

INHIBITION OF NUCLEAR RECONSTRUCTION BY DITHIOTHREITOL

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INTRODUCTION

In echinoderms it is possible to recognize two sequential processes that occur during nuclear reconstitution: first each individual chromosome becomes surrounded by a double membrane with pores characteristic of a nuclear membrane (Harris, 1962; Longo, 1972), then the membrane-bounded chromosome vesicles (karyomeres) fuse to form a single membrane-bounded nucleus containing the full complement of chromosomes (Wilson, 1925; Mazia, 1961; Longo, 1972).

This sequence of events suggests that it should be possible to obtain chromosomes separated during interphase, each in its own "nuclear" membrane, by inhibiting the second step of nuclear reconstitution, the fusion of chromosomal vesicles. Such a cell would then possess many small micronuclei instead of one large nucleus; in fact, as many micronuclei as there are chromosomes.

In an experiment involving the application of dithiothreitol (DTT) to cleaving sea urchin embryos, we noticed a distinct change in the morphology of all interphase nuclei. In each blastomere of two- or four-cell embryos that had been incubated in the presence of DTT from before the first cleavage, the nucleus appeared multivesiculated. It occurred to us that the multivesiculated nucleus might actually be an aggregate of unfused karyomeres.

Earlier studies on dividing cells and of the effects of compounds that block the formation of disulfide bonds have noted interferences with the normal dynamic behavior of the nuclear membrane at mitosis (Hughes, 1949; Mazia, 1961). It therefore seemed reasonable to hypothesize that DTT was producing a multivesiculate nucleus by in-

hibiting the second step of nuclear reconstitution, the fusion of membrane-bounded karyomeres.

This report communicates the results of experiments which lend credibility to such an interpretation.

MATERIALS AND METHODS

Sea Urchins

Two species of Pacific sea urchins were used in this study. *Lytechinus pictus* and *Strongylocentrotus purpuratus* were obtained from the Pacific Bio-Marine Supply Co., Venice, Calif. Sea urchins were maintained at 12°C in an Instant Ocean System (Aquarium Systems, Inc., Eastlake, Ohio) in artificial sea water prepared with Instant Ocean Salts (Aquarium Systems, Inc.). Equivalent results were obtained with both species.

Obtaining Gametes

Eggs were obtained by shedding induced by the coelomic injection of 0.5 ml of 0.55 M KCl. Sperm were obtained from surgically removed gonads of those urchins shedding sperm upon KCl injections. Eggs were shed into and washed through several changes of artificial sea water (Hinegardner, 1967). Eggs were fertilized with a dilute sperm suspension, excess sperm removed by gentle centrifugation, and the embryos incubated at 15°C in artificial sea water containing 80 U of penicillin and 25 mcg of streptomycin per ml. Cultures were maintained in 250-ml Nalgene centrifuge bottle (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) revolving at 12 rpm in a tissue culture roller apparatus (New Brunswick Scientific Co., Inc., New Brunswick, N. J.).

DTT

Unless otherwise indicated, DTT (Clelands reagent, grade A, Calbiochem, San Diego, Calif.) was used at 2 mM concentration.

Light and Electron Microscopy

Embryos at the two- and four-cell stage of development were fixed in Carnoy's fixative (ethanol to acetic acid, 3:1) and cleared by the procedure of Mazia et al. (1960). Fixed samples were examined with a Zeiss RA microscope fitted with Nomarski phase-interference optics.

For electron microscopy, embryos were fixed for 2 h in 2% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) in Millipore-filtered sea water, pH 7.8 (Millipore Corp., Bedford, Mass.), and postfixed for 1 h in 1% osmium tetroxide (Ladd Research Industries, Inc.). After dehydration through an acetone series, embryos were embedded in Epon in predried BEEM capsules. Sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

RESULTS

The hypothesis that DTT blocks the second step of nuclear reconstitution, karyomere fusion,

was tested by manipulating the time of addition of DTT to the embryo culture, taking advantage of the natural division synchrony that exists in the early cleavage stages. It was found that 1 h incubations with DTT after fertilization but before the first cleavage or after the first cleavage but before the second, failed to produce multivesiculated nuclei. However, incubations of comparable duration which included a mitotic event (either the first or second) reproducibly yielded multivesiculated nuclei.

