AN INDIRECT METHOD TO ASSAY FOR MITOTIC CENTERS IN SAND DOLLAR (*DENDRASTER EXCENTRICUS*) EGGS

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ABSTRACT

It is possible consistently to induce sea urchin and sand dollar eggs to cleave directly from one cell into four cells. This is done by exposing the fertilized eggs to benzimidazole for 20 to 30 min beginning about early metaphase. The mitotic apparatus regresses, the cells do not cleave, and shortly after they are returned to normal sea water an early-prophase–appearing nucleus is present in each cell. Each cell then organizes a tetrapolar tetrahedral mitotic apparatus de novo, instead of transforming a bipolar mitotic apparatus into a tetrapolar figure, and cleaves one-to-four. In another type of experiment, it appears that sand dollar eggs exposed to mercaptoethanol during the first period of mitotic center duplication have only half as many centers by first cleavage metaphase as the normal controls. This is consistent with an earlier report by Mazia et al (1960). Using this same experimental technique, it was demonstrated that benzimidazole, on the contrary, does not interfere with mitotic center duplication in sand dollar eggs. A labeling experiment demonstrated that benzimidazole does not interfere markedly with the normal pattern of incorporation of C^{14}-thymidine into the DNA of sea urchin eggs. The data reported here suggest that judicious treatment of sand dollar eggs (and probably sea urchin eggs, too) with benzimidazole can induce the eggs to cleave into as many cells as there were mitotic centers sometime earlier, for example at early metaphase of the first cleavage division. This provides a very useful tool for studies on the process of mitotic center duplication.

INTRODUCTION

The mitotic apparatus (Mazia and Dan, 1952) of animal cells frequently seem to be organized around discrete, functional entities located at the spindle poles. These are termed mitotic centers (Mazia, Harris, and Bibring, 1960) and very likely are equivalent to centrioles.

Mazia, Harris, and Bibring (1960) suggest that a pair of mitotic centers exists at each pole of a normal, bipolar first cleavage mitotic apparatus in sea urchins and sand dollars. This postulated functional duplicity of mitotic centers is consistent with the structural picture determined by light and electron microscopy. Many investigators have described paired, full-sized centrioles in one spindle pole (Boveri, 1901; Huettner, 1933; Nagano, 1959; Schreiner and Schreiner, 1905; Griffin, 1896; Bělař, 1929; Gall, 1961; Szollosi, 1964).

The primary purpose of this paper is to report what seems to be a method, employing benzimidazole, that reliably permits each mitotic center present at a specific time in a cell to organize its own spindle pole some time later. Although mercaptoethanol can also do this (Mazia, Harris, and

Bibring, 1960), the results are much more unequivocal when benzimidazole is used. It is important to the success of the technique, to be described, that the mitotic centers have adequate time to become widely enough separated so that each single center can organize its own spindle pole, without allowing the cell to cleave before the separation is complete. The result is that, when this treatment is applied to a normal cell, a beautiful tetrahedral tetrapolar mitotic apparatus is constructed and the single cell cleaves directly into four cells.

A future publication will describe the effects of various chemicals on mitotic center duplication using the technique described herein to assay for mitotic centers.

MATERIALS AND METHODS

Obtaining Gametes

The sand dollars were induced to release their gametes by injecting 0.5-M KCl into the perivisceral coelom (Tyler, 1949).

Treatment with Benzimidazole and Mercaptoethanol

Benzimidazole (from Nutritional Biochemicals Corporation, Cleveland) was always used at a final concentration of 0.5 mg/ml of sea water. The mercaptoethanol (from Eastman Kodak Company, Rochester) was used at two different concentrations, depending upon the effect to be achieved: (*a*) it was used at 0.35 ml/100 ml sea water to inhibit mitotic center duplication without blocking the cell at metaphase or seriously retarding development through the first cleavage division; (*b*) when it was necessary to block the cell at metaphase, a concentration of 0.70 ml/100 ml sea water was used. These solutions were always prepared the same day they were used, and the unadjusted pH was that of normal sea water. The eggs were always fertilized in normal sea water.

The cells were incubated in about 8 to 10 ml of medium in covered Syracuse dishes at room temperature (16–19°C). The living eggs were observed directly, in the Syracuse dishes, with the 10× objective of a Tiyoda binocular, in order to determine the mitotic stages and the appearance of the mitotic apparatus (whether bipolar or tetrapolar).

