Effects of Heavy Water (D₂O) on the Length of the Mitotic Period in Developing Sea Urchin Eggs

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ABSTRACT. The sequence of mitosis in sea urchin eggs was investigated in the presence and absence of D₂O. Direct observations of living cells under a polarizing microscope and observations with fixation-staining procedures were used. The duration of mitosis was extended by the presence of D₂O. The slight extension of anaphase was due to elongation of the spindle in D₂O, but the period from prophase to metaphase was clearly prolonged in the deuterated condition. These results indicate that D₂O does not suppress anaphase chromosome movement, but does affect prometaphase and delays the alignment of chromosomes on the equatorial plane of the mitotic spindle at metaphase.

The stability of the isolated mitotic apparatus against Ca ions and low temperature also was investigated. There was no difference in the deterioration of isolated spindle birefringence under normal and deuterated conditions. The implications of these results are discussed in relation to the enhancement effect of D₂O on the volume and birefringence of the living mitotic spindle.

Among the many reported physiological effects of heavy water (D₂O) (1, 4, 15), Inouè and Sato (2) have reported its effects on the mitotic spindle of living eucaryotic cells. D₂O at a concentration of less than 45% enhances the volume and birefringence of the mitotic spindle. Its maximum effect is at 45% D₂O (2, 12). The mechanism of this effect, however, has yet to be determined.

As to the physicochemical properties of D₂O, protein molecules are thought to increase their hydrophobicity in a D₂O solution (3). Because hydrophobic interaction also is said to be involved in the assembly of microtubules (2), the effect of D₂O on the mitotic spindle probably is a direct reflection of strengthened binding between tubulin molecules.

In fact, D₂O at a concentration of more than 60% stabilizes the metaphase spindle *in vivo* and inhibits cell division (1). But D₂O at a concentration of less than 45% does not arrest cell division (2, 12); parallel increases in the D₂O concentration and the enhancement of the mitotic spindle take place at concentrations below 45% (12). At a concentration of more than 45%, the increase in volume stops and spindle birefringence decreases, depending upon the increase in the concentration. For this reason, we have defined the effect of D₂O described in this paper as the action

Abbreviations used: FSW, filtered sea water; ASW, artificial sea water; CFSW, calcium-free sea water; CMFSW, calcium, magnesium-free sea water; EGTA, ethylene-glycol-bis-(β -Amino-ethyl ether) N, N'-tetraacetic acid; DTT, dithiothreitol; MES, 2-(N-Morpholino) ethanesulfonic acid; PIPES, piperazine-N, N'-bis (2-ethanesulfonic acid); TAME, p-tosyl arginine methylester HCl.

performed by D₂O at concentrations of less than 45%.

If the D₂O effect on the living mitotic spindle is related to strengthened hydrophobic interaction between tubulin molecules, microtubules under deuterated conditions would be protected from depolymerization by the effect of the reinforced hydrophobic bonds between the molecules.

At anaphase, spindle microtubules depolymerize and the distance between the pole and chromosome is shortened. These events are essential to mitosis in terms of the mutual segregation of duplicated chromosomes. If the effect of D₂O is related to modulation of hydrophobic interaction, at anaphase inhibition of the disassembly of microtubules would occur in the pole region of the mitotic apparatus. This inhibition is thought to delay mitosis.

We here report the effects of D₂O on the length of mitosis in developing sea urchin eggs.

MATERIALS AND METHODS

The first and second mitoses of the sea urchins, *Mespilia globulus* and *Hemicentrotus pulcherrimus*, were studied. To obtain mature eggs, we injected a 0.56 M KCl solution into the body cavities of these sea urchins. Testes were removed from males and stored as dry sperm at 4°C. Spawned eggs were washed three times with filtered sea water (FSW) then inseminated with a diluted sperm suspension and cultured at the constant temperature optimum for normal development (25°C for *M. globulus* and 18°C for *H. pulcherrimus*).

D₂O with a purity of 99.86 M percent was purchased from Bio-Rad (Bio-Rad Laboratories, Richmond, California, U.S.A.). Artificial sea water (ASW) was prepared according to the MBL formula (MBL formula V, Woods Hole, Mass., U.S.A.). Carmine was obtained from Merck Ind. (E. Merck AG, Darmstadt, West-Germany). The quality of all the other reagents used was of special grade.

Carnoy's fixative was prepared by mixing absolute alcohol, acetic acid and chloroform 6: 1: 3. To make the aceto-carmine solution, we dissolved 0.5 g of carmine in 100 ml of boiling 45% acetic acid. After cooling it, the solution was filtered through filter paper no. 1 (Toyo Kagaku Sangyo Co., Ltd., Japan), then several drops of 2% iron alum were added to the filtered solution.

