This study was initiated to determine the concentration of magnesium and calcium in dividing eggs of the sea urchin Stronglyocentrotus purpuratus and to explore the relationship between these ions and mitotic events.

Samples of developing eggs, maintained at 15°C, were taken at ten-minute intervals from fertilization through the second cleavage, cytologically monitored, and analyzed for magnesium and calcium content. The analytical procedure was spectrophotometric, utilizing a modification of the method of Lamkin and Williams which employs Arsenazo \(= 0-[(1,8\text{-dihydroxy-3,6-disulfo-2-naphthyl})\text{-azo}]\) benzene arsonic acid and EGTA \([(\text{ethylene bis (oxyethylenetriolo})\text{tetraacetic acid})\].

Each division cycle was characterized by fluctuations in cellular content of magnesium and calcium which showed for each ion two peaks of uptake and release. The first magnesium and calcium uptakes occurred at very early
prophase and mid-prophase, respectively. These peaks were interpreted to be related to the gelation of the 3.5 S soluble protein component of the mitotic apparatus prior to the actual assembly of the apparatus.

The second magnesium and calcium uptakes occurred at meta-anaphase and anaphase-cleavage, respectively. These peaks were interpreted to be related to the gelation of the proteins associated with cleavage furrowing and the increase of blastomeric cell surface.

At fertilization, the base levels of magnesium and calcium rose to approximately twice the amounts characteristic of the unfertilized egg, i.e., from 11.0 to 22.2 mM for magnesium and 4.0 to 8.0 mM for calcium per Kg of water in the egg. At peak levels during mitosis, the magnesium and calcium concentrations reached 35.0 and 14.0 mM/Kg, respectively, approximately 1.5 times the post fertilization base levels.
Calcium and Magnesium Flux in Dividing Sea Urchin Eggs

by

Stephen John Kleinschuster

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Typed by Erma McClanathan for Stephen John Kleinschuster
ACKNOWLEDGMENTS

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Thanks are also due to Dr. A. Owczarzak and Dr. M. Williams for their advice regarding certain problems encountered in this study.
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CALCIUM AND MAGNESIUM FLUX
IN DIVIDING SEA URCHIN EGGS

INTRODUCTION

This study was initiated to explore the relationships between the divalent cations calcium and magnesium, and the mitotic events in developing sea urchin eggs.

For many years, divalent cations have been implicated in cellular and physiological phenomena. Among the earlier investigations were those of Heilbrunn (1921) who showed cyclic viscosity changes in developing eggs of Cumingia, Nereis and Arbacia. These consisted of a viscosity increase at prophase, a decrease at metaphase, and an increase at mitotic elongation. Later work showed similar changes in the eggs of Chaetopterus (Heilbrunn and Wilson, 1948). Heilbrunn (1956) suggested that these viscosity changes reflect the so-called mitotic gelation of the protoplasm preceding spindle formation. He held that this phenomenon was brought about by the migration of calcium from the cortex to the interior of the cell.

Mazia (1937) showed that in Arbacia eggs fertilization was followed by a 15% decrease in bound calcium with a significant increase in free calcium. The total cellular calcium remained constant. However, an actual loss of both calcium and magnesium following fertilization in
Arbacia was reported by Monroy-Oddo (1946).

Hultin (1950) showed that low concentrations of calcium added to sea urchin eggs which had been homogenized in calcium and magnesium free sea water cause a general gelation reaction. Concentrated homogenates under the influence of calcium became very viscous and elastic strands could be formed.

Kane and Hersh (1959) isolated a major soluble protein fraction from the eggs of Stronglyocentrotus and Arbacia which contained two ultracentrifugal components having sedimentation constants of 7 and 20 S. The addition of small amounts of divalent cation to this soluble fraction caused a gelation which could be reversed by dialyzing it against water or a mild salt solution. The redissolved solution contained only the 7 S component. Eggs of Stronglyocentrotus differed from those of Arbacia with respect to relative amounts of the 7 S component. Further, the 7 S component in Arbacia decreased during mitosis, showing a low level at metaphase. No such decrease was seen in Stronglyocentrotus.

