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CELL DIVISION: COMBINED ANTI-MITOTIC EFFECTS OF COLCHICINE AND HEAVY WATER ON FIRST CLEAVAGE IN THE EGGS OF *ARBACIA PUNCTULATA*

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THERE have been many studies on the anti-mitotic effects of colchicine (cf. [7, 10, 14]), and the same is true with reference to the action of heavy water (cf. [3, 12, 15]). The combined effects of D_2O and colchicine have not been studied, however. Thus it is not known whether these agenices may act synergistically or antagonistically. This could be an important consideration since it could provide some clues as to the basic molecular mechanisms involved.

Perhaps there is a basis for predicting that deuteration and colchicinization might be antagonistic as to their anti-mitotic effects. D_2O is believed to foster polymerization and strengthen the gel structure of the mitotic spindle [12], whereas colchicine appears to have the opposite effect [10]. The situation, however, is very complex and to make such a prediction would be somewhat hazardous.

MATERIALS AND METHODS

Biological procedures.—The eggs, shed by means of the KCl injection method, were washed in two changes of fresh sea water and then inseminated in 50 ml of sea water previously equilibrated to the experimental temperature. The fertilized eggs were maintained in an incubator at $20 \pm 0.1°C$ from the time of insemination until the end of the experiments. Also all solutions and glassware were kept in the incubator [8].

Immersion media.—Each immersion medium was prepared by mixing varying proportions of natural (H_2O) sea water, artificial D_2O-sea water and 0.01 *M* colchicine solution (made up in natural sea water) yielding a total volume of 10 ml, including a standardized sample of egg suspension. The diameter of the immersion

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dishes was 50 mm, thus assuring that the developing eggs were distributed in a shallow well-aerated layer of solution.

The colchicine, designated as Colchicine Alkaloid, U.S.P. (amorphous) was obtained from Fisher Scientific Co and the D_2O, with a specified purity of 99.86 *M* per cent, was prepared by Bio-Rad Laboratories, Richmond, California. The artificial D_2O-sea water had a salt content approximating that of the waters in the vicinity of Woods Hole, Mass. (see p. 55, M.B.L. Formula (1)). Artificial H_2O-sea water, prepared by the same formula, was substituted for natural sea water in some of the experiments, but no difference was discernible in these results.

Time schedule.—Timing proved to be a very critical factor in the experiments. In the first few experiments immersion in the experimental solutions was initiated 45 min following insemination, but this procedure gave considerable variation in the results. Some "fast" batches of eggs required only 57 min to reach the stage when furrowing started, whereas other "slow" eggs required 66 min. Inhibition of cleavage in the D_2O and colchicine solutions, used separatedly or in combination, proved to be relatively low in the former case and relatively high in the latter. Accordingly it was necessary to make a preliminary timing of each new batch of eggs. On the average the first appearance of furrows occurred at 62 min and with such eggs immersion was executed exactly 45 min subsequent to insemination. However, if the time of furrowing was less than this, immersions were started an equivalent number of minutes sooner, or if the time was more, immersions were done an appropriate number of minutes later. In other words, immersion was done exactly 17 min before furrowing was expected and in most of the experiments the timing was not off schedule by more than 1 min. Thus at the time of treatment the eggs had reached early prophase [2].

Controls.—Four samples of eggs were followed in each of the combination experiments. In addition to the experimental sample immersed in a medium which contained both colchicine and heavy water, there were three controls: (1) eggs in natural sea water, (2) eggs in colchicinized medium and (3) eggs in deuterated medium.

Counting procedure.—Two counts were made to determine the percentage of the eggs that had succeeded in reaching the two-cell stage. The preliminary count came 1 h subsequent to the first appearance of furrows in the untreated controls and the final count was 1 h later. In less heavily deuterated media the two counts tended to be identical, but with more heavily treated eggs which displayed a significant delay (up to 30 min) in the onset of cleavage, the second count tended to be higher than the first. In the more heavily colchicinized media, on the other hand, the second was sometimes lower, indicating that some furrows which almost had succeeded in cleavage, subsequently underwent recession.