In another experiment DTT was added in prophase after chromosome condensation had begun. When mitosis was completed, multivesiculate nuclei were evidenced. This rules out the possibility that DTT was influencing an event before mitosis that had its later effects on mitosis.

Furthermore, multivesiculate nuclei appeared even when DTT was added after anaphase migration had begun. This serves to limit the action of

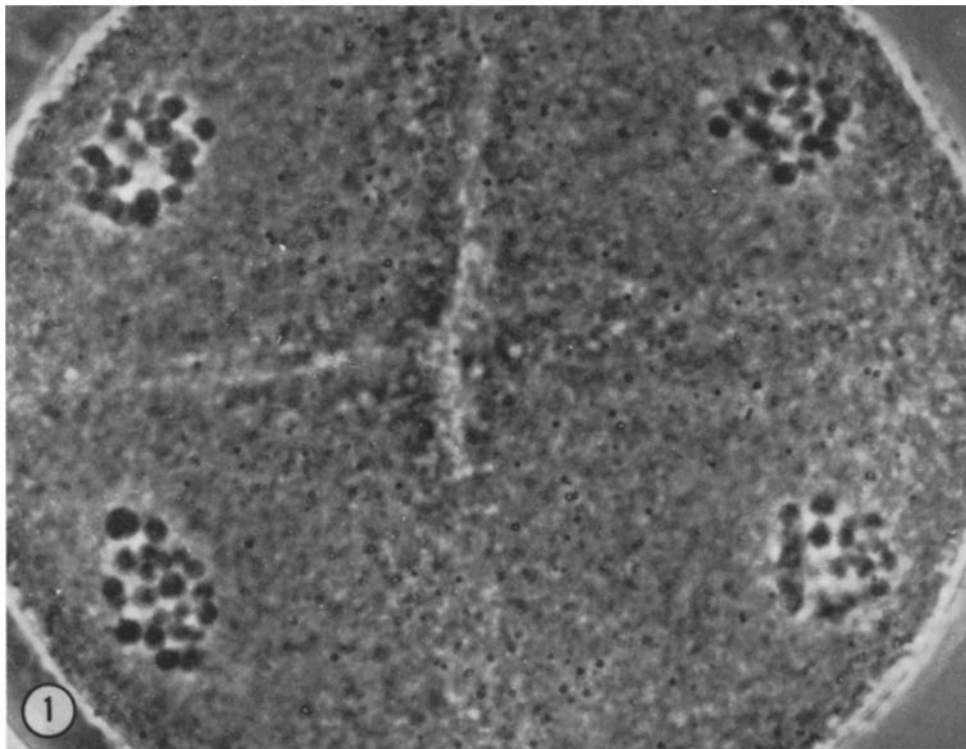


FIGURE 1 Clusters of micronuclei are seen replacing the interphase nucleus in each blastomere of a four-cell embryo (*Strongylocentrotus*) incubated with 2 mM DTT. Micronuclei are visualized by perfusing a slide of living embryos with a clearing solution. The clearing solution consisted of 0.25 M sucrose, 1.5 mM MgCl₂, 0.01 M Tris pH 7.2, and 1% Triton X-100. The micronuclei appear as separate vesicles, each resembling a nucleus but being much smaller. $\times 260$.