Sakai (1960a), using 0.1M MgCl₂, isolated large quantities of egg cortices for the first time. From these cortices, Sakai (1960b) isolated a 0.6M KCl soluble contractile protein which later showed electron transfer activity of SH groups with a calcium insoluble protein (Sakai, 1965). Another instance of electron transfer was
also shown by Sakai (1966) between the 0.6M KCl fraction of the isolated cortices and a calcium insoluble protein of the mitotic apparatus. Yazaki (1968), as reported by Kane and Stephens (1969), divided the original Kane and Hersh protein, which she called Ca ppt. 1, into two fractions. One of these fractions, called the HyS protein, has properties which are chemically and physically similar to those of the Kane and Hersh protein. The other fraction, which she called Ca ppt. 2, is the protein which undergoes cyclic changes in oxidation via electron transfer activity of SH groups. Consequently, it is this fraction which is responsible for the SH activity in the calcium insoluble protein of the isolated mitotic apparatus and whole cell homogenates of Sakai.

Stephens and Kane (1966) found a calcium insoluble protein in cortices of unfertilized sea urchin eggs. Kane and Stephens (1969) showed this protein to be identical with the Kane and Hersh protein. However, the quantity and distribution of the protein throughout the egg was species specific and great variations were found between species. Yazaki's experiment also showed that an antibody of the HyS protein (Kane and Hersh) stained the periphery of the unfertilized eggs and the hyaline layer of the fertilized eggs. This implicated the cortical granules of the unfertilized eggs as a major source of the Kane and Hersh protein. Stephens and Kane (1970) have recently
succeeded in identifying the protein of the hyaline layer of the fertilized egg and have found that the major protein component is the calcium insoluble protein of Kane and Hersh.

Ohnishi (1962) isolated an actinomyosin-like protein from the cortex and cytoplasm of sea urchin eggs. The protein was soluble in 0.6M KCl and showed magnesium dependent ATPase activity. Miki (1963b) also studied ATPase activity of the 0.6M KCl fraction of the cortex upon fertilization and found ATPase activity increasing after fertilization, with a doubling of activity at metaphase and decreasing at cleavage. Weisenberg and Taylor (1968) found a 13 S magnesium and calcium dependent ATPase in both the cytoplasm and isolated mitotic apparatus of sea urchin eggs.

Dornfeld and Owczarzak (1958), investigating the response of cells on exposure to versene, were able to demonstrate certain relationships between divalent cations and mitotic events. Interphasic fibroblasts grown in vitro and exposed to versene respond immediately by contraction of their cellular processes and a rounding of the cells, followed by surface blebbing. These events are similar to those seen in normal fibroblasts as they enter metaphase and anaphase. This response was reversible by removal of the chelating agent. Contraction and blebbing could also be induced by isotonic solutions lacking calcium and
magnesium. Such a response was not seen in cells exposed to calcium and magnesium versenate or in cells which had entered metaphase prior to exposure to versene. Thus the contraction and blebbing phenomena were attributed to the removal of divalent cations from the cell cortex by the chelating agent. Cells after a two-hour exposure to versene eventually cease blebbing and remain in a rounded condition. This was attributed to the depletion of the divalent cation pool of the cell medulla as well as the cortex, as the interphasic form could be restored by the addition of calcium and magnesium. Dornfeld and Owczarzak hypothesized that similar events in normal dividing fibroblasts were induced by the translocation of divalent cations from the cell surface to the developing gel system of the mitotic apparatus. This interpretation would appear to correspond to Heilbrunn's gelation theory respecting ionic requirements of newly forming protein complexes.