Counts were made at a magnification of 200 ×, using a long range 20 × objective, focussing directly upon the immersed eggs in the embryological dishes. Each count included 150–200 eggs. With some of the lesser treatments, many of the eggs had reached the four-cell stage by the time of the second count and these were counted as having undergone successful first cleavage.

RESULTS

Colchicine and heavy water, employed separately

Although similar analyses of the anti-mitotic action of colchicine and of D_2O have been made previously [9, 10], none has employed precisely the same procedures and materials as were used in the present experiments. Accordingly the concentration/inhibition curves for D_2O alone (Fig. 1) and for colchicine alone (curve C, Fig. 2) are presented to provide a basis of comparison with the combination experiments in which the eggs were subjected to both deuteration and colchicination.

Combination treatments with both D_2O and colchicine

The results of all these experiments are presented graphically in Fig. 2. Here it may be seen that the combined anti-mitotic activity of these two agencies cannot be described in very simple terms. Depending upon the concentrations employed, their mutual action may be antagonistic, synergistic or neutral.

At relatively lower concentrations of heavy water, namely 25 per cent and 35 per cent, the presence of D_2O antagonized the colchicine action to a very marked extent (compare curve C, Fig. 2, with E and F). With 25 per cent D_2O, in fact, the release from colchicine blockage averaged 66 per cent, reaching a maximum of 87 per cent 5.0×10^{-4} *M* colchicine. With 35 per cent D_2O, the release from colchicine inhibition was somewhat less, averaging 48 per cent over the full range of concentration with a maximum of 81 per cent, again at 5.0×10 *M*.

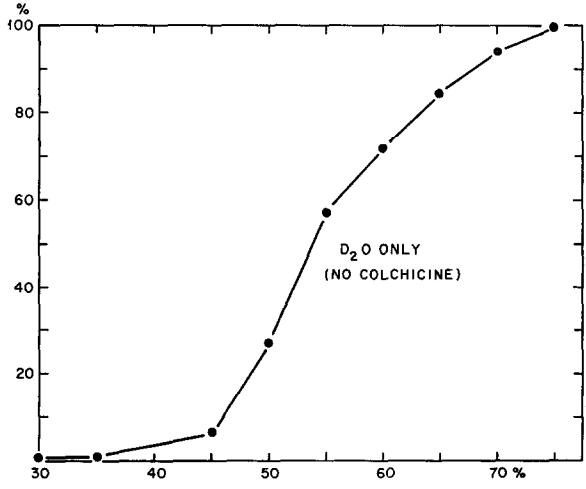

Fig. 1.—Anti-mitotic effects of D_2O. Deuteration was initiated at early prophase—17 min prior to the first appearance of cleavage furrows in untreated *Arbacia punctulata* control eggs maintained at 20 ± 0.1°C. Each point represents an average derived from a minimum of 10 experiments in each of which a minimum of 150 eggs were counted. Extremes of variation of point values, from higher to lower D_2O concentrations, were, respectively: ± 0.1, ± 1.1, ± 2.3, ± 3.6, ± 7.7, ± 4.1, ± 1.2, ± 0.5, ± 0.0, *Abscissa*: D_2O concentration; *ordinate*: inhibition, 1st cleavage.

At an intermediate range of D_2O concentration, namely 45 per cent, the anti-mitotic effect of heavy water was additive to the colchicine effect in the range of concentration below 6.0×10^{-4} *M*, even though the inhibition by D_2O alone at this level amounted to only 7 per cent (Fig. 2, curves C and D). At 6.0×10^{-4} *M*, however, 45 per cent D_2O had little or no influence on the colchicine effect. But above this point a small antagonism appeared (Fig. 2, D).

