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<th>THE SPINDLE MICROTUBULE “紡錘体の微小管”</th>
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<td><strong>Author(s)</strong></td>
<td>Yukiko Sato (佐藤 幸子)</td>
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THE SPINDLE MICROTUBULE

Yukiko Sato

Much information on the physiology and biochemistry of microtubules appear in many publications, and there is no doubt in designating the microtubule as the major component of the mitotic spindle. Considering the importance of this birefringent structure and the fragile nature of spindle microtubules, a dynamic equilibrium model was proposed twelve years ago. Based on the observation of electron-micrographs of dividing cells, the zipper theory on the observation of the lateral interaction of microtubules was presented by Bajer et al. (1975). McIntosh et al. (1969) thought the sliding action between the neighboring microtubules generated the force required for anaphase chromosome movement; meanwhile, Fujiwara and Pollard (1976) considered the active involvement of actomyosin for chromosomal movement as the force generator. Dynein may also be involved to control the anaphase chromosomal movement (Sakai et al. (1976)). We may need all these hypotheses to interpret the whole process of mitosis in eukaryotes, although it can be said that none of them are strong enough to disqualify the other hypotheses. More information is needed on the physicochemical nature of tubulin molecules as well as the physiology of living and dividing cells.

I would now like to point out the following phenomena as a point of interest:

Chromosomal movement during mitosis is primarily controlled by polymerized microtubules and cytokinesis primarily controlled by polymerized actin. These two classic contractile proteins perform important roles in the living cell in a cooperative manner with definitely programmed coordination.
INTRODUCTION

The mitotic spindle is a cellular organelle newly assembled in a eukaryote at mitosis and disassembled after the completion of cell division. The major function is to orient and align chromosomes, then transport them to the two daughter cells. The inner structure of the living mitotic spindle is difficult to visualize with phase-contrast or differential interference microscopy (Nomarsky microscopy) due to the minute difference of refractive indices encountered (Fig. 1). However, owing to the optical anisotropy of spindle structure, we can visualize the fibrous components of the spindle under a sensitive polarization microscope (Fig. 1). This anisotropy, or birefringence, could reflect the amount of orderly aligned fibrous structure within the spindle. Various conditions and agents have been found that systematically and reversibly alter spindle morphology and fiber birefringence. From a series of observations and experiments, we postulated that spindle fibers are composed mainly of parallel arrays of microtubules formed by a reversible association of tubulin molecules. Thus, there exists a dynamic equilibrium between the labile spindle fibers and a pool of unassociated tubulin dimers.

Fig. 1. CELL DIVISION of HAEMANTHUS

Nomarsky microscopy  Polarization microscopy  Phase contrast microscopy
(Inoue and Sato (1967)). The assembly of microtubules is more than likely controlled by the orienting centers such as centrioles and kinetochores, and the available concentration of polymerizable tubulin dimers (Fig. 5).

In this paper, I shall discuss the following subjects:

1. Changes of spindle birefringence during mitosis.
3. Effect of heavy water on the mitotic spindle.
4. Tubulin immunofluorescence.

CHANGES OF SPINDLE BIREFRINGENCE DURING MITOSIS

Mitosis in a tissue culture cell of salamander (Taricha granulosa) lung epithelium is shown in Fig. 6. Primary cultures of amphibian tissue have advantages for polarization microscopy because of their size, clearness, spindle birefringence and moderate speed of chromosome movement at room temperature. Mono-layer cultures can also be achieved in a Rose-chamber (Rose et al. (1958)) according to Seto and Rounds' method (1968) with some modifications (Sato et al. (1975)).

From time-lapse cinematographic records using rectified polarizing optics, we found that the prometaphase spindle, which is composed mainly of continuous spindle fibers and astral rays, elongates past the nuclear envelope into the nucleus and rapidly gains in birefringence and size. The speed of prometaphase spindle growth was 1.4-4 μm/min at 24°C and almost the same value as obtained in endosperm cell division of Haemanthus (Fig. 1) and first and second division in fertilized eggs of Japanese winter sea urchins (Fig. 2). The advancing spindle pole occasionally undergoes saltation, tilts, and swings. Birefringence is high in the elongating prometaphase spindle but decreases in the wider late prometaphase spindle. The prometaphase spindle is not a stable structure; it twists, jerks, swims, oscillates and sometimes migrates around in the
cell. These movements are rather common and even exaggerated forms of spindle movement has been noted in many other cell types.

