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Effect of Demecolcine upon Cleavage of Sea Urchin Eggs and their Reversibility[1)2)]

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Colchicine has been known as a chemical agent which prevents formation of the spindle and aster, and also destroys these structures in dividing cells to block both the nuclear and the cell division in consequence. The effect of colchicine upon the cleavage of sea urchin eggs has been tested by NEBEL and RUTTLE (1938), BEAMS and EVANS (1940), CORNMAN and CORNMAN (1951), ZEUTHEN (1951) and SWANN and MITCHISON (1953). In their experiments, the mitotic apparatus was destroyed as in plant cells but there was no record of an animated embryo produced after the treatment. In the preliminary experiments (1957a, b) the present author applied colchicine of various concentrations to eggs of several kinds of Japanese sea urchins and obtained quite similar results to these works in many respects. Being acquainted with the fact that demecolcine affects the mitosis in a similar way to colchicine and is less toxic than the latter, the author tested its effect upon the cleavage of sea urchin eggs and compared its toxicity with that of colchicine. The results obtained were remarkable in the reversibility of cleavage inhibiting effect of the agent.

The author wishes herewith to express his hearty thanks to Professor Kenji NAKAMURA for suggesting this investigation as well as for constant guidance in the course of the work. He is greatly indebted to the directors and the staffs of Seto Marine Biological Laboratory of Kyoto University and Sugashima Marine Biological Station of Nagoya University, where the present study was mainly carried out. His deep gratitude is also due to Dr. Eizo NAKANO of Nagoya University as well as to the authorities of Ciba Products Ltd., Osaka, who generously supplied him with samples of demecolcine.

Materials and Methods

The experiments were performed with eggs of *Mespilia globulus*. Those of

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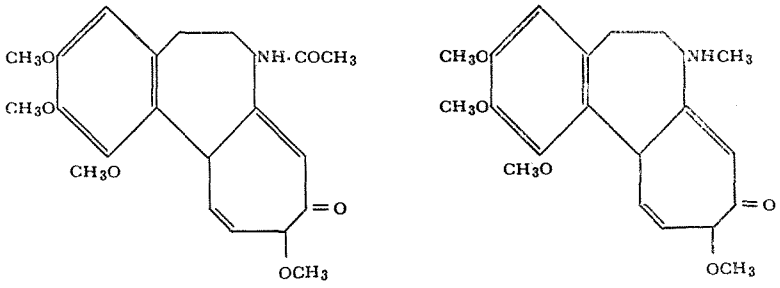
Hemicentrotus pulcherrimus were also employed for comparison. Eggs shed by means of the current KCl method were washed through several changes of sea water and inseminated. When the fertilization membrane was elevated in more than 80% of eggs (it took 5 minutes after fertilization), either demecolcine or colchicine was added to the egg suspension. The final concentrations graded from 1×10^{-3}% to 1×10^{-5}%, intermediates being diluted at 5 times each. In the present paper, the terms 'demecolcinized eggs' and 'colchicinized eggs' denote the eggs in which the cleavage was suppressed by the action of demecolcine and colchicine respectively.

Demecolcine used in the present work was one of derivatives of colchicine produced by Ciba Ltd., Basle. The chemical structures of colchicine and demecolcine were illustrated in Fig. 1. The demecolcine solutions applied to eggs



Colchicine $C_{22}H_{25}O_6N$

Demecolcine $C_{21}H_{25}O_5N$
(Desacetyl-N-methyl-Colchicine)

Fig. 1. Chemical structures of colchicine and demecolcine.

were prepared by diluting Colcemid (commercial name of demecolcine) with sea water, and contained 0.1% of demecolcine, 10% of propylene glycol and 5% of ethanol. Therefore, the latter two agents were contained in the demecolcine solutions at concentrations lower than 0.1% and 0.05% respectively. In preliminary experiments the latter two agents were found to be non-effective with regard to the cleavage of sea urchin eggs at least at these concentrations.

In purpose of finding whether the agents inhibit the cleavage reversibly or irreversibly, and to measure the toxicity at the same time, demecolcinized eggs and colchicinized ones were washed thoroughly and transferred to the normal sea water when the eggs in the control accomplished the first cleavage.

Morphological changes of the eggs were observed *in vivo* with the aid of a phase contrast microscope. Materials fixed with CAROTHERS' mixture, embedded in paraffin, cut at 7μ in thickness and stained with HEIDENHAIN's haematoxylin, were also brought to the microscopical examination.