The experiments with living sand dollar eggs were performed during the months of July and August, in 1964 and 1965, in the aquarium room of the Agassiz Laboratory at the Hopkins Marine Station, Pacific Grove, California.

Benzimidazole-Induced One-to-Four Cleavage in Normal Sand Dollar and Sea Urchin Eggs

The eggs were fertilized and allowed to develop normally to prometaphase or early metaphase of the first cleavage division. Then they were transferred to sea water containing benzimidazole. The sand dollars were left for 20 to 25 min, and the sea urchins for 25 to 30 min; the cells were then returned to normal sea water. In the case of the sand dollars, tetrapolar tetrahedral mitotic apparatus appeared and the cells divided one-to-four 25 to 45 min later (see Fig. 1). The cells were observed intermittently from the time they were removed from the benzimidazole–sea water until tetrapolar mitotic apparatus were clearly evident in most of the cells. In sea urchins, one-to-four division occurred about 1 hr after removing the benzimidazole.

In all cases, the *initiation* of exposure to any chemical agent was primarily dictated by the stage of development and only secondarily by the clock time. The *duration* of exposure was determined by elapsed time and seemed to be less critical than the onset of the exposure period.

Effect of Benzimidazole on C^{14}-Thymidine Incorporation into DNA

(Only one experiment was performed using the sea urchin *Strongylocentrotus purpuratus*.) Eggs from a single urchin (late in the reproductive season) were suspended in about 400 ml of sea water, inseminated, and incubated at 15°C with continuous agitation. There was essentially 100% fertilization. Ninety-two min following insemination, the suspension was carefully divided into two equal aliquots: one was resuspended in normal sea water and the other in sea water with 0.5 ml/mg benzimidazole (see Fig. 5). Seven min later, C^{14}-thymidine was added to both suspensions to give a final level of activity of 0.1 μc./ml. Two series of 1.0-ml aliquots were removed at 4- or 5-min intervals from each cell suspension and fixed in Carnoy's (3 volumes absolute ethanol and 1 volume glacial acetic acid). One series of cells was used for cytological examination. The other series of cells was assayed by the method described by Hinegardner, Rao, and Feldman (1964) for the incorporation of C^{14}-thymidine into DNA, except that the cells were plated on planchets and counted in a Nuclear-Chicago (Desplaines, Illinois) counting system with automatic sample changer equipped with a model D47 gas flow detector using "Q" gas, rather than in a liquid scintillation counter. Thus, the cells were plated and dried without ever being dissolved in hydroxide of hyamine.

The cells for cytological examination were transferred to 45% acetic acid (Mazia, Harris, and Bibring 1960), sealed vaseline mounts were prepared, and examined under phase contrast. Vaseline-sealed

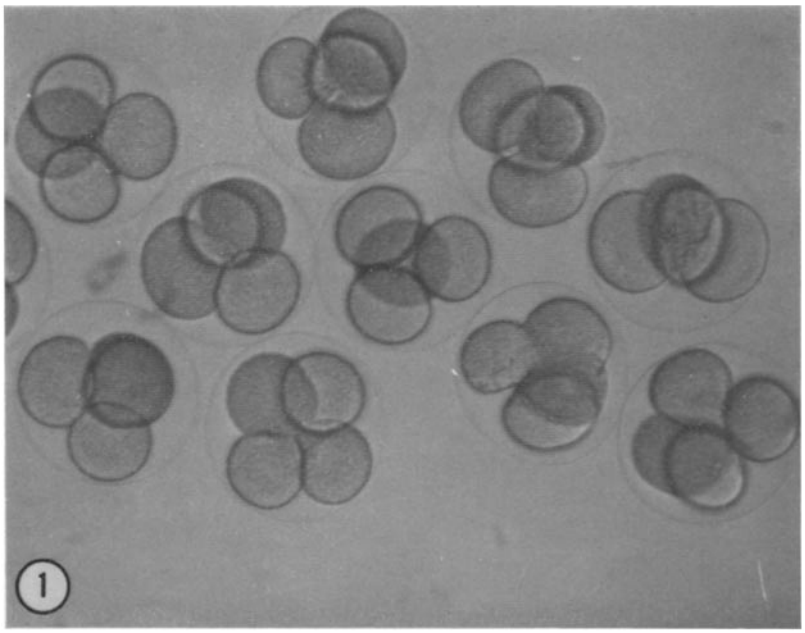

FIGURE 1 Most of these sand dollar eggs had just completed a benzimidazole-induced one-to-four cleavage. Notice the tetrahedral orientation of the blastomeres, reflecting the tetrahedral configuration of the tetrapolar mitotic apparatus. Although the blastomeres appear to be only loosely associated, the functional relationship among them was intimate since one rarely observed the production of twin, triplet, or quadruplet blastulae within a common fertilization membrane. Incidental to the manipulations experienced by these embryos the jelly layer had been removed. Approximately × 200.

