechanism *a* has been discussed by Inoué (1964), and *b* and *c* have been discussed in section 1 of this paper.

FIGURE 9. Electron micrographs of arrested metaphase spindle of a *Pectinaria* oocyte under various experimental alterations. A new fixation procedure utilizing a mixture of glutaraldehyde and hexylene glycol was developed. This quantitatively maintains the birefringence of the spindle during fixation and, hence, preserves the ordered fine structure of the mitotic spindle (Inoué, Kane, and Sato, unpublished data). *A*, control. *B*, enlarged spindle in 45% D_2O. The total number of microtubles in the spindle is increased, but their density remains approximately the same as in the control. *C*, loss of spindle filaments. Metaphase chromosomes are pulled to the cell surface in a slowly chilled cell. *D*, process of spindle filament disintegration in 10^{-5} M Colcemid. Some fragments of the filaments are detectable around the chromosomes, which have been moved to the cell surface by the contracting spindle.

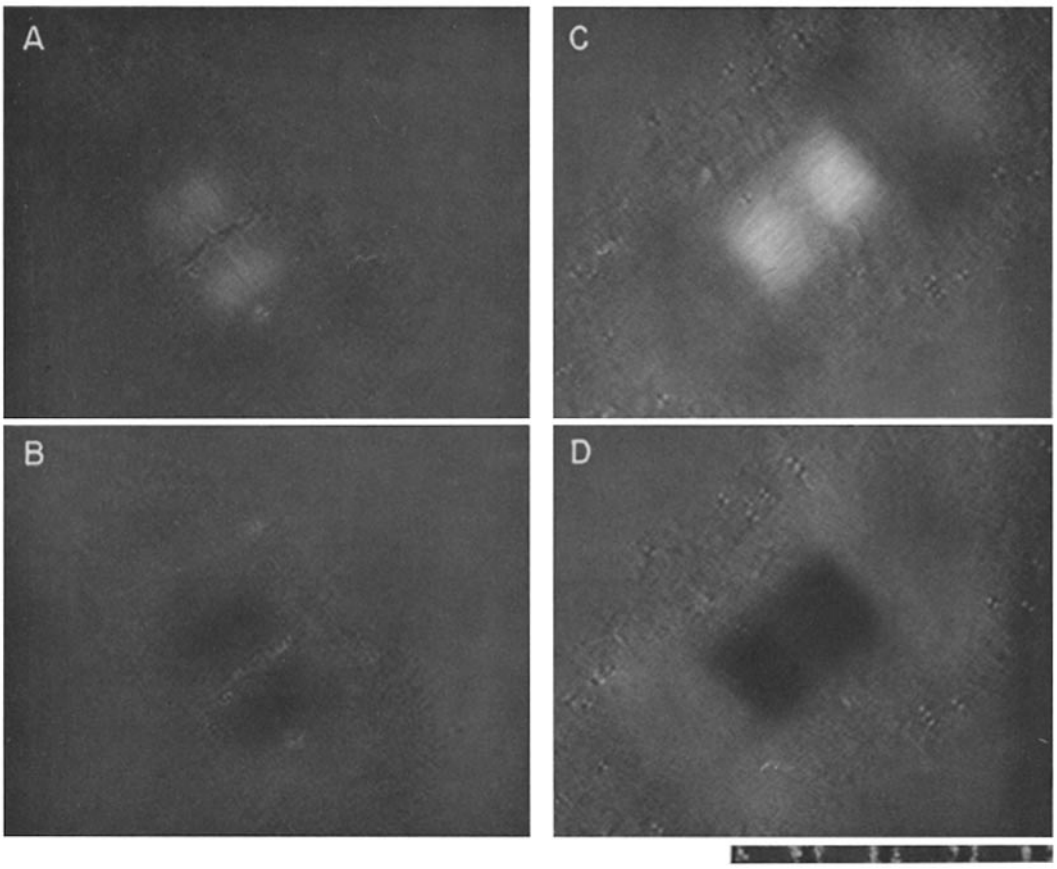


FIGURE 11. Mitotic spindles isolated with hexylene glycol (Kane, 1965) from eggs of *Arbacia punctulata* at first cleavage metaphase; polarization microscopy. *A*, control, chromosomal fibers appear bright. *B*, control, compensator axis reversed, chromosomal fibers dark. *C*, spindle isolated in 40% D_2O–sea water. Note increase of both spindle volume and birefringence. *D*, Same as *C*, compensator axis reversed. Scale interval, 10 μ.

In general, fibrogenesis may follow a pattern suggested by Rees (1951) and by Mercer (1952), or some slight modification of this mechanism: namely, by the linear or helical association of globular proteins to form filaments, which, in turn, condense into tactoids and then become cross-linked by chemical bonds to form coarser fibers. It is important, in the case of spindle fibers, not to have fibrogenesis proceed too far to establish stably cross-linked fibers, but to leave the oriented material in the labile intermediate state described before. With acids (Lewis, 1923; Kane, 1962), with dehydration (Molé-Bajer, 1953), and in the presence of cadmium salts or other heavy

metals (Wada, 1965), one tends to push the equilibrium too far and acquire coarse aggregations, which then make the fibers unfunctional.

8. EXTENSION AND CONTRACTION OF SPINDLE FIBERS

We contend that the spindle fibers and filaments can elongate simply by condensation and polymerization of the spindle molecules. We believe that the elongation can provide forces to push chromosomes or to deform cell surfaces.

Many examples of elongation of spindle fibers with attendant chromosome movement occur naturally during formation of the spindle. It also occurs during recovery from cold, Colcemid, UV microbeam irradiation, and other treatments.

The polymerization that elongates the filaments could be brought about by a simple shift of equilibrium from free molecules to filaments by increased concentration of the free molecules, by the activation of orienting centers, or by mild dehydration. Increased polymerization by heavy water and elevated temperature have been described above. A slightly lower pH also tends to encourage further association, as suggested by Kane's data on the condition required for isolation of the spindle (Kane, 1962, 1965; Lewis, 1923; also see Anderson, 1956, and Klotz, 1958, for interesting relevant discussions).

Contraction, on the other hand, is believed to be brought about by slow removal of the molecules from the polymerized filaments. The fact that chromosomes are pulled poleward by the dissolving fibers in low concentrations of colchicine and by slow cooling has been described above and earlier (Inoué, 1952 *a*, 1964). Slow removal of the molecules from the filaments allows the filaments to reach a new equilibrium position rather than falling apart, thereby effectively shortening the fibers and resulting in a slow contraction. If the molecules were pulled out too fast, then the filaments would fall apart, the structure would simply collapse, and one would not achieve contraction. This was observed with rapid cooling or in the presence of higher concentrations of colchicine (Inoué, 1952 *b*).