Results

In *Mespilia* eggs, under the laboratory conditions at 26°C, the first cleavage

was accomplished within 50 minutes after fertilization, the subsequent cleavage taking place approximately at intervals of 30 minutes. The eggs developed to morulae in 3.5 hours and nearly 95% of the eggs grew into blastulae at about the 5th hour of development.

When fertilized eggs were exposed to demecolcine at concentrations above $5\times10^{-5}\%$, the cleavage was completely suppressed. After several hours of the treatment, cytolysis commenced and the eggs disintegrated afterwards. In a $10^{-5}\%$ solution, however, the cleavage was not affected; the eggs cleaved successively at nearly the same intervals with the control eggs and developed quite normally to swimming blastulae. The only effect recognizable was lowering of the survival rate from 95% of the control to 80% on an average.

The effect of demecolcine was discovered removable by washing and transferring the eggs into the normal sea water. The recovery rate of the eggs varied according to the time of exposure of the eggs to demecolcine and concentration of the agent applied. The results were illustrated in Fig. 2, in which

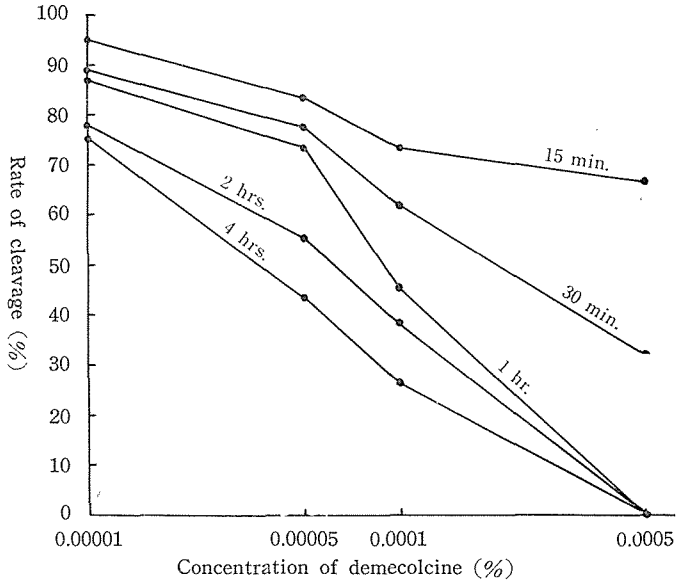


Fig. 2. The cleavage rate of the demecolcinized *Mespilia* eggs in 3 hours after being washed and transferred to the normal sea water at 26°C.

the recovery rate was indicated with percentage of cleaved eggs at the end of the 3rd hour after removal of the agent. As was expected, the recovery rate decreased as either the time of exposure to the agent was prolonged or the concentration of the agent increased. It is noticeable that the recovery rate at a concentration of $10^{-5}\%$ remained almost at the same level with the survival rate of the control. At this concentration a prolonged exposure, at least up to

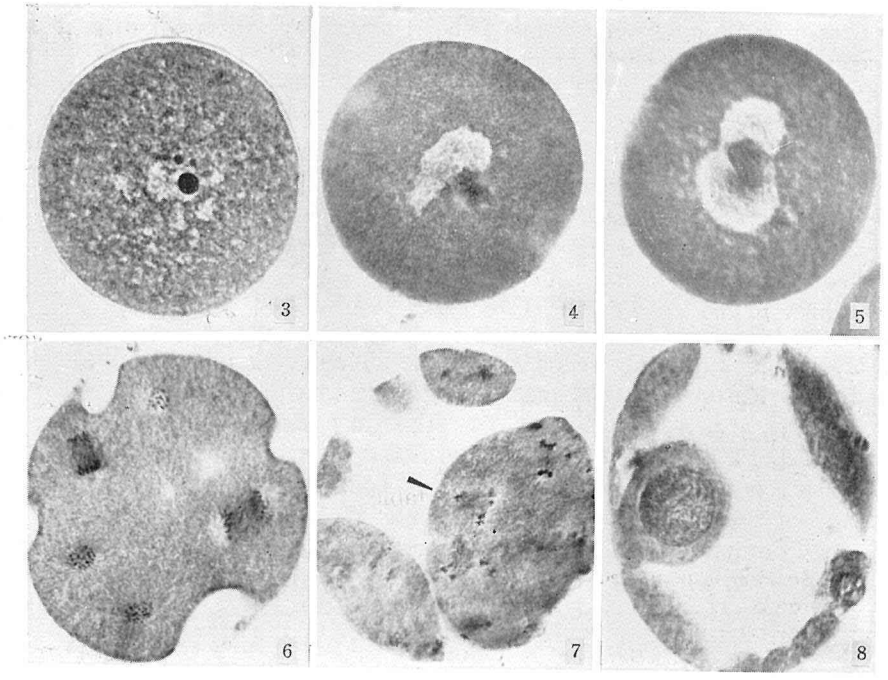
4 hours, yielded no remarkable decrease in the recovery rate. However, the effect was found to be irreversible when the eggs were exposed to the agent at a concentration of $5\times10^{-4}\%$ for more than 1 hour.