mounts were necessary to prevent evaporation of the 45% acetic acid medium and to avoid any flattening of the cells. This technique was used whenever it was necessary to make cytological observations on cells.

RESULTS

Benzimidazole-Induced One-to-Four Cleavage in Normal Sand Dollar Eggs

Cytological observations on fixed material (both sand dollar and sea urchin) revealed that, during the course of exposure to the benzimidazole, the mitotic apparatus regressed and the chromosomes gradually became less distinct. This continued after the return to normal sea water until a single, early prophaselike nucleus was formed. The cells had not cleaved. Then during the following 15 to 35 min each cell organized a single tetrapolar tetrahedral mitotic apparatus and cleaved one-to-four (*see* line *1*, Fig. 3). These cells were never seen to attempt an abortive one-to-two cleavage at any time prior to the direct one-to-four cleavage.

This artificially induced one-to-four cleavage is not unique to the first cleavage division. If the benzimidazole treatment was delayed until prometaphase of the second cleavage division, one could equally easily induce the analogous two-to-eight cleavage (*see* line *2* Fig. 3, and Fig. 2).

If cells were exposed to two successive 20- to 25-min treatments with benzimidazole, with the first exposure beginning at early first cleavage metaphase and the second exposure beginning when tetrapolar tetrahedral mitotic apparatus were clearly evident, it was possible to obtain a cleavage pattern approaching one-to-eight. That is, some cells actually cleaved one-to-eight; but most cleaved one-to-six or one-to-seven (*see* line *3*, Fig. 3).

The experiments of lines *2* and *3* (Fig. 3) have not been attempted on sea urchin eggs.

Not infrequently it had been observed that considerable numbers of cells, following benzimidazole treatment during the first cleavage mitosis, developed two separate mitotic apparatus and then cleaved one-to-four. In all instances, this could be traced to the tardy onset of treatment with ben-

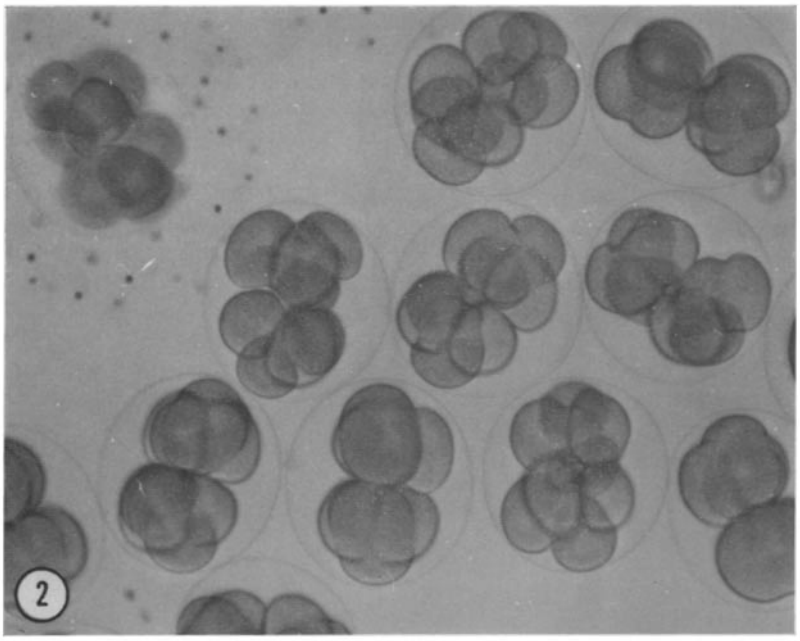

FIGURE 2 This figure shows a population of sand dollar eggs that underwent a normal first cleavage before being exposed to benzimidazole for about 20 min beginning at about prometaphase of the second cleavage division. All of the two-celled stages shown were cleaving or had successfully cleaved two-to-eight. In the upper left hand corner can be seen an embryo with an intact jelly layer containing many conspicuous pigment granules. Approximately × 200.

zimidazole. These cells completed karyokinesis during and following benzimidazole treatment, but cytokinesis was suppressed, yielding a binucleate cell.

