Effects of D₂O on the Movement of Chromosomes and the Shortening of Kinetochore Spindle Fibers in Anaphase in Dividing Spermatocytes of the Grasshopper, *Mongolotettix japonicus*

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ABSTRACT. Spindles in anaphase of dividing primary spermatocytes of the grasshopper, *Mongolotettix japonicus*, were examined with a sensitive polarizing microscope combined with a time-lapse video recorder and a cinematographic apparatus. The pole-to-pole distance of the meiotic spindles was increased and the kinetochore fibers were more birefringent in the presence of 40 % D₂O. However, the rate of shortening of the kinetochore fibers in anaphase was not affected by D₂O. This indicates that D₂O did not inhibit microtubule disassembly in anaphase, supporting the earlier observations (3, 18) that D₂O did not “stabilize” the spindle microtubules at concentrations below 45 %. We confirmed that D₂O, at the concentration mentioned above, neither promotes nor inhibits the anaphase A. However, the overall sequence of anaphase was considerably extended in the presence of D₂O, presumably due to the increased pole-to-pole distance.

When D₂O is applied to the dividing eukaryotic cells at concentrations less than 45 %, both the spindle volume and birefringence (BR) are increased (3, 13, 16), but mitosis is not inhibited (3, 18). Based on experiments using the dividing sea urchin eggs, Takahashi and Sato (17, 18) considered the extension of anaphase duration in D₂O-treated cells could be caused by the elongation of the spindle axis in the presence of D₂O. In the present experiment, we intended to reexamine their observations using dividing primary spermatocytes of the grasshopper, *Mongolotettix japonicus*, whose anaphase is 5 to 6 times longer than the anaphase of dividing sea urchin eggs. Spindles of these cells are large, clear (5, 6, 7) and provide a prominent image of kinetochore fibers under a sensitive polarizing microscope (6, 7). We considered that spermatocytes, which possessed ample amounts of polymerizable tubulin for the spindle assembly in meiosis I and II, allowed us more precise tracing of their anaphasic sequences. We expected to obtain better information concerning the effect of D₂O on the microtubule disassembly in the dividing eukaryote.

Through the present study, we found that while the BR of the non-kinetochore fibers increased little, BR of the kinetochore fibers was greatly enhanced by D₂O. The spindles were elongated and in many occasions their poles reached the cell membrane. In this paper, we shall report the process of shortening of the kinetochore-to-

pole distance, the rate of chromosomal movements and the change of the pole-to-pole distance during anaphase. Results are discussed in relation to the biological effect of D₂O on the spindle assembly and disassembly in the process of anaphase.

MATERIALS AND METHODS

Primary spermatocytes of the Japanese grasshopper, *Mongolotettix (Chrysochraon) japonicus*, were used. They have large and more birefringent spindles with relatively few chromosomes as compared to spermatocytes of other species of grasshoppers. Their centrioles are often detectable from late prophase through telophase in life by light microscopy. Preparations for live observation were made with fluorocarbon oil, FC-47 (Minnesota Mining & Mfg. Co., St. Paul, Minnesota) as described previously (7, 8) with slight modifications. Testicular follicles from individual grasshoppers were divided into two groups. One group was immersed in 0.9 X Carlson's solution (1, 6) as the control. The other group was immersed in 40 % D₂O-0.9 X Carlson's solution. After 20 min of immersion, the testicular follicles were placed onto a slide glass, and their distal tips were cut with a sharp knife. The germ cells contained in the small amount of body fluid were then carefully taken out onto the same slide glass. Drops of FC-47 were then placed onto the cells before a coverslip (18 × 18 mm²) was mounted on the latter. A piece of teflon tape (ASF-110, Chukoh Chemical Industries LTD.) with a square hole (15 × 15 mm²) in the middle was used as a spacer. Finally, after absorbing the excess of FC-47 by filter paper, the edges of the coverslip were sealed with VALAP (vaselin : lanolin : paraffin = 2 : 2 : 1).

The D₂O, purchased from the Bio-Rad Laboratories, Richmond, Ca. U.S.A., had specific purity of 99.86 M percent and was used in this experiment at a final v/v concentration of 40 % D₂O in 0.9 X Carlson's solution.