Clothier (1961) showed three periods of calcium 45 uptake during the first mitotic division in sea urchin eggs. A definite relationship was shown to exist between the periods of uptake and specific mitotic events. The first peak of calcium 45 uptake occurred at mid-prophase and was related to gelation of spindle precursor. The second uptake, occurring at mid-metaphase, was related to gelation of interzonal spindle precursor. The third peak, at mid-cleavage, was related to the gelation of cortical
Borei and Björklund (1953), using centrifugation methods, studied the effects of versene on unfertilized sea urchin eggs and noted a decrease in viscosity in both cortical and medullary cytoplasm, which they attributed to the loss of divalent cation. Hanson (1968) investigated the effects of short versene exposures on dividing sea urchin eggs. It was noted that cleavage times were delayed by different amounts when versene was applied to the developing eggs at different ten-minute periods during the first mitotic division. Two periods showed maximum sensitivity to versene (maximum delay of cleavage). The first of these occurred at the streak stage and was related to gelation of the protein spindle of the achromatic figure. The second occurred at telophase and was associated with the formation of interzonal fibers and new cortical protein.

Robbins and Micali (1965) investigated the effects of hypotonic solutions and calcium solutions on dividing mammalian HeLa cells in tissue culture. Their experiment suggests that Heilbrunn's theory of calcium release from the cortex of the cell may be valid for the mammalian cell. However, instead of causing a simple gelation of endoplasm as stated by Heilbrunn, Robbins and Micali suggest that during mitosis, calcium replaces sodium or potassium ions on chromosome sites, thereby condensing the chromatin.

Robbins, Pederson, and Klein (1970), using hypertonic
calcium solutions, induced interphasic HeLa chromosomes to resemble prophase chromosomes. Whitfield and Youdale (1966) proposed that calcium accelerates mitosis by promoting chromosome coiling in irradiated populations of rat thymocytes. In a later experiment, Whitfield, Rixon, Perris, and Youdale (1969) showed that calcium increases mitotic activity by stimulating cells to enter the (S) phase of the mitotic cycle.

Tyler and Monroy (1959) have shown that only one-fifth of the total cellular potassium is exchangeable in the unfertilized sea urchin egg. However, after fertilization, three-fourth of it is readily exchangeable. This is a substantial amount of ion, since the egg contains almost 21 times more potassium than does sea water. The total cellular potassium has been found to change in a rhythmic fashion during the first cleavage (Monroy-Oddo and Esposito, 1951). In this experiment, the total cellular potassium rose for ten minutes after fertilization, then fell to 75% of the control levels after 40 minutes, then rose again to control levels until cleavage. The temperature of the experiment was not given, but the 40-minute level was said to correspond to the streak stage of development.

Rothschild and Barnes (1953) were critical of all measurements of ions in sea urchin eggs performed previous to their experiments. Their criticism of previous
investigations stems from the lack of consideration given interstitial sea water surrounding the eggs when measurements and volumes were being calculated. Previous determinations of the same ion, magnesium, for example, have varied by as much as 6,100%. These authors proposed a method for eliminating this error, although they still relied on gravimetric methods of calcium and magnesium analysis.

The present study was undertaken to determine, by improved methods, the calcium and magnesium levels in dividing sea urchin eggs at specific times in the mitotic cycle. It was hoped that such a quantitation would be heuristically useful in determining the role of these ions in cell division and that the information obtained might clarify previous findings in this area.
METHODS AND MATERIALS

The Pacific Coast Purple Sea Urchin, *Stronglyocentrotus purpuratus*, was used in this study. All specimens were collected at Yaquina Head near Newport, Oregon, and kept in a closed sea water system maintained by the Zoology Department of Oregon State University.

Sea water for experimental use was obtained from the Marine Science Center of Oregon State University and stored at 15°C. Prior to use, the sea water was filtered, aerated, and the pH adjusted to 7.9. The density of the sea water was determined volumetrically. Calcium and magnesium levels were also determined at this time by the method of Lamkin and Williams (1965), as described below.

All experiments, with one exception at 14.5°C, were carried out at 15°C. Constant temperature of developing egg suspensions was maintained by use of a water bath equipped with a refrigeration unit and a thermostatically controlled heater. Two stirring units were used to promote adequate circulation.