With regard to the inhibition of the cleavage, colchicine was found to be less effective than demecolcine. Complete inhibition was gained at concentrations above $10^{-3}\%$. Even at this minimal concentration there were observed a few eggs taking a long time to accomplish the first cleavage, and a subsequent cleavage inevitably suppressed. The effect vanished completely at concentrations below $5\times10^{-4}\%$, where the cleavage was not delayed but the survival rate did not exceed 60%. Colchicine exhibited a striking contrast to demecolcine in irreversibility of the effect. Eggs exposed to colchicine for 1 hour at the minimal concentration to block the cleavage, i.e. $10^{-3}\%$ in the present experiment, were found not to be liberatable from the effect by mere washing with sea water. No sign of nuclear division was observed in these eggs and they disintegrated afterwards. In a series of experiments the author was unable to find any concentration of the agent which arrested the cleavage completely but reversibly. The results are tabulated in the following table.

Table 1. Inhibiting effects of both demecolcine and colchicine upon cleavage of *Mespilia* eggs applied for 1 hour and their reversibilities by removal of the agents.

	Concentration (%)	10^{-3}	5×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}
Demecolcine	inhibition	+	+	+	+	—
	recovery	—	—	+	+	+
Colchicine	inhibition	+	—	—	—	—
	recovery	—	—	—	—	—

Structural changes of eggs caused by both agents were essentially similar. In both demecolcinized and colchicinized eggs, the mitotic apparatus did not develop, or disappeared if present. The cytoplasm is densely granulated, and around the nucleus there always existed a zone distinguishable from the surrounding cytoplasm. The zone seemed to correspond to the "lake like body" described by BEAMS and EVANS (1940) in colchicinized eggs, but the feature of the zone varied to some extent according to the stage of cleavage, in which the mitotic structures were destroyed by the agent. In some eggs containing a compact nucleus of a small size, the zone was thin and somewhat granulated (Fig. 3). The contour of the zone was not well defined but clear spots scattering in the cytoplasm. In eggs with a swollen nucleus, the zone thickened and looked rather hyaline, being less granulated than in the eggs described above. The zone was well defined and the spots in the cytoplasm less evident. Sometimes, the hyaline zone was thickened on one side of the nucleus (Fig. 4) or dumb-bell in shape having a nucleus in the middle (Fig. 5). These features



Figs. 3–8. Sectioned preparations of eggs recovered from the effect of demecolcine.
Figs. 3–5. Hyaline zone around the nucleus of the demecolcinized egg.—3. The mitotic structures destroyed at an early stage.—4. At the monoaster stage.—5. At the amphiaster stage of the first cleavage.
Figs. 6–8. Showing nuclear divisions and a polyploid nucleus.—6. Equatorial plates and anaphase chromosomes in a multinucleate eggs.—7. Division figures in multinucleate blastomeres, a multipolar division figure being indicated with an arrow.—8. A morula containing a polyploid blastomere.

reminded the author that the eggs were in monoastral and amphiastral stages of the first cleavage. The eggs remained in such states for some hours and then necrotic changes gradually set in. With lapse of time, the nucleus became pycnotic, the hyaline zone around the nucleus thinner, and deeply stained granules of various sizes appearing in the cytoplasm. There was a tendency of cytoplasm condensing into masses giving a snaggy appearance to the egg, and sooner or later the whole egg disintegrated into round or oval masses of various sizes.