It is proposed, in anaphase movement of chromosomes, that the slow removal of the material from the chromosomal spindle fibers, particularly toward the spindle pole region, is responsible for the shortening of the chromosomal spindle fibers and, thus, for the movement of the chromosomes (outside of the movement contributed by the elongation of the central spindle). The reason for the belief that depolymerization takes place primarily toward the pole arises from the observation of the distribution of birefringence during the normal course of division, and from the results of UV microbeam irradiation of the chromosomal spindle fibers (Forer, 1965, 1966; Inoué 1964; also see interpretation of Forer's work by Wada, 1965).

9. CHEMISTRY OF THE SPINDLE PROTEIN MOLECULES

Kane (1967) has prepared a pure 22S protein from isolated mitotic apparatuses and from whole cells. Stephens (1965, 1967) has characterized this protein. It is monodisperse, has a particle weight of 880,000, and can be broken down into units of 110,000 particle weight. The 22S material makes up more than 90% of the KCl-soluble proteins of the spindle after isolation with hexylene glycol. Its quantity in the spindle varies parallel with spindle birefringence and with the number of microtubules during D_2O treatment. It is, therefore, a likely candidate for the spindle fiber material. The 22S proteins extracted from the unfertilized whole egg and from the spindle show identical amino acid compositions (Stephens, 1965, 1967).

On the other hand, Sakai (1966) and Kiefer et al. (1966) isolated a 2.5S protein from the mitotic apparatus with a particle weight of approximately 34,000. This particle would appear to have a dimension better fitting the electron microscope periodicity seen in microtubules of spindle and other cellular structures. At this writing, it is not clear whether these are two distinctly different proteins or are the same material polymerized to different degrees (see Introduction by Mazia).

10. RELATION OF SPINDLE FIBER BIREFRINGENCE TO MICROTUBULES AND TO FILAMENTS OBSERVED BY ELECTRON MICROSCOPY

Earlier electron micrographs of dividing cells often failed to show any fibrous elements in the spindle region. The number of filaments or microtubules that were observed in the spindle fiber region was often very small. With the recognition that low temperature can rapidly destroy spindle birefringence and, hence, the oriented spindle material, and following introduction of improved fixatives, increasing numbers of microtubules and filaments have been observed in spindle regions which are birefringent in life. There now exists a good correlation with the distribution of spindle fiber birefringence and density of intact microtubules or filaments (compare Inoué, 1964, with de-Thé, 1964; Harris and Bajer, 1965; Porter, 1966; and Robbins and Gonatas, 1964). Also, when birefringence declines in isolated spindles, a parallel disruption of filaments in the electron micrographs is found (Kane and Forer, 1965).

Even with glutaraldehyde fixation, which generally shows large numbers of microtubules in the electron micrographs, we find that the *Pectinaria* oocyte spindle birefringence had declined by 50% in the fixative. Further reduction was found with neutral osmium fixation. However, mixture of these fixatives with the spindle-isolating medium, hexylene glycol, prevented the

decline of birefringence. Furthermore, the elevated birefringence in cells treated with D_2O and the reduced birefringence of cells treated for a short period with Colcemid retained their values exactly when fixed in this new fixative (Inoué, Kane, and Sato, unpublished data). The number of spindle filaments thus observed with the electron microscope and the birefringence of the spindle fibers observed in the polarizing microscope showed a close

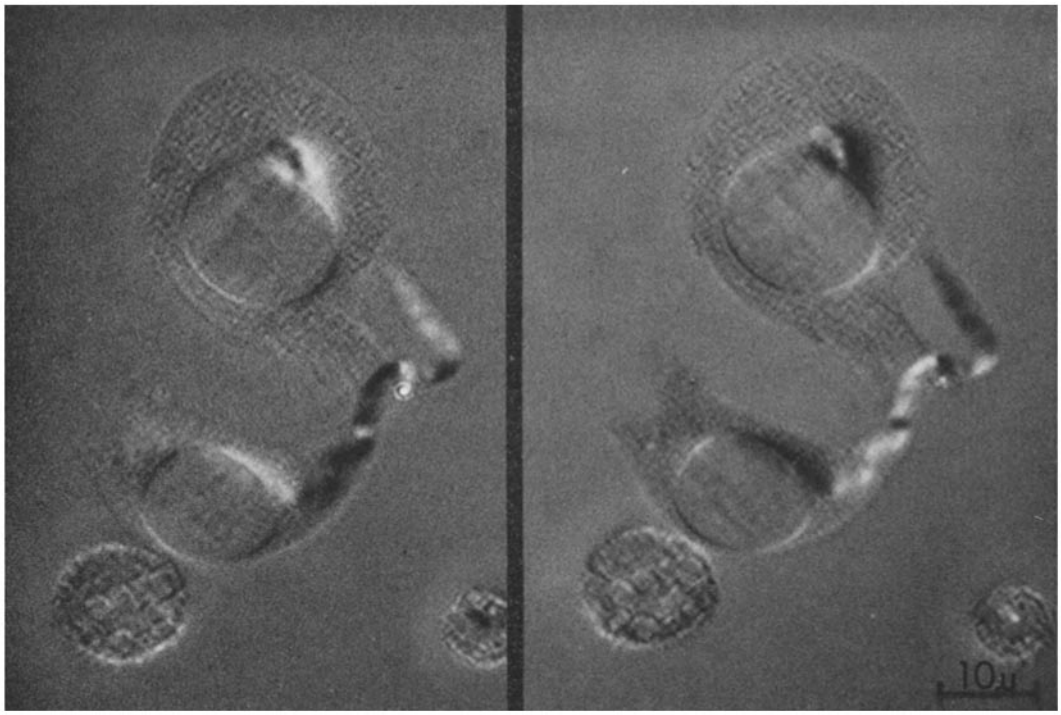


FIGURE 12. Polarization microscopy of Balb/C^+ renal tumor cell from the mesenteric vein of a mouse. Live cells were embedded in Kel-F 10 oil. The fibrous structures seen near the nucleus and cytoplasmic process have a positive sign of birefringence. They correspond to the aggregated bundle of microfilaments demonstrated in electron micrographs in Fig. 13.

parallel for D_2O-treated eggs (Fig. 9 *B*) and their untreated controls (Fig. 9 *A*).