For polarization microscopy and birefringence measurement we used an Ortholux Pol polarizing microscope (E. Leitz, Inc., Rockleigh, N. J., U.S.A.) equipped with rectified strain-free optics (Nippon Kogaku K. K., Tokyo, Japan) and an Osram HBO 200W L-2 mercury arc lamp (Osram GmbH, Berlin-München, West-Germany). Detailed methods for polarization microscopy have been described previously (14).

An Olympus BHC type microscope (Olympus optical Co. Ltd., Tokyo, Japan) was used for phase contrast observations.

To prepare a time table of the mitotic process and to determine the length of time required for each mitotic stage, we used two procedures, direct observation of living cells and the examination of a series of fixed and stained materials.

Methods for direct observation. *M. globulus* was chosen as the experimental material because of the transparency of its egg cytoplasm. A batch of fertilized eggs was divided in two; one part was developed in normal ASW, the other, after a 10-min post-insemination, was developed in 45% D₂O ASW. Each sample was maintained at 25°C and was observed continuously under a polarizing microscope. When more than half of the eggs had gone through a particular stage, we designated that time as the point at which the eggs developed

to that stage. The time table for mitosis then drawn.

Methods for fixation and staining. *H. pulcherrimus* was used in this procedure. Thirty minutes after insemination, a batch of fertilized eggs was divided in two. One part was developed in normal ASW and the other in ASW containing 40% D₂O under the constant temperature of 18°C. Forty minutes after insemination, small portions of each series of developing eggs were fixed with Carnoy's fixative every 2 min. The fixative was changed once then replaced with aceto-carmin solution. The fixed samples were incubated for 1 h at 40°C.

After incubation, the aceto-carmin solution was replaced with 45% acetic acid and the samples observed under a phase contrast microscope. The mitotic figure was examined, and the number of eggs at each mitotic stage recorded. The time table then was drawn using the criteria given in the direct observation method.

Isolation of the mitotic apparatus. To isolate the mitotic apparatus, we immersed artificially denuded fertilized eggs in an isolation medium during the metaphase of the first division (7–11). Eggs about 0.5 ml were suspended in 10 ml of FSW and inseminated in a conical centrifuge tube, then quickly collected in a hand centrifuge; 45 sec after insemination a 1 M urea solution was poured into the tube, and the egg suspension gently centrifuged. The supernatant was discarded and fresh 1 M urea solution was injected into the tube. The eggs were precipitated and the supernatant removed, after which the eggs were suspended in Ca⁺⁺-free ASW (CFSW) and precipitated again. To remove the urea completely, we repeated these processes three times. At metaphase, CFSW was replaced by Ca⁺⁺, Mg⁺⁺-free ASW (CMFSW). The eggs again were collected and the supernatant removed. Isolation medium then was added until it reached halfway up the centrifuge tube.

Two types of isolation medium were used for the successful separation of the mitotic apparatus. One consisted of 1 M glycerol, 1 mM EGTA, 10 mM DTT, 1 mg/l soy bean trypsin inhibitor and 10 mM MES, pH 6.2 (MES isolation medium); the other of 1 M glycerol, 5 mM EGTA, 1 mM DTT, 0.5 mM MgSO₄, 10 mM TAME, 0.5% Triton X-100 and 25 mM PIPES, pH 6.9 (PIPES isolation medium). Ten parts of an isolation medium were added to one part packed eggs. The mixture was shaken quickly by hand then the isolated mitotic apparatus was collected by slow centrifugation (500 g × 3 min).

RESULTS

Direct observation. Each mitotic stage was identified by the distribution pattern of birefringence in the cell (Fig. 1). The transparency of the egg cytoplasm of the *M. globulus* made it easy to classify its living eggs according to mitotic stage.

The time table of the mitotic sequence for the first and second divisions in *M. globulus* with and without 45% D₂O is shown in Fig. 2. The length of mitosis was extended in the presence of D₂O. Prophase up to the onset of cleavage at 25°C was extended 8 min in the first division and 6 min in the second. The period from the onset of anaphase to the onset of cleavage was extended from 4 to 6 min in the first division, but there was no extension in the second division. Prophase to the onset of anaphase also was extended in the presence of D₂O, from 7 to 13 min in the first division and from 5 to 11 min in the second. No morphological abnormalities were found during mitosis in the presence of D₂O.

Fixation and staining. Although the egg cytoplasm of *H. pulcherrimus* is not transparent in living cells, we could make the mitotic figures visible by using the fixation and staining procedures described above. The profile of the nuclear envelope,

Fig. 1. Propagation of the first division in *Mespilia globulus* shown by polarization microscopy. A) early prometaphase, B) prometaphase, C) metaphase, D) onset of anaphase, E) anaphase, F) late anaphase, G) onset of cleavage and H) completion of cleavage. Arrows P and A indicate the polarizer and analyzer axes. One division of the scale represents 10 μm .