For cytological monitoring, eggs were fixed in Schaudinn's fluid which contains 66 parts by volume of cold saturated mercuric bichloride, 33 parts 95% ethyl alcohol, and one part glacial acetic acid. This fixative, when used in a 10:1 sample (eggs plus sea water) to fixative ratio, caused no deformation of the eggs and allowed
the nuclei, asters, spindles, and cleavage furrows to be microscopically examined without sectioning or staining.

Mitotic stages were determined cytologically by noting their percentages in a sample of 100 eggs from one ten-minute period. These percentages were then compared with those of the preceding and following ten-minute periods. From these comparisons, extrapolations showing the beginning of any particular stage, the appearance and disappearance of the 50% level of that stage, and the end of that stage was possible. Such an analysis gives a relatively accurate timed sequence of mitotic events.

Collection of Gametes

Shedding of eggs was induced by the method of Tyler (1949). Approximately 2 ml of 0.53 molar potassium chloride were injected hypodermically through the peristomal membrane into the coelomic cavity. The animals were placed aboral side down over a beaker filled with filtered sea water at 15°C and allowed to shed their eggs. When the shedding was complete, the eggs were passed through bolting silk and washed several times. This effectively removed and eliminated the jelly layers.

Shedding of sperm was induced by the same method. However, sperm was collected "dry" in a Syracuse watch glass and stored at 8°C until use.
Fertilization and Culture of Eggs

After washing, the eggs from one gravid sea urchin were transferred to a two-liter beaker containing one liter of sea water at 15°C, where they were allowed to "round out" and equilibrate for 30 minutes. The eggs were then fertilized by adding 10 ml of a dilute sperm suspension (five drops of dry sperm in 10 ml of sea water) and mixing well. Only those batches of eggs showing 99% activation, determined microscopically, were used for experimentation. After fertilization, the eggs were allowed to settle and the supernatant sea water, along with most of the sperm, was decanted. The eggs were then transferred to a battery jar containing at least 2.5 liters of filtered sea water maintained at a constant temperature of 15°C by immersion in a water bath. The sea water was continuously stirred to keep the eggs uniformly suspended.

Sampling and Quantitation of Eggs

At ten-minute intervals 100 ml aliquants were taken from the uniformly distributed egg suspension with a 100 ml pipette. Of each aliquant, 2 ml were immediately fixed with Schaudinn's fluid for cytological monitoring and egg counting. The remaining 98 ml, to be used in calcium and magnesium determinations, were transferred to a 100 ml Goetz centrifuge tube and centrifuged for 30 seconds at
Following centrifugation, which loosely packed the eggs, the supernatant was drawn off by aspiration. The centrifuge tube plus the compacted eggs were then weighed on a Mettler model H16 balance. The weight of the eggs plus the interstitial sea water was calculated by subtracting the weight of the empty tube. The compacted eggs were then lysed by addition of demineralized water, and the entire content of the tube was carefully washed into a sample vial and frozen for later analysis.

The total volume of eggs in the sample (x in the equation of Rothschild and Barnes (1953), described below) was found by the method of Shapiro (1935). In this method, the mean diameter of 25 eggs from the 2 ml of fixed egg suspension was used to determine the volume (\(\bar{v}\)) of an average egg. The remainder of the egg suspension, containing approximately 15,000 to 25,000 eggs depending on the batch, was used to estimate the number of eggs (\(\bar{n}\)) in the 98 ml sample. This was accomplished by placing the 2 ml of fixed eggs in a graduate cylinder and making a convenient dilution (A). After inverting the cylinder several times to suspend the eggs uniformly, a 0.2 ml capillary tube was quickly lowered and withdrawn from the cylinder. The eggs in the capillary were counted with a dissecting microscope. This operation was repeated until a reliable average, determined by the standard deviation, was obtained. This average was extrapolated to the number of eggs in the
98 ml sample by multiplying by the dilution factor (A). Thus the total egg volume in the sample to be analyzed is equal to Ax, where x = nν.