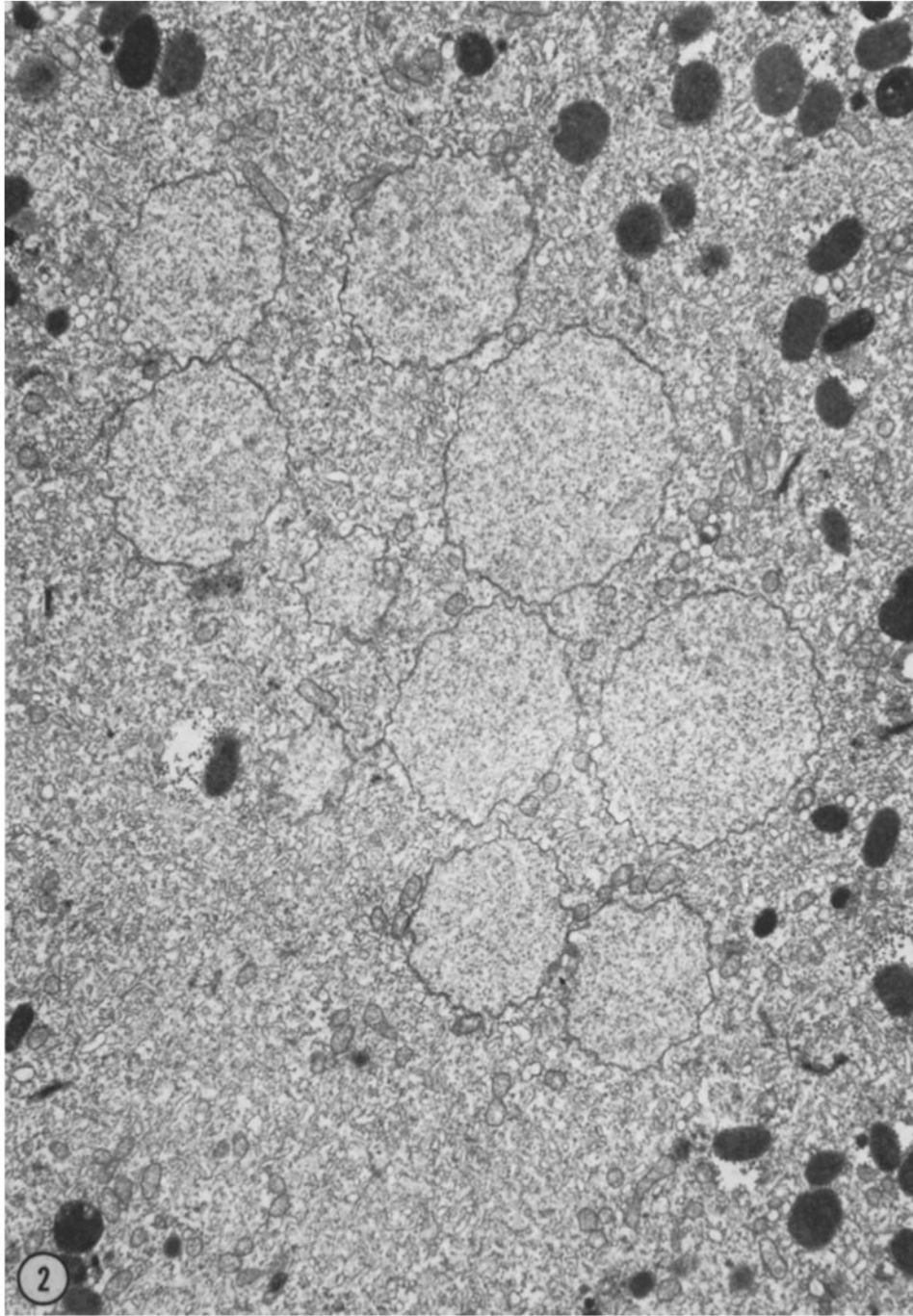


FIGURE 2 Electron micrograph of the nuclear area of a DTT-treated embryo (*Strongylocentrotus*). Instead of one nucleus, several separate and distinct micronuclei are seen. Each micronucleus is surrounded by a pore-bearing double membrane. $\times 8,200$

DTT to a critical time corresponding to late anaphase and telophase, the time of nuclear re-constitution.