Effect of Mercaptoethanol and Benzimidazole on Mitotic Center Duplication

MERCAPTOETHANOL: The experiments performed by Mazia et al. (1960), to show that mercaptoethanol suppressed mitotic center duplication but did not interfere with the separation of the duplicated centers, were repeated with essentially the same results.

However, another experiment provided independent evidence that mercaptoethanol can interfere with the duplication of mitotic centers. Two treatment schedules (lines *4* and *5* in Fig. 3) were examined. In line *4* the cells were exposed to mercaptoethanol (0.35 ml/100 ml sea water) beginning 5 min after insemination and continuing until prometaphase. They were then treated with benzimidazole and returned to normal sea water. Thirty-six min later, 75% of the cells had cleaved and all of these had divided one-to-two. These cells were exposed to the mercaptoethanol during most of the 1st period of mitotic center duplication and *no one-to-four cleavages were ever observed among them.* The treatment schedule for the cells in line *5* differed from that of those in line *4* only in the onset of exposure to mercaptoethanol, such that the 1st period of mitotic center duplication was not included. These cells cleaved one-to-four, and at no time did they attempt to cleave one-to-two.

BENZIMIDAZOLE: The effect of this agent upon center duplication was examined by exposing one batch of cells to benzimidazole for most of the 1st period of mitotic center duplication (line *1*, Fig. 4), and another batch of cells to this agent for the period which did not include the 1st period of mitotic center duplication (line *2*, Fig. 4). In both cases, the cells were transferred directly from the benzimidazole into the mercaptoethanol, in which they remained for 69 min. Fifteen min following removal from the mercaptoethanol to normal sea water, about 50% of the cells in *each* culture contained tetrapolar mitotic apparatus. That is, the two batches of cells behaved similarly.

Effect of Benzimidazole upon C^{14}-Thymidine Incorporation into DNA

This experiment was performed only with sea urchin (*Strongylocentrotus purpuratus*) eggs. The results of this experiment are graphed in Fig. 5. During the first 25 min of exposure to the C^{14}-thymidine, the pattern of incorporation into the perchloric acid–insoluble fraction was identical in the control and benzimidazole-treated cells; but thereafter the level of incorporation into the experimental cells was consistently about 15% above that of the controls. At 118 min following fertilization, chromosomes were clearly visible in the treated cells but no mitotic apparatus could be seen, while the control cells contained normal-looking metaphase and anaphase stages. However, by 124 min, neither chromosomes nor nuclei could be seen in the treated cells, while most of the control embryos appeared to be two-celled, although a few cells with condensed chromosomes were present. By 159 min, late prophase-to-prometa-