The final volume of eggs plus interstitial sea water in the sample to be chemically analyzed was calculated. The weight or mass of eggs in the sample was found by multiplying their density, obtained from Harvey (1956), by their volume. This weight, subtracted from the total weight of the eggs and interstitial sea water, provided the weight of the interstitial sea water. By use of the formula \( D = \frac{M}{V} \), the volume of the sea water was calculated, which, when added to the egg volume, gave the final volume of the egg suspension (\( V_F \) in the equation below).

**Analytical Techniques for Calcium and Magnesium**

The concentration of calcium and magnesium in the eggs was calculated by the formula of Rothschild and Barnes:

\[
(1) \quad Ca_F = Ca_{SW} (V_F - Ax) + ca Ax,
\]

where:

- \( V_F \) = the final volume of the egg suspension (egg volume plus interstitial sea water volume)
- \( Ca_F \) = the amount of calcium (or magnesium, \( Mg_F \)) in \( V_F \)
- \( Ca_{SW} \) = the concentration of calcium in the sea water
ca = the unknown concentration of calcium in the egg

Ax = the volume of the eggs in the sample being analyzed

solving for ca:

\[
ca = \frac{Ca_F - Ca_{SW} V_F}{Ax} + Ca_{SW}
\]

The analytical procedure for calcium and magnesium was that of Lamkin and Williams, modified to use blood sera with known calcium and magnesium concentrations as standards. One ml of normal blood serum (Lab-Trol, obtained from Dade Reagents, Inc.) was pipetted into a 100 ml volumetric flask. To this was added 10 ml of pH 9.6 glycine buffer solution and 15 ml of the acidic form of Arsenazo \([\text{0-}[(1,8\text{-dihydroxy-3,6-disulfo-2-naphthyl})\text{-azo}]\text{ benzene arsenic acid}].\) This solution was diluted to volume with demineralized water and the absorbance at 580 m\(\mu\) was read against a blank \((b_1)\) prepared by the same procedure less calcium reagent. The reading obtained is the absorbance for calcium plus magnesium.

Fifty ml of the above solution were pipetted into another 100 ml volumetric flask containing 5 ml of EGTA \([\text{ethylene bis (oxyethylene nitrilo)}\text{tetraacetic acid} ],\) and diluted to volume. This solution was used against a blank \((b_2)\) prepared by taking 50 ml of the previous blank \((b_1)\), adding 5 ml of EGTA, and diluting to volume. The absorbance reading is for magnesium alone.
Using the above procedure with normal blood serum samples which have known concentrations of calcium and magnesium, it is possible by means of Beer's Law to calculate the constants $K_1$ for the combined calcium and magnesium in the sample and $K_2$ for magnesium alone. Using Beer's Law and solving for $K$:

$$K_1 = \frac{c_1}{A_1} \quad \text{or} \quad K_2 = \frac{c_2}{A_2}$$

where

- $c_1 =$ the concentration of calcium plus magnesium
- $A_1 =$ the absorbance of calcium in a 1 cm cell
- $c_2 =$ the concentration of magnesium
- $A_2 =$ the absorbance of magnesium in a 4 cm cell

This method was tried experimentally using pathological blood serum (Path-Trol, from Dade Reagents, Inc.), with known concentrations of calcium and magnesium. In all cases the calculated results were well within the tolerances supplied by the manufacturer.

The egg samples were treated in a similar manner as the blood serum. After thawing, each sample and its washings were placed in a beaker and ten drops of 20% trichloroacetic acid were added. When the protein had precipitated, the solution was transferred to a 100 ml Goetz centrifuge tube, diluted to approximately 70 ml, and centrifuged at 2000 XG for three minutes. After centrifugation and decantation into a beaker, the precipitated
protein was washed twice with a TCA acidified solution. These washings were then added to the original supernatant. The pH of this solution was adjusted to that of the original sea water with one normal sodium hydroxide, transferred to a 100 ml volumetric flask and diluted to volume. After thorough mixing, 10 ml of this solution was pipetted into a 100 ml volumetric flask and used to determine the absorbances $A_1$ and $A_2$ of the sample. The values were multiplied by the constants $K_1$ and $K_2$, respectively, in order to obtain $c_1$ and $c_2$. Thus $c_2$ is the concentration of magnesium in the sample and the difference between $c_1$ and $c_2$ is the concentration of calcium in the sample.