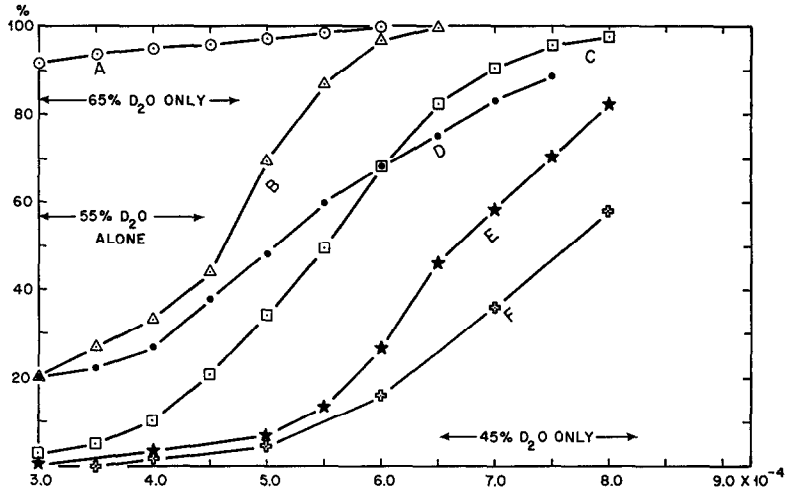

Fig. 2.—Combined anti-mitotic effects of colchicine and D_2O. Initiation of treatments was the same as specified in Fig. 1. Curve *A*: colchicine plus 65 % D_2O; *B*: colchicine plus 55 % D_2O; *C*: colchicine only; *D*: colchicine plus 45 % D_2O; *E* and *F*: colchicine plus 35 and 25 % D_2O, respectively. Note antagonism with lower D_2O concentrations, synergism with higher and both effects in the intermediate range (45 % D_2O). The points on curve C (colchicine only) represent, in each case, an average from at least 7 expts. Other points are averages from a minimum of 3 expts. Inhibition, 35 % D_2O = 1.0 %; 25 % D_2O = 0.3 %.

Extremes of variation of point values, from higher to lower colchicine concentrations, were, respectively: Curve *A*: ±0.0, ±0.3, ±0.3, ±0.9, ±1.0, ±1.7, ±1.6; *B*: ±0.1, ±0.7, ±3.5, ±5.8, ±4.2, ±2.9, ±1.1, ±1.4; *C*: ±1.0, ±1.8, ±2.6, ±2.4, ±5.2, ±6.0, ±2.7, ±3.0, ±2.1, ±1.8, ±0.3; *D*: ±4.0, ±3.6, ±3.4, ±4.1, ±5.6, ±3.9, ±4.2, ±2.3, ±1.5, ±1.9; *E*: ±5.1, ±3.8, ±6.0, ±3.1, ±4.8, ±2.1, ±2.1, ±1.1; *F*: ±6.7, ±5.3, ±4.9, ±2.0, ±0.9, ±0.0.

Abscissa: Colchicine, molal concentration; *ordinate*: inhibition, 1st cleavage. ○—○, *A*, colchicine + 65 % D_2O; △—△, *B*, colch. + 55 % D_2O; □—□, *C*, colch. only; ●—● *D*, colch. + 45 % D_2O; ★—★, *E*, colch. + 35 % D_2O; ✠—✠, *F*, colch. + 25 % D_2O.

The synergism between 45 per cent D_2O and colchicine in concentrations below 6.0×10^{-4} was quite marked. In fact, the enhancement of inhibition was considerable greater than the D_2O effect alone, being 14 per cent on the average, with a maximum of 17.2 per cent in 4.5×10^{-4} *M* colchicine solution.

With higher concentrations of D_2O (55 and 65 per cent) the anti-mitotic effects of colchicine were intensified, over the whole range of colchicine contraction (Fig. 2, curves A, B and C).

In the case of 55 per cent D_2O, the increased inhibition as compared to the effect of colchicine alone, averaged 24 per cent, with a maximum value of 36 per cent at 5.0×10 *M* colchicine. With 65 per cent the combined inhibitory effects of D_2O and colchicine were almost complete event at the lowest concentration of colchicine which, by itself, caused almost no inhibition.

The case of 55 per cent D_2O differs from the others in one respect, if we look at the situation from a different angle. Fig. 2 (B and C) shows that the presence of colchicine in the range of concentration below 5.0×10^{-4} *M* exerted a definite antagonism to the D_2O inhibition. At the same time, however, the presence of D_2O definitely intensified the action of colchicine.