Occasionally, over-elongated spindle poles split and form a smaller but more birefringent spindle with one or two tandem satellite spindles. Spindle axis is determined in metaphase and all oscillatory movement of spindle ceases at this stage. In anaphase, chromosomes move to the poles at constant velocities, 1–2 μm/min on the average, independent of the absolute value of spindle birefringence. It suggests the existence of definite control mechanisms of anaphase chromosome movement which is the major subject of this chapter.

Chromosomes are led by birefringent kinetochore fibers to the poles and the overall birefringence decreases gradually during anaphase. The birefringence adjacent to kinetochores and the large part of chromosomal spindle fibers remain unchanged during the major part of anaphase.

In summary, with the sensitive polarization microscope, we are able to follow the transition of birefringent material from one spindle component to another throughout mitosis. The directional segregation of centrioles contribute to the subsequent orientation of spindle fibers in
prometaphase. The oscillatory movement of prometaphase spindle fibers appears to assist the coordination and alignment of chromosomes on the metaphase plate and to establish the metaphase equilibrium. The events in anaphase can be interpreted as the process of controlled dissociation of microtubules or the polymerized tubulin dimers.

**NATURE OF SPINDLE FORM BIREFRINGENCE**

The birefringence (BR) of the mitotic spindle can be yielded by a system of highly oriented molecules, fine structures such as microtubules and microfilaments, or secondary molecules trapped in between subunits in the microtubules.

\[
\text{Wiener's equation} \quad n_2^2 - n_0^2 = \frac{\theta_1 \theta_2 (n_1^2 - n_2^2)^2}{(\theta_1 + 1) n_2^2 + \theta_2 n_1^2}
\]

Table 1. Wiener's curve (solid line) with Pisaster imbition data. Broken line corresponds with the estimated value of the intrinsic birefringence of subunits in the microtubules.

- \( n_1 \): the refractive index of the spindle.
- \( n_2 \): the refractive index of the medium.
- \( \theta_1 \): the density of the spindle. (the volume fraction)
- \( \theta_2 \): the density of the medium. (\( \theta_1 + \theta_2 = 1 \))
- \( n_0 \): the refractive index of the extraordinary ray.
- \( n_1 \): the refractive index of the ordinary ray.
fine structures. Preliminary calculations for the form birefringence by Wiener's equation (Wiener (1912)) and our data are shown in Table 1.

The metaphase-arrested meiosis I spindle can be isolated in quantity from the star fish Pisaster ochraceus. These isolates are relatively clean and the BR are uniform.

The BR of mixed bodies composed of oriented rodlets or platelets whose thicknesses are well below the wave length of light is believed to arise from two sources: a) An intrinsic BR due to the intramolecular anisotropy of the rodlets or platelets, and b) A form BR due to the anisotropic arrangement of the fine structure. Weiner (1912) and Bragg and Pippard (1953) have derived equations which relate the form BR in terms of dielectric anisotropy to the volume fraction occupied by the rodlets or platelets, their refractive indices or dielectric constants and the refractive index of the second phase in which the rodlets or platelets are immersed.

Isolated spindles with 12% hexylene glycol, pH 6.3, were mounted in the Rose chamber, fixed with 3% glutaraldehyde, then perfused with solutions with various refractive indices. Spindle retardations (BR) were measured with a Brace-Köhler compensator using mercury green light as the illuminating source. The volume fraction of microtubules calculated from electron microscopy, X-ray diffraction and hydrodynamic data.

Table 1. shows the best-fit Wiener curve with Pisaster spindle imbibition data. From this execution, we obtained the rodlet refractive index \(n_1=1.512\), rodlet volume fraction \(f=0.0206\), coefficient of intrinsic BR=\(4.7\times10^{-5}\), and coefficient of BR of spindle=\(5\times10^{-4}\) (Sato, Inoue and Ellis, 1975).