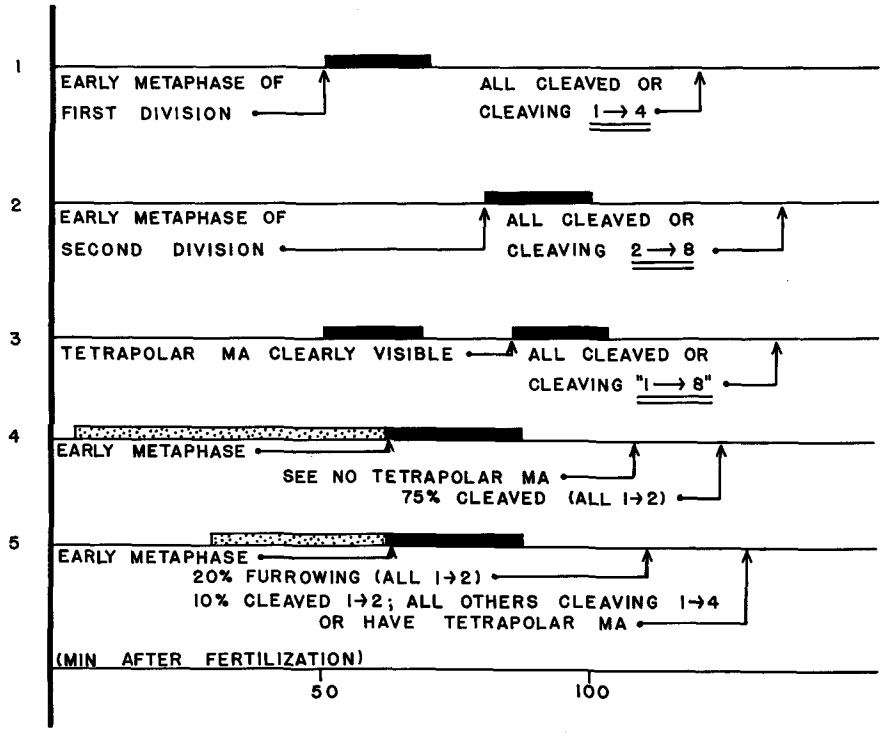

FIGURE 3 This figure shows various treatment schedules and the results obtained with sand dollar eggs. Line *1* shows the treatment schedule for benzimidazole-induced one-to-four cleavage. The 1st period of mitotic center duplication was from just after fertilization to about 25 min postfertilization, and the 2nd period of mitotic center duplication extended from about first cleavage telophase into the following interphase. Line *2* shows that a two-to-eight cleavage could be obtained when two periods of center duplication preceded the benzimidazole treatment. Line *3* shows that, when fertilized eggs were exposed to two successive exposures of benzimidazole in such a manner that no cleavage occurred before the end of the second treatment, they would subsequently cleave directly into six, seven, or even eight cells. Lines *4* and *5* show that mercaptoethanol (0.35 ml/100 ml sea water) inhibited mitotic center duplication. Cells exposed to mercaptoethanol during most of the 1st period of center duplication (line *4*), cleaved one-to-two; *no* one-to-four cleavages were observed. When the 1st period of center duplication was not included (line *5*), 80% of the cells cleaved directly one-to-four without first ever attempting a one-to-two cleavage. The bottom line gives the time scale in minutes after fertilization (vertical black line). The *solid black bars* identify the periods of exposure to benzimidazole; the *stippled bars* identify the periods of exposure to mercaptoethanol.

phase tetrapolar mitotic figures could be seen in the benzimidazole-treated cells.

DISCUSSION

In order to achieve the direct one-to-four cleavage of cells, it was necessary to give the existing mitotic centers enough time to separate. Mercaptoethanol blocks karyokinesis at metaphase, thereby giving the centers time to separate. Benzimidazole does this by inducing regression of the mitotic apparatus, thereby postponing cytokinesis. When both sand dollar and sea urchin eggs were treated with benzimidazole in accordance with the basic schedule shown in line *1* of Fig. 3, the invariable result was a direct one-to-four cleavage by the cells.

To demonstrate that one-to-four cleavage was not unique to the 1st cleavage division, the benzimidazole treatment was delayed in other experiments until the corresponding time interval of the 2nd cleavage division, with the result that the two-celled stages cleaved two-to-eight. These results clearly suggested that *each cell was cleaving directly into as many blastomeres as there had been mitotic centers present at the onset of benzimidazole treatment.*

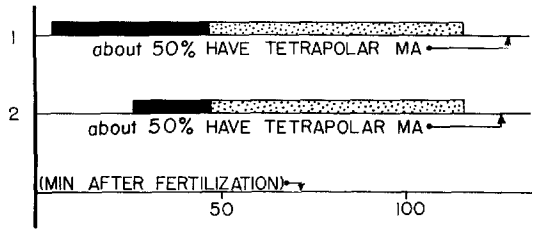

FIGURE 4 Here is shown the treatment schedule of an experiment which indicates that benzimidazole did not inhibit mitotic center reproduction in sand dollar eggs. In line *1*, the period of exposure to benzimidazole included most of the 1st period of center duplication while none of it was included in the benzimidazole exposure in the exposure schedule of line *2*. In spite of this, about the same frequency of one-to-four cleavages was observed in each culture. If benzimidazole had interfered with mitotic center duplication, *no* one-to-four cleavages at all should have occurred in the culture treated according to the schedule of line *1*. The *solid black bars* identify the period of exposure to benzimidazole; the *stippled bars* identify the period of exposure to mercaptoethanol (0.70 ml/100 ml sea water). The bottom line gives the time scale in minutes after fertilization (vertical black line).