Polarization microscopy. A Leitz polarizing microscope (Ortholux-II Pol-BK, Leitz, West Germany) modified to be equipped with strain free, rectified condensers (Nikon, N.A. 0.52 or N.A. 1.15) and rectified objectives (Nikon, 40X, N.A. 0.65 and 100X, N.A. 1.25) was used for polarization microscopy. A Brace-Köhler type compensator ($\lambda/30$, Leitz) was routinely used for retardation measurements. A Glan-thomson prism and a Leitz plastic polarizing filter were used as a polarizer and an analyzer, respectively. Strong illumination was provided by a super-high-pressure mercury arc lamp (HBO 200 W, Osram, West Germany) from which the 546 nm green light was isolated using a filter system which consisted of a heat absorbing glass filter, a 5 cm thick liquid layer containing 20 % ferrous ammonium sulfate solution, and an interference filter (Toshiba, Tokyo, Japan). The anaphasic movement in dividing primary spermatocytes was followed and recorded utilizing either a time-lapse video recorder or cinematographic apparatus.

Time-lapse video recording. The polarization microscope was equipped with a sensitive video camera (Hamamatsu, C-1000, type 12) connected to a time-lapse video cassette recorder (National, AG-6010). Microscope images were recorded on Sony or National 1/2 inch high density VHS video tape. The time-lapse rate was either 150 or 75 frames per minute. The outlines of the cells, chromosomes, kinetochore spindle fibers and the position of centrioles during anaphase were traced on transparent plastic sheets mounted on the monitor screen. Inter kinetochore distance, length of kinetochore fibers as well as interpolar distance were measured on these tracings and plotted against time.

Time-lapse cinematography. Cinematographic records were made on Kodak Plus-X 16 mm negative film at 7.5 frames per minute. From a positive copy of the film, every fifth frame was traced utilizing a Shinko 16 m/m halogen viewer. The method of analysis was the same as that of the images recorded on the video tapes.

RESULTS

The metaphase spindle of the primary spermatocyte immersed in 40 % D₂O was longer and more birefringent as compared with the spindle in control cells (Fig. 1). BR of the kinetochore spindle fibers was markedly increased, while the change of BR of non-kinetochore fibers was not conspicuous (Fig. 1B). As a result of spindle elongation, the spindle poles of D₂O-treated cells were often observed to reach the cell membrane (Fig. 1B; Fig. 2A). This kind of image was never seen in the control cells. The spindle in the treated cells appeared to be somewhat stiffened judging from the fact that its movement was rather restricted.

Preliminary observation showed that the D₂O effects were detected but less prominent in spindles of secondary spermatocytes than spindles of the primary spermatocytes. Therefore, we decided to analyze the D₂O effects only on meiosis I spindles of the spermatocytes.

The exact time of the onset of anaphase both in the control and the D₂O-treated cells was determined from measurements of the distance between bivalent kinetochores (KKD). We considered the onset of anaphase as the time when KKD started to show a gradual increase (Fig. 3). In most cells, the shortening of kinetochore-to-pole distance (KPD) began a few minutes prior to the increase of KKD. This phenomenon remained unchanged in the presence of 40 % D₂O in the Carlson's medium (Fig. 4). The rate of chromosome movement at the beginning of anaphase was slow and then became progressively faster, reaching a maximum in early to mid-anaphase. The movement slowed down at the ending part of anaphase

Fig. 1. A: The spindle of a control cell in metaphase as seen by polarization microscopy. B: The spindle of a cell immersed in 40 % D₂O-Carlson' solution in metaphase. The poles of the spindle in D₂O-treated cells often reached the cell membrane at metaphase, denoting a longer spindle in cells affected by D₂O. Temperature was 26°C.

(Fig. 3). The presence of D_2O made the chromosome movement slower in early or early to mid-anaphase. The speed was $1.3 \pm 0.3 \mu\text{m}$ per minute in the control cells, and $0.9 \pm 0.2 \mu\text{m}$ per minute in 40 % D_2O -Carlson' solution (Table 1). As a consequence of the spindle elongation in D_2O -treated cells, when the cell proceeded through anaphase, the chromosomes had to move a longer distance to reach the poles. In fact, the anaphase took approximately 9 min longer than the control (Table 1). Despite the longer time course of anaphase sequence and the slower

Fig. 2. Spindle transition from metaphase up to late anaphase (A–D) in a D_2O -treated cell. During anaphase (B, C and D) the kinetochore spindle fibers had prominent BR, however BR was lower near the poles.

Fig. 3. Representative changes of the spindle parameters during anaphase of a control primary spermatocyte (upper figure) and a D₂O-treated primary spermatocyte (lower figure). PPD: Pole-to-pole distance (p1p2). KKD: Kinetochore-to-kinetochore distance or distance between kinetochores of homologous half-bivalents (k1k2). KPD: Kinetochore-to-pole distance $\left(\frac{KPD_1 + KPD_2}{2}\right)$. The start of chromosome separation is indicated by larger arrows. Temperature was 26°C.

TABLE 1. DURATION OF ANAPHASE, RATE OF CHROMOSOME MOVEMENT, RATE OF KINETOCHORE-TO-POLE DISTANCE SHORTENING (mean $\pm$ SD).