EFFECT OF HEAVY WATER (D\(_2\)O) ON THE MITOTIC SPINDLE
Inoue and Sato (1967) found an 8-fold increase in spindle volume
and a doubling in the retardation using heavy water (D$_2$O) on the metaphase-arrested meiosis I spindle of the oocyte of Pectineria gouldi. (Fig.3). The increase of volume and retardation depend on the concentration of D$_2$O and the stage of mitosis. Maximum increase is obtained by applying 45% D$_2$O during metaphase to the onset of anaphase. This particular concentration of D$_2$O is common for metaphase spindle in various organisms. The changes are rapid, with the new state of dynamic equilibrium being reached within 2 min. in Pectinaria oocyte, 90 sec. in developing Japanese sea urchin egg, and 5 min. in Pisaster ochraceus oocyte. The D$_2$O effect is completely reversible and repeatable on the same spindle.

In many respects, the D$_2$O effect is quite similar to elevating the temperature within an optimum range. However, the spindle will be over stabilized or frozen by the application of high concentration of D$_2$O.

To compare the effect of temperature and D$_2$O, we analyzed the association-dissociation reaction of the spindle with a thermodynamic approach (Inoue and Morales (1959), Inoue et al. (1975)). Metaphase arrested meiosis I spindle of the mature oocyte of Pisaster ochraceus was used as the material. Thermodynamic parameters were calculated from retardation measurements at various temperatures with or without D$_2$O (Sato and Bryan (1968)). In sea water, we obtained $\Delta H = 58.9$ Kcal/mol, $\Delta S = 205.9$ eu*, and $\Delta F = -1.1$ Kcal/mol. In 45% D$_2$O the values were $\Delta H = 29.55$ Kcal/mol, $\Delta S = 106.3$ eu, and $\Delta F = -0.9$ Kcal/mol. These values are similar to those obtained for Chaetopterus spindle and Pectineria spindle. Both association and dissociation processes appear to follow first-order kinetics. $E_{act}$* for the association reaction are 45 Kcal/mol. and 39 Kcal/mol. with D$_2$O. $E_{act}$ for the dissociation reaction for

* eu; entropy unit.
* $E_{act}$; activation energy.
removing D$_2$O is 15 Kcal/mol. These data support the hypothesis that the spindle reaction is the reversible association of tubulin dimers into the linearly aggregated polymers in a first-order reaction. However, the difference of both $\Delta H$ and $\Delta S$ in control and D$_2$O spindle and their

\[ \begin{align*}
\Delta H &= 58.9 \text{ Kcal/mol} \quad \Delta H = 29.55 \text{ Kcal/mol} \\
\Delta S &= 205.9 \text{ eu} \quad \Delta S = 166.3 \text{ eu} \\
\Delta F &= 1.1 \text{ Kcal/mol} \quad \Delta F = 0.9 \text{ Kcal/mol}
\end{align*} \]

Table 2. A thermodynamic analysis of the effects of D$_2$O and temperature on the spindle association reaction. Material: Metaphase-arrested spindle in Pisaster ochraceus oocytes.

<table>
<thead>
<tr>
<th>Condition of isolation</th>
<th>13°C, 12%HG</th>
<th>45%D$_2$O, 13°C, 12%HG</th>
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<tr>
<td>Pol. M.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retardation(BR), nm</td>
<td>3.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Spindle diameter(d), µm</td>
<td>8.0</td>
<td>12.4</td>
</tr>
<tr>
<td>$(n_e-n_o)=\Delta x/d$</td>
<td>$5\times10^{-4}$</td>
<td>$5\times10^{-4}$</td>
</tr>
<tr>
<td>E. M.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimension of microtubules, Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD</td>
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<td>240</td>
</tr>
<tr>
<td>ID</td>
<td>150</td>
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</tr>
<tr>
<td>Density of microtubules per µm</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>Total number of microtubules per spindle</td>
<td>4,200</td>
<td>10,000</td>
</tr>
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Table 3. Comparison of D$_2$O-spindle versus normal spindle. Material: Pisaster ochraceus.
similar Eact suggest that $D_2O$ is in fact elevating the concentration of polymerized tubulin dimers.

The coefficients of $BR (n_n - n_o)$ of both control and $D_2O$ spindle remains constant at $5 \times 10^{-4}$. This means that there is a significant increase in microtubules in $D_2O$ spindle without any difference of the original population density. From the electron micrographs, we found the total number of spindle microtubules is increased from 4,200 in the control

![Normal vs 45% D2O](image)

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Fig. 3. 45% $D_2O$ PERFUSION TEST.