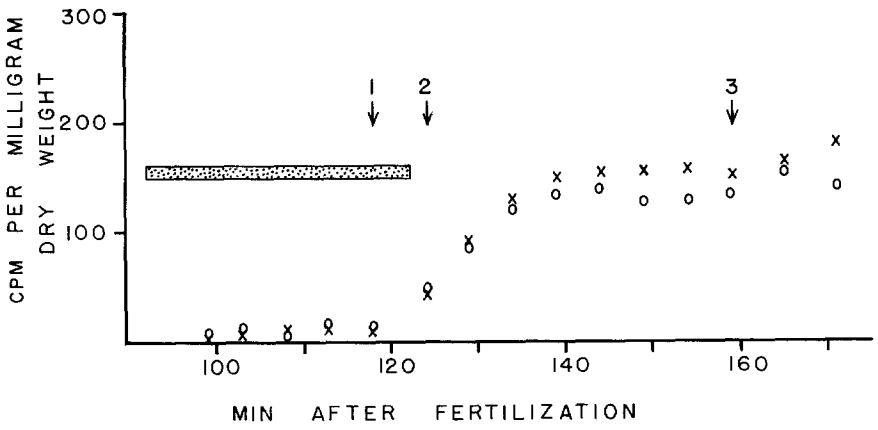

FIGURE 5 This figure shows the time course of C^{14}-thymidine (0.1 μc/ml of egg suspension) incorporation, expressed in counts per minute per milligram air-dried weight, into the DNA of benzimidazole-treated sea urchin eggs (*X*) and untreated control eggs (*O*) continuously exposed to the labeled DNA precursor beginning 97 min after fertilization. The *stippled bar* indicates the duration of treatment with benzimidazole (0.5 mg/ml of egg suspension). Arrow *1* (118 min) identifies the time of the last sample, before the onset of rapid incorporation of C^{14}-thymidine, in which chromosomes were clearly visible in *both* the control cells and the benzimidazole-treated cells. At the time indicated by arrow *2* (124 min), chromosomes could not be seen in either group of cells. Arrow *3* (159 min) identifies the time when tetrapolar prometaphase was visible in the benzimidazole-treated cells. By 171 min the control cells were in second cleavage prophase, and at 177 min metaphase to four-celled stages were visible. At this time (177 min) the benzimidazole-treated cells had tetrapolar mitotic apparatus in anaphase.

This was tested by subjecting the cells to two periods of benzimidazole treatment according to the time schedule of line *3*, Fig. 3. The cells had not cleaved by the end of the 2nd period of benzimidazole treatment. The prediction was that these uncleaved cells would contain eight mitotic centers and, therefore, should cleave one-to-eight. This prediction was essentially substantiated, in that many cells cleaved one-to-six, some one-to-seven, and a few one-to-eight. The fact that only a few cells cleaved one-to-eight was ascribed to inadequate spatial separation of all eight spindle poles, with the result that two poles were frequently incorporated into a single cell.

Mazia, Harris, and Bibring (1960) present evidence that mercaptoethanol inhibits mitotic center duplication. Thus, exposure to mercaptoethanol during the 1st period of center duplication (the first 20 to 30 min following fertilization) should produce cells that have only two mitotic centers instead of the normal number of four. If benzimidazole treatment does, indeed, cause the cells to cleave into as many blastomeres as there were mitotic centers at an earlier time, these cells should cleave one-to-two following treatment with benzimidazole. It can be seen from line *4*, Fig. 3, that this occurs. This one-to-two cleavage is superficially indistinguishable from that of normal, untreated eggs. Line *5* of the same figure is the control to line *4* and shows that, when the 1st period of center duplication is not included in the exposure period to the mercaptoethanol, the preponderance of the cells cleave one-to-four, indicating that they had the normal number of mitotic centers.

So far, the data clearly suggest that benzimidazole will induce a cell to cleave into as many blastomeres as there had been mitotic centers present at the onset of the benzimidazole treatment, but tell us little about what effect benzimidazole has on the mitotic centers themselves. To learn something more about this, it was necessary to design an experiment that did not depend upon benzimidazole to assay for mitotic centers. Mercaptoethanol was selected, for it was already known (Mazia et al., 1960) that this did not prevent the separation of mitotic centers, although it clearly inhibited their duplication. Thus, in the experiment designed to examine whether or not benzimidazole interfered with center duplication (see Fig. 4), mercaptoethanol was used operationally to induce the cells to cleave into as many blastomeres as there had been centers present at the beginning of the mercaptoethanol treatment. Following exposure to benzimidazole for an interval including the 1st period of center duplication (line *1*, Fig. 4), the frequency of cells containing tetrapolar mitotic apparatus was the same as that observed in cells not exposed to benzimidazole during the 1st period of center duplication. This implies that benzimidazole does not prevent the reproduction of mitotic centers.