As in most studies in which quantitations are made, precisions of measurement are subject to error. In the procedures used in this study, the largest possibility of error lies in the estimation of egg numbers ($\bar{n}$) from which the total egg volume ($x$) is calculated. Shapiro (1935) found that cell volumes obtained by his dilution method were within approximately 5-6% of the volumes obtained by the centrifuge method. Harvey (1956) considered the dilution method as the best of three methods she reviewed.

Error could also be introduced in the weighings of the samples and in the analytical techniques. However, the precisions of the instruments employed should limit the combined errors to 1-2%.
Absorbances were measured on a Beckman Du-2 spectrophotometer in matched 1 cm silica cells. A Beckman Expandomatic pH meter was used for all pH measurements, and class A pipettes were used for all pipetting.
RESULTS

Samples of developing sea urchin eggs were chemically analyzed for their calcium and magnesium content at ten-minute intervals. The results of four experimental and two control series (unfertilized eggs) are shown in Table 1 for calcium and Table 2 for magnesium. Cursory inspection shows that fertilization is followed by immediate elevation of both calcium and magnesium content. The cell division cycle is further characterized by additional uptake and release of these ions, in accordance with rhythmic patterns which show peaks at two specific periods in the cycle. The occurrence of these peaks with respect to time in the division cycle is shown in Tables 3 and 4.

The total concentration of each ion in the cells, as related to time and stage of development, is seen in Figures 1, 2, 3, and 4. The ionic levels in these graphs are expressed as millimoles of ion per kilogram of water in the eggs. Figures 1 and 2 show the fluctuations of calcium and magnesium, respectively, for eggs maintained at 15°C. The base lines of the experimental series of each ion is approximately double the base line of the control series (Figures 1 and 2). The concentration of calcium in the first and second peaks is very similar in series A, B, and C, maintained at 15°C (Figure 1). This is also true for the magnesium peaks of series A, B, and C (Figure 2).
Both calcium and magnesium peaks of experimental series A, B, and C are approximately 3.5 times the level of the base line of the controls (Figures 1 and 2). No appreciable fluctuation in ionic content is seen in the control series (Figures 1 and 2). In Series D (Figures 3 and 4), maintained at 14.5°C, the peaks of calcium and magnesium are slightly higher and are reached five to ten minutes later than series A, B, and C (Figures 1 and 2).

The calcium and magnesium peaks could be consistently correlated with specific cytological stages of the division cycle. Thus, in the experimental series A, B, and C, maintained at 15°C, the first calcium peak occurred at 60 minutes. This period corresponded cytologically to mid-prophase (Figure 1) which was recognized by the formation of the asters and the beginning of nuclear membrane breakdown (Figure 5a).

The second calcium peak occurred at 110 minutes. This period cytologically corresponded to very late anaphase and early cleavage (Figure 1). Late anaphase was identified by the elongated spindle and early cleavage by the appearance of the cleavage furrow (Figure 5b).

The first magnesium peak occurred at 40 minutes and corresponded cytologically to early prophase (Figure 2). This period was characterized by an enlarged prophase nucleus which was slightly elliptical in shape (Figure 5c). No spindle or asters were present at this stage.
The second magnesium peak occurred at 90 minutes and corresponded cytologically to late metaphase and early anaphase (Figure 2). This period was characterized by a fully developed spindle which was beginning to elongate (Figure 5d).

Similar cyclic uptake of both calcium and magnesium was seen in the second cleavage (Figures 1, 2, 3, and 4). However, since this division was much more accelerated than the first, the ten-minute intervals of sampling did not provide the resolution needed for satisfactory analysis.
Table 1. Calcium Levels in Dividing Sea Urchin Eggs.
(mg/liter eggs)

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Table 2. Magnesium Levels in Dividing Sea Urchin Eggs.  
(mg/liter eggs)

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Table 3. Peaks of Calcium Uptake in Relation to Temperature and Time after Fertilization.