Pectinaria oocyte (Polarization micrography)
to 10,000 in D$_2$O spindle, holding the average population density of 130. Length of each spindle microtubules also increased by D$_2$O. Estimated rate of tubulin association was calculated as $1 \times 10^{-2}$ monomers $\text{sec}^{-1}$ microtubule in Pisaster oocyte. The comparison of D$_2$O spindle and control spindle is summarized in Table 2, 3 and Fig. 3.

D$_2$O effect on the mitotic spindle was thought to occur only in specialized cells such as the mature oocytes or unfertilized eggs which hold a large amount of polymerizable tubulin. However, we found that the D$_2$O effect can clearly be demonstrated even in a tissue culture cell. Knowing that the artificially induced tubulin paracrystals by the vinblastine can represent almost all available tubulin in a cell, we calculated tubulin concentration photometrically. We estimate that at most 18% of tubulin is used for spindle assembly in a dividing Pt-K$_2$ cell. The same value is also obtained in a dividing tissue-cultured salamander lung epithelial cell. Order of magnitude of tubulin consumption for spindle assembly in these cells is quite comparable to the case of developing zygotes such as in the case of the fertilized sea urchin egg where about 5-8% of cytoplasmic tubulin reserve is used for each mitosis.

**TUBULIN IMMUNOFLUORESCENCE**

Tubulin is a polymorphic protein and can be crystalized in vivo into two alternate crystalline forms; we call them SM-crystal and VB-crystal. The birefringent SM-crystals induced in echinoderm gametes are labile liquid crystals and can be transformed from, or to, functional mitotic spindle, but they are difficult to stabilize and isolate. The VB-crystal, the irreversibly formed tubulin paracrystal (Fig. 4) is obtained by incubating the materials with $10^{-4}$M Vinblastine and $10^{-4}$M Colcemid. VB-crystals are produced with a much larger yield than SM-crystals. VB-crystals have little contaminant, can be stabilized and isolated in
quantity. Therefore, rabbit antiserum directed against tubulin is prepared using VB-crystals as an immunogen. Purity and specificity are examined; then antiserum is used for direct or indirect tubulin immunofluorescence. Using images of spindles observed in vivo as the critical standard, we have endeavored to improve the preservation and staining techniques.

As shown in Fig. 7, the pattern of fluorescence of the stained spindle is similar to in vivo spindle image observed in a tissue-cultured rat-kangaroo Pt-K₂ cell. In the dividing cells, the fluorescent image provides clean differentiation between tubulin organized into spindle fiber, and astral ray microtubules, besides that present as unstructured amorphous material in the non birefringent area around the spindle.

The amount of amorphous material increases during prometaphase.
Metaphase.

A) Prophase.  

B) Metaphase.  

C) Ana-Telophase.  

D) The Phase contrast image for B.  

Fig. 6. TUBULIN IMMUNOFUORESCENT IMAGE.  

Fig. 7. TETRAPOLAR MITOSIS (rat-kangaroo Pt-K5 cell)  
A) Phase contrast image.  B) Immunofluorescent image. (← shows the centriole)
Rather intense fluorescence appears to surround the prophase nucleus corresponding to the perinuclear clear zone. For the chromosomal and continuous spindle fibers in metaphase and anaphase, these fluorescent images are similar to the in vivo images obtained from polarization microscopy. However, the astral rays, which occasionally reach the cell membrane, appear more distinct than in the polarized light image. Chromosomes show no affinity for the antiserum.

In prophase, the number of cytoplasmic filament decreases and the amorphous fluorescence increases. Amorphous fluorescence sharply decreases at prometaphase then later increases during anaphase again. This suggests that most of the cellular tubulin accumulates around the nucleus in preparation for assembly into the mitotic spindle. The increase in amorphous fluorescence during anaphase obviously reflects the disassembly of spindle microtubules. (Fig. 6).

In telophase, as soon as spindle fibers disappear, full of amorphous fluorescence comes out with two daughter nuclei, then the cleavage finishes for these daughter cells.

In the above, I have briefly mentioned about spindle microtubules. Actin filaments and other decorating systems have been found both inside and outside the spindle. Of course, the analysis of the mechanism which regulates the interactions between them will be the next step in explaining the chromosome movement and cell division.

REFERENCE


Ser., 30, Raven Press, New York.