If fusion of telophase karyomeres is inhibited

by DTT, then the multivesicular nucleus should be resolvable as separate and distinct nuclear structures. This is possible at both the light and electron microscope levels.

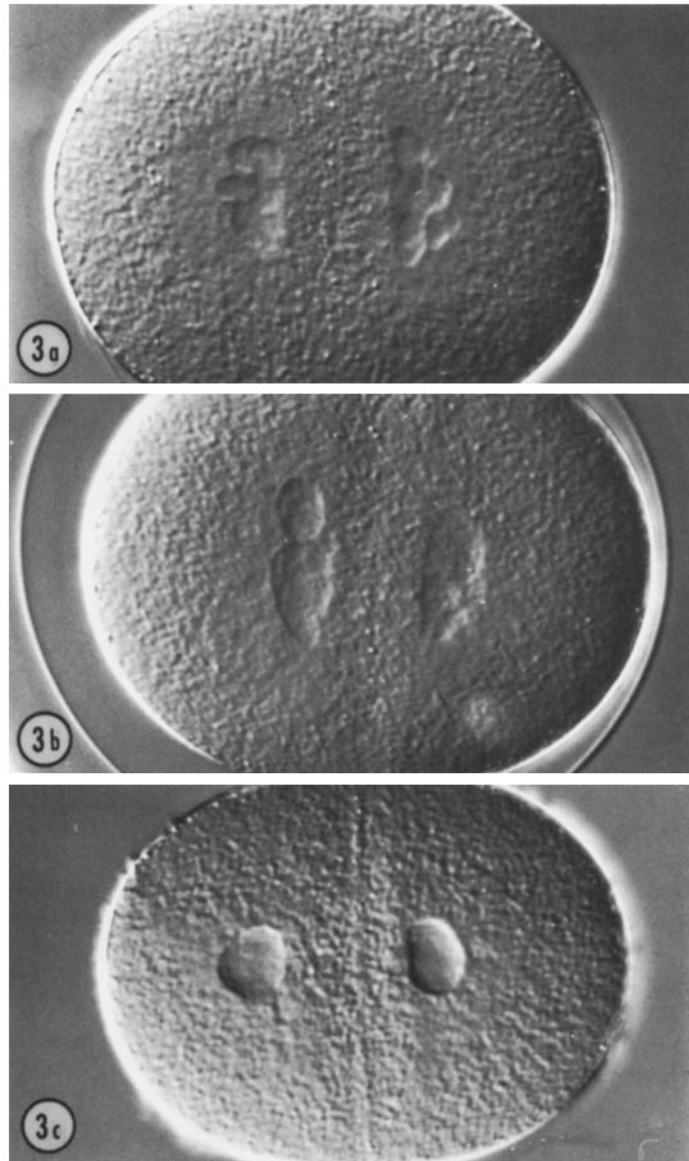


FIGURE 3 The separation of chromosomal vesicles during interphase can be readily reversed by washing the embryos (*Lytechinus*) free of DTT. Within minutes, fusion of the vesicles into a single interphase nucleus is complete. Embryos fixed and cleared by the procedure of Mazia et al. (1960). Nomarski optics, each $\times 180$.

FIGURE 3 a Immediately after removal of DTT, vesicles are still separate.

FIGURE 3 b 2 min after removal of DTT, partial fusion has occurred. Instead of many small vesicles, a small number of large vesicles is seen.

FIGURE 3 c 4 min after the removal of DTT, the interphase nucleus is completely reconstituted.

Fig. 1 shows numerous small "nuclei" in each blastomere of a four-cell embryo. The micrograph was prepared by perfusing a slide of DTT-treated sea urchin embryos, grown to the four-cell stage, with a solution that served to decrease the opacity of the cytoplasm while stabilizing the nuclei (see figure legend for details). The embryos had been exposed to DTT continually, beginning 30 min after fertilization. The micrograph demonstrates the presence of numerous micronuclei. The total number of micronuclei is not visible in the micrograph, others being located above or below the plane of focus. It can be seen, however, that the micronuclei are distinct and separate, with no apparent continuities or connections.