From this, one can postulate that at each spindle pole of a benzimidazole-induced tetrapolar mitotic apparatus there is a *pair* of potential mitotic centers, while each pole of a mercaptoethanol-induced tetrapolar mitotic apparatus should contain a *single* mitotic center.

Bucher and Mazia (1960) demonstrated that during the mercaptoethanol-imposed metaphase block in sea urchin eggs no H^3-thymidine was incorporated into the DNA. It should be recalled that the chromosomes remained condensed during the mercaptoethanol-imposed block. Thus, in mercaptoethanol-treated cells, the chromosomes never decondensed between the onset of treatment with the mercaptoethanol and cleavage (either one-to-two or one-to-four, depending upon the treatment time schedule). On the other hand, chromosome decondensation does occur between the onset of treatment with benzimidazole and cleavage. From this cytological observation, one could predict that benzimidazole might not interfere with the incorporation of C^{14}-thymidine into the DNA of the treated cells. The results plotted in Fig. 5 support this prediction. Although these are the data of one experiment, performed upon cells developing abnormally slowly, the internally consistent pattern of incorporation, the good agreement in *onset* and *duration* of incorporation with the data of Hinegardner, Rao, and Feldman (1964), and the expected one-to-four division of the benzimidazole-treated cells led the author to conclude that the data are reliable.

Another significant fact is that the incidence of gastrulation was less than 1% among the blastulae which developed from eggs following a benzimidazole-induced one-to-four division. This is probably the consequence of the unequal distribution of the chromosomes at the time of the one-to-four division, and is supported by cursory cytological observations.

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BIBLIOGRAPHY

BĚLAŘ, K., 1929, Beitrage zur Kausalanalyse der Mitose. II. Untersuchungen an den Spermatocyten von *Chorthippus* (*Stenobothrus*) *lineatus* Panz, *Arch. Entwcklngsmechn. Org.*, **118**, 359.

BOVERI, T., 1901, Über die Natur der Centrosomen. Zellen-Studien, Jena, Gustav Fischer, **4**.

BUCHER, N. L. R., and MAZIA, D., 1960, DNA synthesis in relation to duplication of centers in dividing eggs of the sea urchin *Strongylocentrotus purpuratus*, *J. Biophysic. and Biochem. Cytol.*, **7**, 651.

GALL, J. G., 1961. Centriole duplication. A study of spermatogenesis in the snail *Viviparus*, *J. Biophysic. and Biochem. Cytol.*, **10**, 163.

GRIFFIN, B. B., 1896, The history of the archoplasmic structures in the maturation and fertilization of *Thalassema*, *Tr. New York Acad. Sc.*, **2**, 163.

HINEGARDNER, R. T., RAO, B., and FELDMAN, D. E., 1964, The DNA synthetic period during early development of the sea urchin egg, *Exp. Cell Research*, **36**, 53.

HUETTNER, A. F., 1933, Continuity of the centrioles in *Drosophila melanogaster*, *Z. Zellforsch.*, **19**, 119.

MAZIA, D., and DAN, K., 1952, The isolation and biochemical characterization of the mitotic apparatus of dividing cells, *Proc. Nat. Acad. Sc.*, **28**, 826.

MAZIA, D., HARRIS, P. J., and BIBRING, T., 1960, The multiplicity of the mitotic centers and the time-course of their duplication and separation, *J. Biophysic. and Biochem. Cytol.*, **7**, 1.

NAGANO, T., 1959, Spermatogenesis of the domestic fowl studied with the electron microscope, *Anat. Inst. Med. Fac. Okayama*, **164**, 311 (in Japanese).

SCHREINER, A., and SCHREINER, K. E., 1905, Über die Entwicklung der männlichen Geschlechtszellen von *Myxine glutinosa* (L.), *Arch Biol.*, **21**, 315.

SZOLLOSI, D., 1964, The structure and function of centrioles and their satellites in the jellyfish *Phialidium gregarium*, *J. Cell Biol.*, **21**, 465.

TYLER, A., 1949, A simple, non-injurious method for inducing repeated spawning of sea urchins and sand dollars, *Collecting Net*, **19**, 19.