The delay in the onset of cleavage tended to parallel the magnitude of the final inhibition. In the media containing up to 35 per cent D_2O it did not exceed 6 min, but in media of 65 per cent deuteration there was a lag of 25–30 min.

DISCUSSION

It is not a simple matter to propose a basic interpretation of the foregoing rather complex results. A number of factors must, however, be taken into consideration by any such interpretation.

It is generally believed that colchicine weakens the structure of the microtubular elements of the mitotic spindle by interfering with the processes of polymerization by which elongate protein fibrils are built up from many relative short units present in the cell prior to mitosis [5, 10, 13, 14]. Presumably such interference results from the tendency of colchicine to combine with and block off the bonding sites which otherwise would be free to form the end to end linkages of the polymer structure [14]. Or if the polymer structure be pre-formed at the time of treatment, colchicine would tend to disrupt it by interpolating its molecules into the polymer bonds.

D_2O, on the other hand, appears to favor polymerization [6]. According to the viewpoint of Lauffer [6] and of Marsland [9], the potential bonding sites of the prospective polymer structure are protected by shells of bound water which must undergo dispersion before polymerization can occur. Deuteration is believed to foster polymerization because the electro-strictive forces which tend to bind the aqueous shells at the prospective bonding sites are weaker in the case of D_2O than they are for H_2O. Thus rising temperature, which favors polymerization in such systems by promoting a dispersal of

the protective aqueous shells, is more effective when H_2O is replaced in significant proportion by D_2O [6, 11]. The anti-mitotic activity of D_2O, on the other hand, may represent an over-stabilization of the polymer or gel structure, thus preventing the ebb and flow of break-down and build-up which normally occur during the functioning of the mitotic apparatus [4].

As to the present results one might argue that low concentrations of D_2O tend to counteract the anti-mitotic effects of colchicine by fostering the polymerization of such residues of the mitotic protein subunits as may have escaped the colchicine block. Such low concentrations of themselves produce no significant deuterational inhibition indicating that they do not of themselves effect much interference with the formation and functioning of the mitotic apparatus.

Higher concentrations of D_2O, on the other hand, in the range of 55 per cent to 65 do of themselves produce a high degree of deuterational blockage, indicating an over-stabilization of such of the mitotic protein polymer as may have escaped colchicine disintegration. But why, in the case of 55 per cent D_2O, low concentrations (less than 5.0×10^{-5} M) of colchicine should counteract deuterational blockage, remains an open question. In fact, all of these proposed interpretations must remain open to question until the effects of colchicine and heavy water, used separately and in combination, can be studied with reference to their action upon the isolated mitotic protein polymer system.

A final word as to the timing of the present experiments seems necessary. Initiating the colchicine immersions just 17 min before the first appearance of furrowing (21 min before 50 per cent of the eggs showed furrows) required significantly higher concentrations of colchicine to produce degrees of inhibition equivalent to those reported previously [10]. However, in our previous experiments the immersions were started 10 min earlier. In the present case, the nuclear outlines began to fade some 3 min after immersion, indicating that the mitotic spindle was already partly formed at the time of immersion [2]. This and other evidence indicates that thigher concentrations of colchicine are required to induce spindle disintegration than to interfere with its formation in the first place. The deuterational effect, on the other hand, appears to be much less dependent on timing [12].

SUMMARY

The anti-mitotic effects of colchicine and of heavy water, used separately and in combination, were studied with reference to inhibition of first cleav-

age in the eggs of *Arbacia punctulata*. Treatments were initiated at early prophase which at the experimental temperature (20 +0.1°C) was 17 min before the first appearance of cleavage furrows.

With lower concentrations of D_2O (25–35 per cent) the two anti-mitotic agents antagonized each other over the whole range of colchicine concentrations (3.0–8.5 $\times 10^{-4}$ *M*). At higher concentrations (55–65 per cent D_2O) there was a distinct synergism throughout; whereas at intermediate D_2O concentration (45 per cent) synergism was observed when the colchicine concentration was below 6.0 $\times 10^{-4}$ *M* and antagonism when the colchicine concentration was above this point.

A tentative interpretation, in terms of molecular mechanisms, is proposed.

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