Electron microscopy of DTT-treated embryos reveals that each of the micronuclei is surrounded by a double-unit membrane with pores characteristic of nuclear membranes (Fig. 2). No areas of membrane fusion or other continuities between the micronuclei have been detected at the electron microscope level. It is reasonable to conclude, therefore, that DTT is inhibiting the fusion of karyomeres in telophase. Each membrane-bounded micronucleus may, therefore, correspond to an individual chromosome.

It is of interest that the effect of DTT is also readily reversible. Micronuclei proceed to fuse in a matter of minutes after washing away the DTT. The fusion process that occurs normally at telophase can therefore be separated from mitosis and experimentally controlled. As can be seen in the series of micrographs in Fig. 3, fusion is completed within 4 min at 15°C. At 2 and 3 min after DTT is washed away, several large vesicles are seen, indicating that fusion occurs in a stepwise fashion.

Since fusion of karyomeres depends upon the lability of the vesicle membranes, it is likely that DTT interrupts fusion by altering disulfide interactions within the membrane. To test whether pronuclear fusion is also sensitive to DTT, unfertilized sea urchin eggs (*Lytechinus*) were preincubated with DTT (2 mM) for 15 min and then fertilized. Unexpectedly, pronuclear fusion took place in the presence of DTT. This suggests that there may be some compositional difference between pronuclear membranes and karyomere membranes. Since nuclear membranes are now isolatable in quantities sufficient for biochemical analyses (Kashnig and Kasper, 1969; Franke et al., 1970), it should be possible to soon probe for

developmental modifications in nuclear membranes.

DISCUSSION

The evidence presented here makes it clear that DTT inhibits the completion of nuclear reconstruction after mitosis in cleaving sea urchin embryos. Precedence exists for a role for sulfhydryl interactions in the mitotic behavior of the nuclear membrane. Using chick embryo cells dividing in vitro, Hughes (1949) demonstrated that iodoacetamide could prevent the telophase reformation of the nuclear membrane. Mazia (1961) reported that mercaptoethanol is able to block the degradation of the nuclear membrane at prophase, without barring the condensation of chromosomes. Here DTT prevents neither the dissolution nor the reformation of the nuclear membrane, but does inhibit the fusion of the membrane-coated karyomeres in telophase.

The result of this inhibition is the production of cells with multiple membrane-bounded micronuclei, each of which is physically separated from the other. The chromosomal composition of the micronuclei is not yet certain but from the mechanism of action and the large number of micronuclei evidenced in interphase it seems highly likely that each micronucleus is identical with a chromosome.

If this be true, then a means for the controlled individualization of chromosomes during interphase is at hand. This approach could be useful for the study of the structure and activity of individual interphase chromosomes. It also opens avenues of research on interchromosomal information exchange.

The ready reversibility of the inhibition successfully dissociates the process of nuclear reconstruction from mitosis, permitting experimental control of the fusion process. It is demonstrated that fusion of the micronuclei proceeds stepwise and is completed in a matter of a few minutes. These results are in substantial agreement with those of Aronson (1973) who blocked pronuclear fusion in sea urchins with Colcemid. Upon release of the block, pronuclear fusion took place rapidly, often within 2-4 min.

Interestingly, pronuclear fusion is not arrested by DTT. (It is not known whether Colcemid prevents karyomere fusion in telophase.) Unless the fusion process itself is different, an unlikely possibility, the differential effect of the DTT is most easily seen as reflecting a difference in com-

position of pronuclear membranes and karyomere membranes. The possibility should not be overlooked that molecular restructuring of the nuclear membrane occurs during early embryogenesis. Indeed, because the nuclear membrane can restrict what enters and leaves the nucleus, small changes in the structure of the membrane could have profound influence on the course of development.

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