Microtubules and filaments have been observed by electron microscopy following glutaraldehyde fixation, not only in the mitotic spindle, but also in various other regions of the cell (see review by Porter, 1966). Recently, we had the good fortune of observing renal tumor cells with Professor A. Claude. Examination of living cells under polarized light revealed positively birefringent bundles of material (Fig. 12) running parallel to the bundle of

microfilaments, which Professor Claude had shown in his electron micrograph (Fig. 13) with a special fixative in 1960 (Claude, 1961 *a,b*). Wherever we have tested, we have found that the long axes of such microtubules and filaments lie parallel to the fiber axes of positively birefringent structures in living cells.

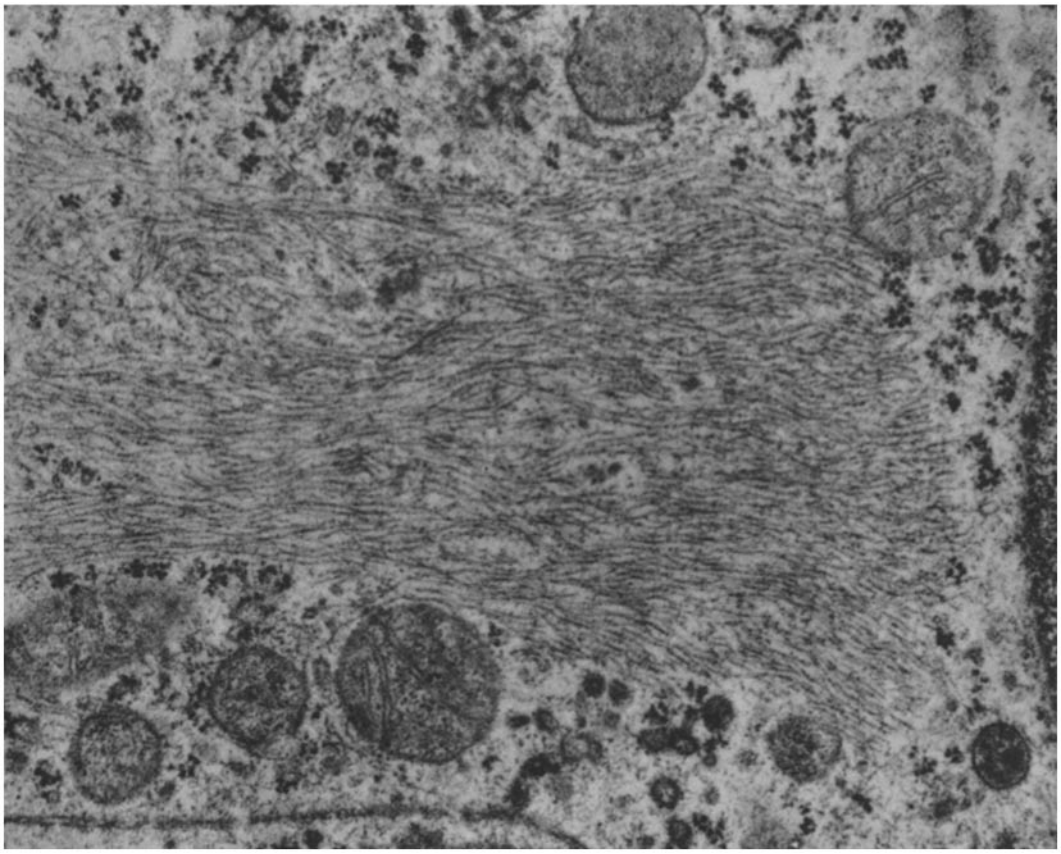


FIGURE 13. Electron micrograph of Balb/C^+ renal tumor cell of a mouse (Claude, 1961 *a,b*, 1965). × 33,400. *Figure reprinted by permission from La Biologie, Acquisitions Récentes, Centre International de Synthèse, Editions Aubier-Montaigne, Paris, 1965, 13.*

In general, conditions which give rise to decreased birefringence also give rise to the reduction of the microtubules and filaments. Conditions which give rise to increase in birefringence give rise to increased microtubules or filaments and, with D_2O, also to the 22S protein in the spindle. This correlation is found in spindle fibers and in axopods of *Actinosphaerium* and in other systems also (see sections 4 and 11).

It now seems reasonable, then, to assume that the measured birefringence

of spindle fibers under a variety of conditions closely follows the number of microtubules or filaments making up the fibers (see Engelmann, 1875, 1906; Schmidt, 1937, 1941; and Picken, 1950, for general discussions relating birefringence or anisotropic fine structure to contraction).

II. CELL MOTILITY AND LABILE ASSOCIATION OF MOLECULES

In the foregoing discussions, the dynamic orientation equilibrium hypothesis was used to explain the organization and function of the spindle. A species of ubiquitous protein molecule available in a pool was reversibly assembled into filaments and fibers upon demand. The material could perform various mechanical functions in the mitotic apparatus during division of the cell, and, when not in division, the same material could be organized in different parts of the cell to perform other functions. It could maintain or alter cell shape. It could partake in pinocytosis, phagocytosis, ameboid movement, streaming, etc. In this connection it is interesting to recall that cells generally stop wandering, and round up, prior to mitosis as though localized mechanical modulations of the cell had disappeared. Cells also generally resume their various mechanical activities shortly following mitosis. The presence of microtubules and filaments in association with these activities has been described, among others: for streaming, by Wolfarth-Bottermann (1964), Nakajima (1964), and Nagai and Rebhun (1966); for melanophore pigment migration, by Bikle et al. (1966); for pinocytosis and ameboid movement, by Marshall and Nachmias (1964) and Nachmias (1964); and in differentiating cells associated with their shape changes, by Arnold (1966), Byers and Porter (1964), Taylor (1966), Tilney and Gibbins (1966), and Tilney et al. (1965, 1966) (also see review by Porter, 1966). In those cases tested, the labile microtubules responded to temperature, hydrostatic pressure, heavy water, and colchicine or their combinations in a manner virtually identical with the response of the spindle material, all being reversible as in the spindle (e.g. Tilney et al., 1965, 1966; Malawista, 1965; Marsland, 1965, 1966; Marsland and Hiramoto, 1966).

We are considering here primarily slow movements such as movements of chromosomes and changes in shape of developing cells. These generally involve velocities of 1 to a few micra per second. In general, the growth and dissolution of the filaments or microtubules are believed to impose an anisotropic modulation of the mechanical properties that could cause slow extension or contraction of local regions of cells. Faster movements may also arise secondarily. For example, cytoplasmic streaming in the slime mold *Physarum* could result from formation and dissolution of the birefringent fibers observed by Nakajima (1964) in their ectoplasm. Local changes in the intracellular pressure, i.e. an interplay between the growth and contraction of the filaments

and the elastic properties of the slime mold surface, could indirectly drive the streaming.