	Control	40 % D ₂ O-Carlson's solution
1. Anaphase duration	28.3 $\pm$ 7.0 min (8)	36.5 $\pm$ 5.0 min (9)
2. Rate of chromosome movement	1.3 $\pm$ 0.3 μ m/min (9)	0.9 $\pm$ 0.2 μ m/min (11)
3. Rate of Kinetochore-to-pole distance shortening	0.6 $\pm$ 0.1 μ m/min (10)	0.5 $\pm$ 0.1 μ m/min (11)

Numbers in parentheses indicate the number of observed cells. The temperature during observation ranged between 25°–27°C. The rate of chromosome movement and the rate of shortening of the kinetochore-to-pole distance were measured in early or early to mid-anaphase when the rates were almost constant.

Fig. 4. Time relationship between the initiation of chromosome separation and beginning of kinetochore-to-pole distance shortening. The time at which bivalent kinetochore fibers of control (solid circles) and D_2O -treated (open circles) cells started to shorten are plotted. Temperatures ranged from 25° to 27°C.

chromosomal movement, KPD decreased at a rate not significantly different from the controls. On the average KPD during early to mid-anaphase, the period of fastest chromosomal movement, shortened to $0.6 \pm 0.1 \mu\text{m}$ per minute in the control cells while in the D_2O -treated cells, it shortened to $0.5 \pm 0.1 \mu\text{m}$ per minute (Table 1). In general, the control spindle elongated in early and mid-anaphase, but shortened at late anaphase (Fig. 3). The pole-to-pole distance (PPD) of the D_2O -treated spindles, in many occasions, did not fluctuate much.

DISCUSSION

In the present study, 40 % D_2O was shown to cause a longer and more birefringent spindle in the primary spermatocytes of the grasshopper, *Mongolotettix japonicus*. The increase of the spindle BR was especially conspicuous in the kinetochore spindle fibers. This observation confirms the result of the previous work obtained in the spermatocyte of the same species (7). In addition, the enhancement of the spindle assembly by D_2O is what could be expected from a number of investigations dealing with the D_2O effects on the living mitotic spindle.

As an explanation of the enhancement of spindle assembly by D₂O, increased hydrophobic interaction between tubulin molecules may be taken into consideration, since protein molecules in the cytoplasm might increase their hydrophobicity proportionally to the given concentration of D₂O (9, 13), and since the hydrophobic interaction of protein molecules is also involved in the assembly of microtubules. Thus, promotion of tubulin polymerization by D₂O *in vitro* may be a direct reflection of the strengthened binding of tubulin molecules (4). In anaphase, kinetochore microtubules depolymerize and the distance between the pole and the chromosomes decreases (anaphase A). The microtubule disassembly might be responsible for driving the chromosomes poleward (3, 10). If D₂O is involved in the modulation of hydrophobic interaction amongst tubulin molecules, D₂O might decrease the speed of anaphase A. Investigation dealing with the effects of D₂O on mitosis in eukaryotes revealed that application at more than 60 % caused a structural stabilization of the mitotic apparatus (11, 12) and suppressed the mitotic events. On the other hand, application of D₂O at concentrations below 45 % did not inhibit anaphasic movement in sea urchin eggs (13, 14). The present experiment on grasshopper spermatocytes using 40 % D₂O have revealed that although the anaphase was prolonged due to the longer spindle axis and slower chromosome movement, the KPD shortened at a rate not significantly different from that of the control. D₂O, therefore, did not affect the progression of anaphase A. In accordance with this result, thermodynamic analysis of the effect of D₂O on the mitotic spindle in echinoderm eggs (15, 17) did not support the postulation that D₂O strengthens the hydrophobic interaction between tubulin molecules. Furthermore, observation on the Ca²⁺-dependent deterioration of BR of isolated spindles in sea urchin eggs (18) showed that the rate of deterioration of the spindles pre-treated with 45 % D₂O was essentially the same as in the control spindle. All these facts seem to be more consistent with the conclusion that D₂O enhances spindle assembly by increasing the number of polymerizable tubulin molecules required for the assembly of the spindle (15, 16, 17), the microtubule of which is assumed to be in a state of dynamic equilibrium with the monomers in the tubulin pool (3).

Although 40 % D₂O did not modify the process of anaphase A, the chromosome movement was retarded in the D₂O-treated spindle. Our results have shown that the PPD did not fluctuate much during anaphase in the D₂O-treated cells. Because spindle poles were already wide apart in metaphase in the presence of D₂O, the separation of the poles (anaphase B) contributed little to the anaphasic chromosome movement. This may explain the slower chromosome movement in anaphase of the D₂O-treated cells.

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