<table>
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<th>Series</th>
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<th>Peak 2</th>
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<td>Series A</td>
<td>15°C</td>
<td>60 minutes</td>
<td>110 minutes</td>
</tr>
<tr>
<td>Series B</td>
<td>15°C</td>
<td>60</td>
<td>110</td>
</tr>
<tr>
<td>Series C</td>
<td>15°C</td>
<td>60</td>
<td>110</td>
</tr>
<tr>
<td>Series D</td>
<td>14.5°C</td>
<td>70</td>
<td>120</td>
</tr>
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</table>

Table 4. Peaks of Magnesium Uptake in Relation to Temperature and Time after Fertilization.

<table>
<thead>
<tr>
<th>Series</th>
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<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
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<tr>
<td>Series A</td>
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<td>90 minutes</td>
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<td>Series B</td>
<td>15°C</td>
<td>40</td>
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<tr>
<td>Series C</td>
<td>15°C</td>
<td>40</td>
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<tr>
<td>Series D</td>
<td>14.5°C</td>
<td>50</td>
<td>90</td>
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Figure 1. Calcium Levels During the First Two Division Cycles (15°C)
Figure 2. Magnesium Levels During the First Two Division Cycles (15°C)
Figure 3. Calcium Levels During the First Two Division Cycles (14.5°C)
Figure 4. Magnesium Levels During the First Two Division Cycles (14.5°C)
EXPLANATION OF FIGURE 5

Mitotic stages of cytologically fixed eggs:

<table>
<thead>
<tr>
<th>Figure reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a Mid-Prophase; 1st Division</td>
<td>the nuclear membrane is breaking down at the poles where early aster formation can be seen.</td>
</tr>
<tr>
<td>5b Late Anaphase-Cleavage; 1st Division</td>
<td>the interzonal spindle is elongated and the cleavage furrow is evident.</td>
</tr>
<tr>
<td>5c Early Prophase; 1st Division</td>
<td>an enlarged, elliptical nucleus is present with no evidence of spindle or asters.</td>
</tr>
<tr>
<td>5d Metaphase-Anaphase; 1st Division</td>
<td>a fully developed spindle apparatus which is beginning to elongate is evident.</td>
</tr>
<tr>
<td>5e Anaphase; 2nd Division</td>
<td>a fully developed elongated spindle apparatus with cleavage furrow is evident.</td>
</tr>
<tr>
<td>5f Prophase; 2nd Division</td>
<td>an enlarged nucleus and cleavage furrow is present with no evidence of spindle or asters.</td>
</tr>
<tr>
<td>5g Metaphase; 1st Division</td>
<td>a fully developed spindle which has not yet elongated is present.</td>
</tr>
<tr>
<td>5h Metaphase-Anaphase; 3rd Cleavage - Polar View</td>
<td>four cells are present with a polar view of the metaphase-anaphase spindle apparatus.</td>
</tr>
</tbody>
</table>
Figure 5. Photomicrographs of cytologically fixed eggs.
Figure 5. Photomicrographs of cytologically fixed eggs (continued).
DISCUSSION

The data presented of the cyclic uptake of Ca and Mg provide evidence for the involvement of these ions in specific mitotic events.

The first magnesium and calcium peaks in dividing sea urchin eggs (maintained at 15°C) occurred at 40 and 60 minutes, respectively, corresponding to very early prophase for magnesium and mid-prophase for calcium, i.e., prior to establishment of the definitive microtubular achromatic figure.

The occurrence of gelation of the endoplasm (viscosity increase) preceding the actual appearance of the spindle has been shown experimentally by Heilbrunn (1956). As noted previously, Heilbrunn postulated that this gelation was calcium-dependent. In 1959, the sea urchin egg was, in fact, shown to contain a calcium insoluble protein, which was isolated by Kane and Hersh. The amount of cation necessary to produce gelation of this protein was approximately 0.8 gm/liter of calcium or 2.4 gm/liter of magnesium.