We have outlined our current hypothesis regarding the relation of the birefringent structures to slow movement of cell parts by reversible association of molecules. An alternative interpretation would describe the observed changes in the birefringent structures as simply the formation and dissolution of the fibrous fine structure necessary to make the movement possible. The motor force itself could result from a separate mechanism, as in the sliding filament model of muscle contraction, or from a mechanism such as that proposed by Thornburg (1967), in which fine pulses travel along the length of the fine anisotropic filaments. The dynamic equilibrium hypothesis would then account for motile structure organization rather than for production of force per se. The distinction may, however, turn out to be more subtle than is apparent and awaits analysis of the various systems in further detail (for example, see Szent-Györgyi and Prior, 1966, for the behavior of actin filaments during muscle contraction.)

POSTSCRIPT

This article portrays the authors' best current picture of the molecular mechanism of reversible fibrogenesis and mitotic chromosome movements. Clearly, much information is still wanting, and the arguments are often less than tight. Nevertheless, we hope that the article may convey enough useful information to stimulate further experimentation and to bring to focus some of the critical questions about mitosis and spindle formation which now may be asked.

Supported in part by NIH Grant CA 10171 (formerly 04552) from the National Cancer Institute and by Grant GB 5120 (formerly 2060) from the National Science Foundation.

Much of our work reported here was done at the Department of Cytology, Dartmouth Medical School, and at the Marine Biological Laboratories, Woods Hole, Massachusetts. The authors gratefully acknowledge the efficient assistance provided by the devoted staff, as well as the encouragement and critical discussions offered by their colleagues and students.

REFERENCES

ANDERSON, N. G. 1956. Cell division. II. A theoretical approach to chromosomal movements and the division of the cell. *Quart. Rev. Biol.* **31**:243.

ARNOLD, J. M. 1966. Squid lens development in compounds that affect microtubules. *Biol. Bull.* **131**:383.

ASAKURA, S., M. KASAI, and F. OOSAWA. 1960. The effect of temperature on the equilibrium state of actin solutions. *J. Polymer Sci.* **44**:35.

BAJER, A. 1957. Cine-micrographic studies on mitosis in endosperm. III. The origin of the mitotic spindle. *Exptl. Cell Res.* **13**:493.

BAJER, A. 1961. A note on the behavior of spindle fibres at mitosis. *Chromosoma.* **12**:64.

BAJER, A. 1965. Cine-micrographic analysis of cell plate formation in endosperm. *Exptl. Cell Res.* **37**:376.

BIKLE, D., L. G. TILNEY, and K. R. PORTER. 1966. Microtubules and pigment migration in the melanophores of *Fundulus heteroclitus* L. *Protoplasma.* **61**:322.

BYERS, B., and K. R. PORTER. 1964. Oriented microtubules in elongating cells of the developing lens rudiment after induction. *Proc. Natl. Acad. Sci. U.S.* **52**:1091.

CAMPBELL, R. D., and S. INOUÉ. 1965. Reorganization of spindle components following UV micro irradiation. *Biol. Bull.* **129**:401.

CARLSON, J. G. 1952. Microdissection studies of the dividing neuroblast of the grasshopper, *Chortophaga viridifasciata* (de Geer). *Chromosoma.* **5**:199.

CAROLAN, R. M., H. SATO, and S. INOUÉ. 1965. A thermodynamic analysis of the effect of D_2O and H_2O on the mitotic spindle. *Biol. Bull.* **129**:402.

CAROLAN, M., H. SATO, and S. INOUÉ. 1966. Further observations on the thermodynamics of the living mitotic spindle. *Biol. Bull.* **131**:385.

CHAMBERS, R. 1924. The physical structure of protoplasm as determined by microdissection and injection. *In* General Cytology. E. V. Cowdry, editor. University of Chicago Press, Chicago. 237.

CLAUDE, A. 1961 *a*. Mise en évidence par microscopie électronique d'un appareil fibrillaire dans le cytoplasme et le noyau de certaines cellules. *Compt. Rend.* **253**: 2251.

CLAUDE, A. 1961 *b*. Problems of fixation for electron microscopy. Results of fixation with osmium tetroxide in acid and alkaline media. *Ext. Pathol. Biol.* **9**:933.

CLAUDE, A. 1965. Naissance de la biologie moléculaire. Vingt années d'invention technique et de progrès dans l'exploration de la cellule. *In* La Biologie, Acquisitions Récentes. Centre International de Synthèse. Éditions Aubier-Montaigne, Paris. 13.

CLEVELAND, L. R. 1938. Origin and development of the achromatic figure. *Biol. Bull.* **74**:41.

CLEVELAND, L. R. 1953. Studies on chromosomes and nuclear division. *Trans. Am. Phil. Soc.* **43**:809.

CLEVELAND, L. R. 1963. Functions of flagellate and other centrioles in cell reproduction. *In* The Cell in Mitosis. L. Levine, editor. Academic Press, Inc., New York. 3.

CONKLIN, E. G. 1917. Effects of centrifugal force on the structure and development of the eggs of *Crepidula. J. Exptl. Zool.* **22**:311.

COSTELLO, D. P. 1961. On the orientation of centrioles in dividing cells, and its significance: a new contribution to spindle mechanics. *Biol. Bull.* **120**:285.

DE-THÉ, G. 1964. Cytoplasmic microtubules in different animal cells. *J. Cell Biol.* **23**:265.

DIETZ, R. 1959. Centrosomenfreie Spindelpole in Tipuliden-Spermatocyten. *Z. Naturforsch.* **146**:749.

DIETZ, R. 1963. Polarisationsmikroskipische Befunde zur chromosomeninduzierten Spindelbildung bei der Tipulide *Pales crocata* (Nematocera). *Zool. Anz.* **26** (Suppl.), 131.

ELLIS, G. W. 1962. Piezoelectric micromanipulators. Electrically operated micromanipulators add automatic high-speed movement to normal manual control. *Science.* **138**:84.

ENGELMANN, R. W. 1875. Contractilität und Doppelbrechung. *Arch. Ges. Physiol.* **11**:432.

ENGELMANN, R. W. 1906. Zur Theorie der Contractilität. *Sitzber. Kgl. Preuss. Akad. Wiss.* 694.

FORER, A. 1965. Local reduction of spindle fiber birefringence in living *Nephrotoma suturalis* (Loew) spermatocytes induced by ultraviolet microbeam irradiation. *J. Cell. Biol.* **25**:95.

FORER, A. 1966. Characterization of the mitotic traction system, and evidence that birefringent spindle fibers neither produce nor transmit force for chromosome movement. *Chromosoma.* **19**:44.