Fig. 2. Time table of the first and second divisions of *Mespilia globulus* in the presence, and absence, of 45% D₂O at 25°C. The mitotic process was extended in the presence of D₂O.

Fig. 3. Phase contrast microscopy of aceto-carmine stained mitotic figures during the first division in *H. pulcherrimus*. a) prophase, b) breakdown of the nuclear envelope, c) metaphase, d) early anaphase, e) mid-anaphase, f) late anaphase, g) onset of cleavage and h) completion of cleavage. One division of the scale represents 10 μ m.

Fig. 4. Time table of the first division of *Hemicentrotus pulcherrimus* in the presence, and absence, of 40% D₂O at 18°C. Elongation of the mitotic period took place in the presence of 40% D₂O.

the outline of the mitotic apparatus and condensed chromosomes were clearly visible in the stained specimen (Fig. 3). In this procedure, the initiation of prometaphase was denoted by the breakdown of the nuclear envelope. No abnormal mitotic figures were observed in stained eggs developed under the deuterated condition. The reconstructed time table of the first division of *H. pulcherrimus*, based on our survey of fixed specimens at various stages of mitosis, is shown in Fig. 4.

Prophase to the onset of cleavage was extended for 18 min in the presence of 40% D₂O at 18°C. The period from the onset of anaphase to the onset of cleavage was extended from 12 to 16 min in the presence of D₂O. Prophase to the onset of anaphase was extended from 18 to 32 min. This 14 min difference was caused only by the extension of prometaphase from 8 to 22 min (Table 1). Breakdown of the nuclear envelope was not delayed in the presence of D₂O.

When eggs that had been developed in deuterated sea water were transferred to

TABLE 1. AVERAGE TIME (MIN) NEEDED TO INITIATE EACH DEVELOPING MITOTIC STAGE AFTER INSEMINATION

		streak stage	pro-phase	prometa-phase	meta-phase	onset of anaphase	mid-anaphase	late anaphase	onset of cleavage
<i>M. globulus</i>									
1st division at 25°C	H ₂ O	18(4)	22(2)	24(3)	27(2)	29(2)	31(2)	—	33
	45% D ₂ O	18(6)	24(4)	28(5)	33(4)	37(3)	40(3)	—	43
2nd division at 25°C	H ₂ O	—	46(1)	47(3)	50(1)	51(2)	53(4)	—	57
	45% D ₂ O	—	60(5)	65(5)	70(1)	71(2)	73(4)	—	77
<i>H. pulcherrimus</i>									
1st division at 18°C	H ₂ O	44(4)	48(4)	52(8)	60(6)	66(4)	—	70(8)	78
	40% D ₂ O	44(4)	48(4)	52(22)	74(6)	80(6)	—	86(10)	96

Parentheses show the period, in minutes, from one mitotic stage to the next.

Fig. 5. Deterioration of the birefringence of isolated spindles at a low temperature. Isolated mitotic apparatuses of *Mespilia globulus* were stored at 4°C with, and without, 45% D₂O. Note the progress of spindle birefringence. The ordinate shows spindle retardation and the abscissa the time after insemination. Open circles (non-deuterated condition) and closed circles (deuterated condition) essentially show the same values.

normal ASW, they developed normally with no further delay in the mitotic period (data not shown).

Stability of the isolated mitotic apparatus.

Low temperature. To study the stability of the isolated mitotic apparatus under low temperature, we used MES isolation medium. Mg⁺⁺ was deleted from this medium because its presence causes overstabilization of spindle microtubules and prevents the degeneration of spindle microtubules and prevents the degeneration

of spindle structures at low temperatures (8). The presence of 10 mM DTT also was essential as an inadequate concentration endangered preservation of the spindle structures.

The isolates were stored in medium containing 1 M glycerol, 1 mM EGTA and 10 mM MES, pH 6.2 with, or without, 45% D₂O. Changes in spindle birefringence were followed with a polarizing microscope.

The deterioration of spindle birefringence is shown in Fig. 5. Thirty minutes after immersion in the isolation medium spindle had an average retardation value of 2.9 nm. After 180 min at 4°C, the value had decreased to 1.8 nm in the presence, or absence, of 45% D₂O. Deterioration in the deuterated medium was essentially the same as in non-deuterated medium.

Ca⁺⁺ sensitivity. We used PIPES isolation medium because it contains Mg⁺⁺ and makes it easier to obtain well preserved mitotic apparatuses. These isolates were not as sensitive to a low temperature; but, they responded well to Ca⁺⁺, in the order of 10⁻⁵ M.