The mode of the cleavage in the eggs, recovered from the effect of demecolcine, differed remarkably from the normal way. A small mitotic apparatus, formed beside the nucleus (Plate I, Fig. 1) and the nuclear division proceeded in quite a usual manner, although the cleavage did not follow the nuclear division. Such nuclear division successively occurred and the egg became multinucleated (Plate I, Fig. 2). Then, furrows appeared on the surface of the egg in close association with the division of nuclei which located near the

peripheral zone of the egg (Plate I, Fig. 3). Sooner or later, the egg cleaved into blastomeres of unequal sizes (Plate I, Fig. 4), which cleaved successively and the egg developing into a morula. Notwithstanding that the mode of cleavage displayed by the multinucleate egg, which showed much irregularity, the morulae were quite normal in every respect, being composed of mononucleate blastomeres of normal iszes (Plate I, Fig. 5). Swimming blastulae were then formed (Plate I, Fig. 6), in which gastrulation took place in the usual manner and finally developed to normal prutei following the normal course of development.

Because of the spindle destroying effect of the agent, polyploid nuclei were expected to be formed by the treatment. In sectioned preparations, a polyploid equatorial plate was rarely found among the diploid, both in the multinucleated eggs and in blastomeres, but there being none in which the equatorial plates were exclusively polyploid (Figs. 6, 7). Abnormalities in nuclear division, such as the multipolar division and the partial defect of spindle, were seldom found. It was also found that some of the morulae contained a few blastomeres with a nucleus of abnormally large size (Fig. 8). Though the fate of such blastomeres was not pursued, they or the embryo which contained abnormal blastomeres might be obliterated in the course of development, because all the swimming blastulae were composed of normal blastomeres, each containing a nucleus of the normal size.

Discussion

In the present work, it has been found that demecolcine destroys the mitotic apparatus and prevents its formation in sea urchin eggs as colchicine does. The similarity of the agents in the effect is also illustrated in morphological changes caused in the cell structures. However, differences lie both in effectiveness to block the nuclear division and in reversibility of the effect. The minimal concentration, which affords complete blocking, is $5\times10^{-5}\%$ or 1.25×10^{-6}M in demecolcine and $10^{-3}\%$ or 2.5×10^{-5}M in colchicine at 26°C. Thus demecolcine seemed to be nearly 20 times stronger than colchicine as far as this effect is concerned.

In previous works of other investigators (*loc. cit.*), the concentrations of colchicine which yielded inhibition of cleavage or destruction of the mitotic apparatus in eggs of other sea urchins, were between 2.5×10^{-5}M and 5×10^{-4}M. No reversibility of the effect was gained in these works. On the other hand, INOUÉ (1952) reported in *Chaetopterus* egg that the mitotic apparatus of the first meiotic division was destroyed with colchicine. It was restituted after the egg was washed in sea water and the egg became fertilizable. In his experiment the concentration of the agent used was 1×10^{-4}M, being 4 times stronger than the minimal concentration in the present work but the time of exposure was less than 10 minutes at 23°C. The reversibility may depend on a short exposure to the agent.

The effect of demecolcine has been studied extensively with malignant tissues and found to be less toxic than colchicine to animals used (*cf.* EIGSTI and DUSTIN, 1955). In the present work, eggs demecolcinized at concentrations below $1\times10^{-4}\%$ for more than 1 hour are able to recover from the cleavage inhibiting effect of the agent. Though the mode of cleavage is quite different from the normal in shape and size. The morphological changes caused in the demecolcinized eggs are similar to those in the colchicinized ones. These facts seem to indicate that both demecolcine and colchicine affect the cleavage of sea urchin eggs in the same way, the former being more effective and less toxic than the latter.

Summary

Demecolcine inhibits the cleavage of sea urchin eggs as colchicine does. The minimal concentration of the agent which yields the complete inhibition is $5\times10^{-5}\%$, being 20 times weaker than that of colchicine. The effect is reversible by washing the egg through several changes of sea water, while that of colchicine is irreversible. Thus, demecolcine is found to be far more effective as regards the blocking of cleavage but less toxic than colchicine is.

In the recovered eggs cleavage does not follow at least early several nuclear divisions and the eggs become multinucleate. Though the mode of cleavage is quite different from the usual way, morulae of normal appearance are formed. They develop to gastrulae through vivid blastural stage. No polyploid embryo is formed.