FREY-WYSSLING, A., J. F. LOPEZ-SAEZ, and K. MÜHLETHALER. 1964. Formation and development of the cell plate. *J. Ultrastruct. Res.* **10**:422.

GAULDEN, M. E., and J. G. CARLSON. 1951. Cytological effects of colchicine on the grasshopper neuroblast in vitro with special reference to the origin of the spindle. *Exptl. Cell Res.* **2**:416.

GRANT, R. J. 1965. Doctorate Thesis. Columbia University, New York.

GROSS, P. R., and W. SPINDEL. 1960 *a*. Mitotic arrest by deuterium oxide. *Science.* **131**:37.

GROSS, P. R., and W. SPINDEL. 1960 *b*. The inhibition of mitosis by deuterium. *Ann. N.Y. Acad. Sci.* **84**:745.

HARRIS, P. 1962. Some structural and functional aspects of the mitotic apparatus in sea urchin embryos. *J. Cell Biol.* **14**:475.

HARRIS, P., and A. BAJER. 1965. Fine structure studies on mitosis in endosperm metaphase of *Haemanthus katherinae* bak. *Chromosoma.* **16**:624.

HIRAMOTO, Y. 1964. Mechanical properties of the starfish oöcyte during maturation divisions. *Biol. Bull.* **127**:373.

IKKAI, T., and T. OOI. 1966. The effects of pressure on F-G transformation of actin. *Biochemistry.* **5**:1551.

INOUÉ, S. 1952 *a*. The effect of colchicine on the microscopic and submicroscopic structure of the mitotic spindle. *Exptl. Cell Res.* Suppl. 2:305.

INOUÉ, S. 1952 *b*. Effect of temperature on the birefringence of the mitotic spindle. *Biol. Bull.* **103**:316.

INOUÉ, S. 1953. Polarization optical studies of the mitotic spindle. I. The demonstration of spindle fibers in living cells. *Chromosoma.* **5**:487.

INOUÉ, S. 1959. Motility of cilia and the mechanism of mitosis. *Rev. Mod. Phys.* **31**:402.

INOUÉ, S. 1960. On the physical properties of the mitotic spindle. *Ann. N.Y. Acad. Sci.* **90**:529.

INOUÉ, S. 1964. Organization and function of the mitotic spindle. *In* Primitive Motile Systems in Cell Biology. R. Allen and N. Kamiya, editors. Academic Press, Inc., New York. 549.

INOUÉ, S., and A. BAJER. 1961. Birefringence in endosperm mitosis. Chromosoma. **12**:48.

INOUÉ, S., and K. DAN. 1951. Birefringence of the dividing cell. *J. Morphol.* **89**:423.

INOUÉ, S., and H. SATO. 1964. See Inoué (1964). 580.

INOUÉ, S., H. SATO, and M. ASCHER. 1965. Counteraction of Colcemid and heavy water on the organization of the mitotic spindle. *Biol. Bull.* **129**:409.

INOUÉ, S., H. SATO, and R. W. TUCKER. 1963. Heavy water enhancement of mitotic spindle birefringence. *Biol. Bull.* **125**:380.

IZUTSU, K. 1961 *a*. Effects of ultraviolet microbeam irradiation upon division in grasshopper spermatocytes. I. Results of irradiation during prophase and prometaphase I. *Mie Med. J.* **11**:199.

IZUTSU, K. 1961 *b*. Effects of ultraviolet microbeam irradiation upon division in grasshopper spermatocytes. II. Results of irradiation during metaphase and anaphase I. *Mie Med. J.* **11**:213.

KANE, R. E. 1962. The mitotic apparatus: isolation by controlled pH. *J. Cell Biol.* **12**:47.

KANE, R. E. 1965. The mitotic apparatus: physical-chemical factors controlling stability. *J. Cell Biol.* **25**:137.

KANE, R. E. 1967. The mitotic apparatus: identification of the major soluble component of the glycol-isolated mitotic apparatus. *J. Cell Biol.* **32**:243.

KANE, R. E., and A. FORER. 1965. The mitotic apparatus: structural changes after isolation. *J. Cell Biol.* **25**:31.

KAUZMANN, W. 1957. The physical chemistry of proteins. *Ann. Rev. Phys. Chem.* **8**:413.

KHALIL, M. T., R. A. SHALABY, and M. A. LAUFFER. 1964. Reversible polymerization of TMV protein in D_2O and versene solutions. Abstracts of the Biophysical Society 8th Annual Meeting. Chicago. TC3.

KIEFER, B., H. SAKAI, A. J. SOLARI, and D. MAZIA. 1966. The molecular unit of the microtubules of the mitotic apparatus. *J. Mol. Biol.* **20**:75.

KLOTZ, I. M. 1958. Protein hydration and behavior. *Science.* **128**:815.

KLOTZ, I. M. 1960. Non-covalent bonds in protein structure. *In Brookhaven Symp. Biol.* **13**:25.

LAUFFER, M. A., A. T. ANSEVIN, T. E. CARTWRIGHT, and C. C. BRINTON, JR. 1958. Polymerization-depolymerization of tobacco mosaic virus protein. *Nature.* **181**: 1338.

LEDBETTER, M. C., and K. R. PORTER. 1963. A "microtubule" in plant cell fine structure. *J. Cell Biol.* **19**:239.

LEWIS, M. R. 1923. Reversible gelation in living cells. *Bull. Johns Hopkins Hosp.* **34**:373.

MALAWISTA, S. E. 1965. On the action of colchicine: the melanocyte model. *J. Exptl. Med.* **122**:361.

MALAWISTA, S. E., and H. SATO. 1966. Vinblastine and griseofulvin reversibly disrupt the living mitotic spindle. *Biol. Bull.* **131**:397.

MARSHALL, J. M., and V. T. NACHMIAS. 1964. Cell surface and pinocytosis. *J. Cell Biol.* **13**:92.

MARSLAND, D. 1965. Partial reversal of the anti-mitotic effects of heavy water by high hydrostatic pressure: an analysis of the first cleavage division in the eggs of *Strongylocentrotus purpuratus. Exptl. Cell Res.* **38**:592.

MARSLAND, D. 1966. Anti-mitotic effects of colchicine and hydrostatic pressure; synergistic action on the cleaving eggs of *Lytechinus variegatus. J. Cell Physiol.* **67**:333.

MARSLAND, D., and Y. HIRAMOTO. 1966. Cell division: pressure-induced reversal of the antimeiotic effects of heavy water in the oocytes of the starfish, *Asterias forbesi*. *J. Cell Physiol.* **67**:13.