The isolates were transferred to Ca-EGTA buffer (1 M glycerol, 5 mM EGTA, 5 mM CaCl₂, 0.5 mM MgSO₄ and 25 mM PIPES, pH 6.9) in which the Ca⁺⁺ concentration was calculated as about 10⁻⁵ M (6, 11). Every 5 min, a small portion of the spindle suspension was mixed with an equal volume of 100 mM EGTA solution (pH 6.9). Further Ca⁺⁺-dependent degeneration of the spindle was arrested by this treatment. Each EGTA-treated sample was examined under a polarizing microscope, and its spindle birefringence was measured.

Fig. 6. Deterioration of the birefringence of isolated spindles in the presence of 10⁻⁵M Ca ions. The Ca⁺⁺-dependent breakdown of the mitotic apparatus isolated from *Hemicentrotus pulcherrimus* was caused by a concentration of about 10⁻⁵M Ca⁺⁺ at 18°C in the presence of 1M glycerol with, and without, 45% D₂O. Spindle retardation was observed by polarizing microscope. The ordinate shows spindle retardation and the abscissa the time after the Ca⁺⁺ addition. The values of the open (non-deuterated condition) and closed circles (deuterated condition) do not differ much.

The Ca^{++} -dependent deterioration of the spindle isolates from *H. pulcherrimus* eggs at 18°C with, or without, 45% D_2O is shown in Fig. 6. The decay of birefringence was uniform over the entire spindle. At 18°C for 20 min, the average retardation value decreased from 3.4 nm to less than 1.0 nm. Deterioration in the deuterated medium was essentially the same as in the non-deuterated solution.

DISCUSSION

Our results show that D_2O at concentrations of less than 45% does not inhibit anaphase chromosome movement. The extension of the anaphase was less than 50% (Table 1). This extension apparently is due to elongation of the spindle in D_2O (2, 12, 14).

If the velocity of anaphase chromosome movement in D_2O sea water is equal to that in normal sea water, the chromosomes of the deuterated spindle would require more time to move from metaphase plate to their goal near the spindle pole region because of the lengthened distance to be traveled. In fact, enhancement of spindle length was as much as 1.5-fold in 45% D_2O .

It is most likely that the migration speed of anaphase chromosomes is regulated by microtubule dissociation (2). Thus, if microtubules are stabilized and prevented from dissociating in the presence of D_2O , anaphase chromosome movement should be suppressed. From our analysis of the mitotic process in D_2O , we conclude that enhancement of spindle birefringence and its volume in D_2O at concentrations below 45% (14) does not signify stabilization of the mitotic spindle.

Marsland and Zimmerman (4) reported structural stabilization of the mitotic apparatus in D_2O , but the concentration of D_2O they used was more than 60%. Differences in concentration are important in studies of the D_2O effect during mitosis, especially in studies of its effect on the mitotic spindle (2, 12). We therefore investigated the effects of D_2O concentrations of less than 45%.

To test the above hypothesis concerning the stability of mitotic microtubules, we studied the stability of isolated mitotic apparatus with, and without, D_2O . We measured the decay process of mitotic spindle birefringence against Ca ions and low temperatures. There was no difference between the normal and deuterated conditions (Figs. 5 and 6). Our results are evidence that D_2O does not strengthen the binding properties between tubulin molecules assembled in microtubules.

In our previous study (14), a thermodynamic analysis of the D_2O effect on the mitotic spindle was done; we concluded that D_2O does not enhance the hydrophobic interaction of tubulin molecules. Our conclusion is consistent with the results of the study reported here. We can exclude the possibility that strengthening of binding properties, other than hydrophobic interaction, is involved in the mechanism of the D_2O effect on the mitotic spindle.

Our results show that D_2O affects the length of time from prophase to metaphase. The time at which the nuclear envelope breakdown (initiation of prometaphase in the time table of *H. pulcherrimus*) was not affected, but establishment of the metaphase plate was delayed by the presence of D_2O .

D_2O is thought to promote microtubule assembly (5), and prometaphase is the stage in which the intracellular conditions must be favorable for tubulin association (2). Therefore, we expected the acceleration of prometaphase, but results show its elongation. The reason for this is not known, but in a previous study (14), we concluded

that D₂O increased the number of polymerizable tubulin molecules. In prometaphase, local association and dissociation of tubulin probably takes place in the mitotic spindle during the latitudinal arrangement of the duplicated chromosomes along the equatorial plane. Therefore, the increased concentration of tubulin molecules is considered to influence the association-dissociation balance of tubulin molecules and to retard establishment of the metaphase plate. This, however, is only speculation, more detailed analyses are necessary to verify whether this is so.

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