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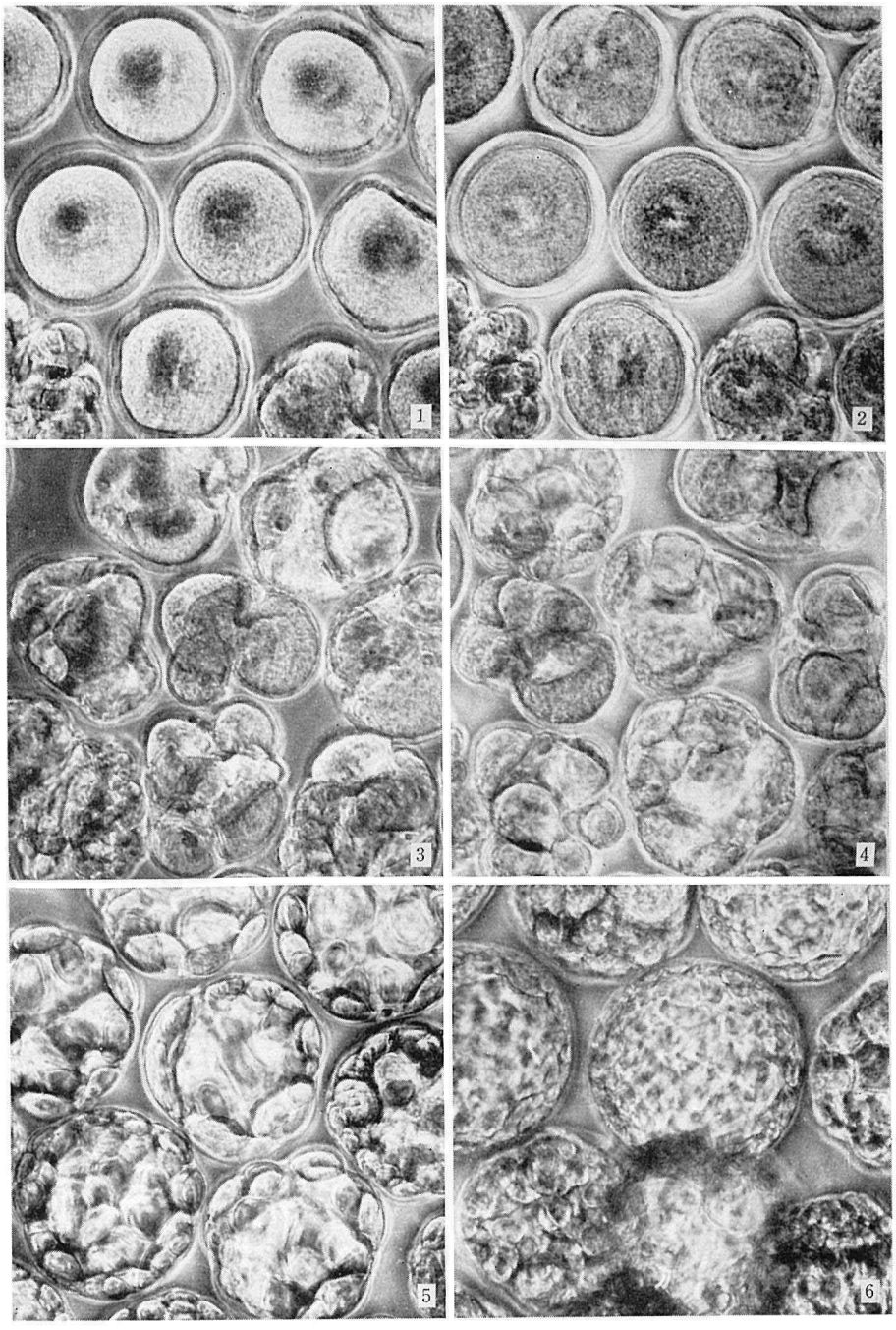
Explanation of Plate I

Cleavage process of demecolcine treated eggs (at $5\times10^{-5}\%$) after washing in normal sea water. Phase contrast micrographs.

Fig. 1. Washing (1 hour after fertilization).—Fig. 2. 1 hour after washing. —Fig. 3. 2 hours.—Fig. 4. 3 hours.—Fig. 5. 3.5 hours, morulae, nearly normal. —Fig. 6. 4 hours, normal swimming blastulae.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