MARSLAND, D., and A. M. ZIMMERMAN. 1965. Structural stabilization of the mitotic apparatus by heavy water, in the cleaving eggs of *Arbacia punctulata*. *Exptl. Cell Res.* **38**:306.

MAZIA, D. 1961. Mitosis and the physiology of cell division. *In* The Cell. J. Brachet and A. E. Mirsky, editors. Academic Press, Inc., New York. **3**:77.

MERCER, E. H. 1952. The biosynthesis of fibers. *Sci. Monthly*. **75**:280.

MOLÉ-BAJER, J. 1953 *a*. Influence of hydration and dehydration on mitosis. II. *Acta Soc. Botan. Polon.* **22**:33.

MOLÉ-BAJER, J. 1953 *b*. Experimental studies on protein spindles. *Acta Soc. Botan. Polon.* **22**:811.

MOLÉ-BAJER, J. 1958. Cine-micrographic analysis of C-mitosis in endosperm. *Chromosoma*. **9**:332.

NACHMIAS, V. T. 1964. Fibrillar structures in the cytoplasm of *Chaos chaos*. *J. Cell Biol.* **23**:183.

NAGAI, R., and L. I. REBHUN. 1966. Cytoplasmic microfilaments in streaming *Nitella* cells. *J. Ultrastruct. Res.* **14**:571.

NAKAJIMA, H. 1964. The mechanochemical system behind streaming in *Physarum*. *In* Primitive Motile Systems in Cell Biology. R. Allen and N. Kamiya, editors. Academic Press, Inc., New York. 111.

NÉMETHY, G., and H. A. SCHERAGA. 1964. Structure of water and hydrophobic bonding in proteins. IV. The thermodynamic properties of liquid deuterium oxide. *J. Chem. Phys.* **41**:680.

NICKLAS, R. B. 1965. Experimental control of chromosome segregation in meiosis. *J. Cell Biol.* **27**:117A.

NICKLAS, R. B. 1967. Chromosome micromanipulation. I. The mechanics of chromosome attachment to the spindle. II. Induced reorientation and the experimental control of segregation in meiosis. *Chromosoma*. **21**:1, 17.

OOSAWA, F., S. ASAKURA, S. HIGASHI, M. KASAI, S. KOBAYASHI, E. NAKANO, T. OHNISHI, and M. TANIGUCHI. 1965. Morphogenesis and motility of actin polymers. *In* Molecular Biology of Muscular Contraction. Igakushoin, Tokyo. 56.

ÖSTERGREN, G. 1951. The mechanism of co-orientation in bivalents and multivalents. *Hereditas*. **37**:85.

PEASE, D. C. 1946. High hydrostatic pressure effects upon the spindle figure and chromosome movement. II. Experiments on the meiotic divisions of *Tradescantia* pollen mother cells. *Biol. Bull.* **91**:145.

PICKEN, L. E. R. 1950. Discussion on morphology and fine structure. Fine structure and the shape of cells and cell-components. *Proc. Linnean Soc. London*. **162**:72.

PORTER, K. R. 1966. Cytoplasmic microtubules and their functions. *Ciba Found. Symp., Principles Biomol. Organ.* 308.

PORTER, K. R., and R. D. MACHADO. 1960. Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tip. *J. Biophys. Biochem. Cytol.* **7**:167.

REES, A. L. G. 1951. Directed aggregation in colloidal systems and the formation of protein fibers. *J. Phys. Chem.* **55**:1340.

ROBBINS, E., and N. K. GONATAS. 1964. The ultrastructure of a mammalian cell during the mitotic cycle. *J. Cell Biol.* **21**:429.

ROBINSON, D. R., and W. P. JENCKS. 1965. The effect of compounds of the urea-guanidinium class on the activity coefficient of acetyltetraglycine ethyl ester and related compounds. *J. Am. Chem. Soc.* **87**:2462, 2470, 2480.

SAKAI, H. 1966. Studies on sulfhydryl groups during cell division of sea-urchin eggs. VIII. Some properties of mitotic apparatus proteins. *Biochim. Biophys. Acta.* **112**:132.

SATO, H., S. INOUÉ, J. BRYAN, N. E. BARCLAY, and C. PLATT. 1966. The effect of D_2O on the mitotic spindle. *Biol. Bull.* **131**:405.

SCHERAGA, H. A. 1967. Contractility and conformation. *J. Gen. Physiol.* **50**(6, Pt. 2):5.

SCHMIDT, W. J. 1937. Die Doppelbrechung von Chromosomen und Kernspindel und ihre Bedeutung für das kausale Verständniss der Mitose. *Arch. Exptl. Zellforsch.* **19**:352.

SCHMIDT, W. J. 1939. Doppelbrechung der Kernspindel und Zugfasertheorie der Chromosomenbewegung. *Chromosoma.* **1**:253.

SCHMIDT, W. J. 1941. Die Doppelbrechung des Protoplasmas und ihre Bedeutung für die Erforschung seines submikroskopischen Baues. *Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol.* **44**:27.

SCHRADER, F. 1953. Mitosis. The Movements of Chromosomes in Cell Division. Columbia University Press, New York. 2nd edition.

SCOTT, E., and D. S. BERNS. 1965. Protein-protein interaction. The phycocyanin system. *Biochemistry* **4**:2597.

SHIMAMURA T. 1940. Studies on the effect of the centrifugal force upon nuclear division. *Cytologia.* **11**:186.

SIDGWICK, N. V. 1950. Chemical Elements and Their Compounds. Clarendon Press, Oxford. **1**:44.

STEPHENS, R. E. 1965. Characterization of the major mitotic apparatus protein and its subunits. Doctorate Thesis. Dartmouth College, Hanover, N.H.

STEPHENS, R. E. 1967. The mitotic apparatus. Physical chemical characterization of the 22S protein component and its subunits. *J. Cell Biol.* **32**:255.

STEVENS, C. L., and M. A. LAUFFER. 1965. Polymerization-depolymerization of tobacco mosaic virus protein. IV. The role of water. *Biochemistry.* **4**:31.

SZENT-GYÖRGYI, A. G., and G. PRIOR. 1966. Exchange of adenosine diphosphate bound to actin in superprecipitated actomyosin and contracted myofibrils. *J. Mol. Biol.* **15**:515.

TAYLOR, A. C. 1966. Microtubules in the microspikes and cortical cytoplasm of isolated cells. *J. Cell Biol.* **28**:155.

TAYLOR, E. W. 1959. Dynamics of spindle formation and its inhibition by chemicals. *J. Biophys. Biochem. Cytol.* **6**:193.

THORNBURG, W. 1967. Mechanisms of biological motility. *In* Theoretical and Experimental Biophysics. A. Cole, editor. Marcel Dekker, New York **1**:77.

TILNEY, L. G., and J. R. GIBBINS. 1966. Microtubules and morphogenesis. The role

of microtubules in the development of the primary mesenchyme in the sea urchin embryo. *Biol. Bull.* **131**:378.

TILNEY, L. G., Y. HIRAMOTO, and D. MARSLAND. 1966. Studies on the microtubules in heliozoa. III. A pressure analysis of the role of these structures in the formation and maintenance of the axopodia of *Actinosphaerium nucleofilum* (Barrett). *J. Cell Biol.* **29**:77.

TILNEY, L. G., and K. PORTER. 1965. Studies on microtubules in heliozoa. I. The fine structure of *Actinosphaerium nucleofilum* (Barrett), with particular reference to the axial rod structure. *Protoplasma.* **60**:317.

TOMITA, K., A. RICH, C. DE LOZÉ, and E. R. BLOUT. 1962. The effect of deuteration on the geometry of the α-helix. *J. Mol. Biol.* **4**:83.

TUCKER, R. W., and S. INOUÉ. 1963. Rapid exchange of D_2O and H_2O in sea urchin eggs. *Biol. Bull.* **125**:395.

WADA, B. 1965. Analysis of mitosis. *Cytologia.* **30** (Suppl.):1.

WENT, M. A. 1966. The behavior of centrioles and the structure and formation of the achromatic figure. *Protoplasmatologia.* **6** (G 1):1.

WOHLFARTH-BOTTERMANN, K. E. 1964. Differentiations of the ground cytoplasm and their significance for the generation of the motive force of ameboid movement. *In* Primitive Motile Systems in Cell Biology. R. Allen and N. Kamiya, editors. Academic Press, Inc., New York. 79.

ZIMMERMAN, A. M., and D. MARSLAND. 1964. Cell division: effects of pressure on the mitotic mechanisms of marine eggs (*Arbacia punctulata*). *Exptl. Cell. Res.* **35**:293.

Discussion

Dr. Bárány: I would like to make two brief comments on the very nice work just presented by Dr. Inoué. You suggested that hydrophobic bonds are involved in the aggregation of the protein isolated from the spindles. The amino acid composition of this protein exhibited a rather polar character. There was no apparent excess in apolar amino acids, like alanine, valine, isoleucine, and leucine. Interestingly, the proline content of this material was extremely high. Do you think this amino acid composition supports the concept of the hydrophobic interaction? Second, you showed that the aggregation phenomenon has a positive temperature coefficient. As you know, hydrophobic interactions have a negative temperature coefficient. Would you care to comment on this?

Dr. Inoué: Let me try and answer this question. It was pointed out by Dr. Bárány that the amino acid composition did not fit in with what one would expect from molecules assembling with hydrophobic interaction. I think we are all right on this point. Van Holde (1966. The molecular architecture of multichain proteins. *In* Molecular Architecture in Cell Physiology. T. Hayashi and A. G. Szent-Györgyi, editors. Prentice Hall, Inc., Englewood Cliffs, N.J. 81) has examined a large number of globular proteins which are known either to form or not to form aggregates naturally, and looked at their amino acid compositions. He showed that all the proteins with more than 31% (and some above 29%) form aggregates without exception. All those

with less than 28% (and some with less than 31%) hydrophobic amino acid did not. The Kane-Stephens spindle protein has slightly above 30% hydrophobic amino acids so that it does fit in the right range. The second point had to do with the temperature effect. Temperature response certainly cannot be an unequivocal clue as to what kind of interaction is taking place. And, furthermore, the situation is much worse because we are not dealing with an obviously isolated closed system, which is the necessary condition for doing thermodynamic analysis. We've taken whole cells and seen if we could make any sense. It is actually surprising that we should get as good a fit to the van't Hoff relation. Again, however, the signs and magnitudes of the thermodynamic parameters are consistent with hydrophobic interaction (see Scheraga, this Symposium). Now, we must obviously do more experiments. One important point I had not gotten around to mentioning is that no person up to now can take isolated spindles and make them respond in ways similar to the spindle in living cells. This is a huge gap and I think we should be very conscious of this limit of our knowledge now. The moment eggs are treated, for example, with hexylene glycol for isolating the spindle, all of the characteristics disappear; that is, the colchicine response, temperature response, heavy water response, and so on. We have no idea as to what factor is missing here, but we're clearly dealing with incomplete systems.

On the other hand, there is some encouragement to be gained from model systems. If one takes the association of tobacco mosaic virus A-protein or G–F transformation of actin and other types of protein subunit association, these respond to temperature, to heavy water, and, I'm told, to colchicine in just the same way.

This is a nice correlation. I really don't know yet what is happening during spindle isolation, but I have a feeling that if we get the right factors we might be able to get the isolates to respond properly. But you are perfectly right in saying that the mechanism we postulate has not yet been proven.

Question from the Floor: I have a question for the Chairman. I wasn't able in the micrograph that you showed of the 40 A globules to see whether or not there were 13. But I was aware of your counting them in that recent paper. Do you still see 13 distinctly at all times?

Dr. Mazia: Not at all times. They bend over on themselves. If one takes unspread ones, one sees 6 or 7; in the best spread ones, one often sees 12 and sometimes 13.

Dr. Jerome J. Freed: I'd like to add a few comments to the discussion on saltatory movements. Last year (1965) we reported at the meetings of the American Society for Cell Biology an experimental approach to the problem, in which we examined the movements of various particles in cultured cells and found that these did have the to-and-fro characteristics so beautifully shown in Dr. Rebhun's pictures taken with the Nomarski microscope. These movements were sensitive to the action of colchicine and colchicine-like drugs. We consider this as evidence that saltatory movements may be associated with the microtubules. We have since looked at the distribution of microtubules in these cells using rather thick methacrylate sections and the stereoreconstruction technique of Francis Ashton and Jack Schultz. The predominant directions of the saltatory movements and of the microtubules seem to coincide to the extent that we can associate them. The interesting part of this, as one might also expect, is that the array of microtubules seems to be centered upon the juxtanuclear region, or "nuclear *hof*," where the centrioles are located.

Dr. Rebhun: I'm sorry I didn't get a chance to mention Dr. Freed's work in the talk. It is discussed in the written paper. Basically, we found that saltatory motion of echinochrome granules in *Arbacia* is colchicine-independent although colchicine gets into the egg.

Dr. Freed: Were you able to confirm that the asters, or microtubular arrangement, had been destroyed by the colchicine treatment?

Dr. Rebhun: No. We have not been able to; I think that practically nobody has seen microtubules as such in unfertilized eggs. We have under certain special conditions of rapid freezing in electron microscopy, but not in other ways. So I don't know quite what to make of this except here is a case in which colchicine does not affect saltatory movements.

Dr. Edwin W. Taylor: I think that at this stage in our understanding of the chemistry of mitosis and the mitotic spindle we have to be prepared to go on disagreeing with each other, at least for a little while to come. And I find it difficult to accept the idea that the 22S protein is in fact the subunit of the spindle microtubule. If this is the subunit, you would expect to get this protein if you started with some other source of tubules and, in particular, if you use cilia.

If you remove essentially only the central pair of microtubules from cilia, under very mild conditions, that is, just by dialyzing at low ionic strength and pH 7, you get essentially one protein and it has a sedimentation constant of 6S. The outer nine go on into solution to give a protein with a sedimentation constant of 4S. The properties of the 4S protein seem to agree fairly well with the protein isolated by Sakai and Professor Mazia from the mitotic apparatus. We do not find the 22S protein in cilia.

Our other evidence is, of course, that if colchicine has the effect of breaking down the spindle, you might expect to demonstrate some interaction between colchicine and this protein subunit. A long series of experiments along these lines done in my laboratory by Dr. Borisy, Dr. Shelanski, and Mr. Weisenberg show that colchicine does in fact form a fairly strong complex with an equilibrium constant of around 10^6 with the 6S protein that we get from cilia, and also from the mitotic apparatus and nervous tissue; that is, other systems which are rich in microtubules. Colchicine shows no affinity whatsoever for the 22S protein.

Dr. Inoué: I should like to report what Stephens has done (1965). He has been able to get the 22S protein from cilia as well as from the spindle of the same species of sea urchins and has looked at their amino acid compositions. The compositions are virtually identical. With different species there is more variation.

Now, I think we all are aware that there are other 22S protein–containing particles that come out of cells, and I think we should distinguish which particular one we're talking about. I have a feeling that the Sakai protein and the 22S are not different, that these are different states of aggregation of the same protein. I believe Dr. Mazia has more or less the same view. The reason why one gets the 22S with Kane's preparation and 3.5 or 6S with Sakai's isolation we don't know, but it might turn out to be a methodological problem rather than a basic difference in materials that we're dealing with. I do think one should define the amino acid composition of material clearly to make the argument meaningful.

Dr. Mazia: I don't know if the audience is interested in this small point. In fact, the difference has to be methodological. Sakai also obtained a lot of 22S when he used the same method as did Stephens and Kane. The 22S can't be a subunit. One need only look at the picture to see that subunit is just too small to be the 22S particle.

Dr. Hayashi: In line with this discussion I would like to comment that in our laboratory Mrs. Forsheit and I have been examining the effect of colchicine on contractile proteins and we can report now that colchicine has no effect on myosin ATPase and on actomyosin ATPase. However, with regard to the temperature-reversible polymerization of G-ADP actin, colchicine has an inhibitory effect but it does not depolymerize already polymerized actin. The monomer-to-polymer change of the actin system does not seem to be on as fine a hairline as the mitotic spindles as described by Dr. Inoué. Making exactly the same assumption as was done for the mitotic spindle, Grant has obtained values for the thermodynamic parameters in which the ΔS and the ΔH for the actin are somewhat larger. This may explain the fact that the colchicine does not seem to bring about a depolymerization, but it does inhibit the polymerization and this is essentially in the same direction as the colchicine effect as presented by Dr. Inoué.

Dr. John J. White: Dr. Mazia, I believe that some years ago you reported the association of adenosinetriphosphatase activity with the spindle tubules. Is this information still valid?

Dr. Mazia: Yes, we did find ATPase in the association with the spindle, but the work was done at a time when we couldn't distinguish tubules from the whole apparatus. I really meant to imply in my introduction that we don't know whether ATPase is associated with the tubule or whether the ATPase could lead us to a second protein which has not been found in a search for a protein having the physical-chemical properties of myosin.

Dr. White: Has there been any evidence that the resolved microtubule protein does have ATPase activity?

Dr. Mazia: The best evidence, speaking of microtubules in general, is the evidence given by Dr. Satir this afternoon; namely, that the ATPase in the form of dynein is associated with the "arms" but not with the structures we would call microtubules proper.

Dr. Rebhun: I would just like to make one comment on the effects of some of the agents which have been used for isolating spindles on the spindle itself. We became interested for a variety of reasons in trying things like hexylene glycol, dimethylsulfoxide, ethylene glycol, dithiodipropanol, ethanol, or acetone, or what-have-you, all of which can be used for isolating spindles under the same conditions on the in vivo mitotic apparatus. If one is going to isolate spindles then one would like to have a reagent which doesn't affect the in vivo spindle, at least to a great extent. It turns out that all of these agents have rather phenomenal effects on the spindles. You can divide the agents in two groups; half of them have the same kinds of effects in general as D_2O, although the concentration of the agent varies. Thus, 2 or 3% hexylene glycol in sea water rather than in a buffer will increase both size and retardation of the in

vivo spindle reversibly. The effects on sea urchin spindles, on *Pectinaria*, and on clam spindles are similar. Dimethylsulfoxide and ethylene glycol at higher concentrations will also have these effects.

On the other hand, acetone, ethanol, dithiodipropanol, and *t*-butyl alcohol have diametrically opposite effects. That is, they disperse the spindle, generally reversibly, the effect looking somewhat like a colchicine type of effect. However, I don't know if it is depolymerization or separation of the mitotic filaments on the ultrastructural level. The time constants of both these effects are rather rapid. That is, the effects begin within about 0.5–1 min of the application of reagent, which is certainly the time during which swelling takes place when these agents are used as isolating media. So I think one has to be a little bit careful, since no matter which of these agents you use, you get very nice birefringent mitotic apparatuses with microtubules if you use it as an isolating medium. Thus, these agents which have been used in spindle isolation are not innocuous with respect to spindle structure. That should be kept in mind and should be a precaution used in interpreting the results of chemical analysis of isolated mitotic apparatuses.

Dr. Arthur Zimmerman: Dr. Inoué, would you care to speculate as to perhaps why there's an accelerated recovery following the Colcemid treatment when you have used actinomycin D? and puromycin?

Dr. Inoué: Why is recovery faster in actinomycin D and puromycin? Well, we have no clear answer. But it is a striking phenomenon. It might be—a real might—that these agents have a high enough binding constant with Colcemid to pull out the Colcemid faster from cells. However, we have no proof of this explanation.