Clothier (1961), who found a sharp calcium 45 uptake at mid-prophase, attributed this uptake to the gelation of a pre-spindle protein composed primarily of the Kane and Hersh material. The first versene sensitive period in Hanson's (1968) experiments likewise corresponds to the
early to mid-prophase period.

It now appears, however, that the Kane and Hersh protein may not be the protein responsible for this divalent ion dependent gelation. Stephens and Kane (1970) found it, rather, to be the main constituent of the hyaline layer of the fertilized egg. In the unfertilized egg, this protein is principally confined to the cortical granules and is expelled on fertilization. These investigators, however, also showed that the intracellular distribution of the protein is species specific and that in some species it may be found in the endoplasm; Stronglyocentrotus, unfortunately, was not one of the species studied in this experiment.

Sakai (1966) isolated a calcium insoluble protein from the mitotic apparatus which showed SH electron transfer activity with a cortical protein. Yazaki (1968), as reported by Kane and Stephens (1969), has shown that the calcium insoluble protein of Sakai is not the same as the Kane and Hersh protein. The Kane and Hersh protein can be divided into two fractions by dialysis against water. The dissolved fraction, HyS, has the gel and physical properties described by Kane and Hersh. The other fraction, which is insoluble in distilled water, and which Yazaki terms Ca ppt. 2, is a totally different protein. Only the latter fraction shows SH electron transfer activity. Consequently, it would appear that the Ca ppt. 2 protein and
the calcium insoluble protein of Sakai are identical.

The Sakai calcium insoluble protein of the isolated mitotic apparatus was found to contain three ultracentrifugal components with sedimentation coefficients of 3.2-3.5, 11-13, and 21-22. What is now referred to in the literature as the major soluble component of the mitotic apparatus is the largest of the three components (Sakai, 1966). This is a protein with the sedimentation coefficient of 3.2-3.5 S and is precipitated by calcium in a concentration of 2.0 gms/liter. This 3.5 S unit was further shown to be a dimer consisting of two 2.3-2.5 monomers linked by a single S-S bond. This monomer is also believed to be the basic structural unit of the mitotic apparatus (Mazia, 1967; Miki-Noumura, 1968).

With reference to the data of the present study, the first calcium and magnesium uptakes are interpreted to reflect a non-localized gelation which precedes compaction of the mitotic apparatus and involves the 3.5 S component of Sakai's mitotic apparatus protein. It is known that in the sea urchin egg the spindle proteins are present, prior to mitosis, in a disassembled condition (Wilt, Sakai, and Mazia, 1967).

Preceding the second uptakes of magnesium and calcium, a fall in the cellular content of each ion to base line levels was noted. The low points occurred at 50 and 80 minutes respectively. The magnesium low point corresponds
to the beginning of the mid-prophase period and the low point for calcium occurs at mid-metaphase. It should be noted that the calcium curve is still rising when that for magnesium, at 50 minutes, has fallen to its lowest level. Consequently, if both ions are being utilized as structural components in a pre-spindle protein gel, it seems that magnesium may be the initial ion involved, only to be substituted by calcium as the gelation progresses. This is further suggested by the fact that the rise and fall of the first magnesium peak extends only over 20-30 minutes, while the first calcium cycle is of 60 minutes duration.

As previously noted, the calcium low point following its first peak was reached at 80 minutes and corresponded cytologically to mid-metaphase, a period when cytological monitoring reveals a well formed spindle and aster. Reference has been made to the observation that the major soluble protein of the mitotic apparatus shows recurrent SH fluctuations throughout the division cycle. If, as postulated by Mazia and Dan (1952), the definitive mitotic apparatus is constituted of subunits which have been polymerized by intermolecular S-S bonds and the degradation of the system is due to the disassembly of the subunits, then the electron transfer reactions should be reflected in the available SH groups presented during the mitotic cycle. Such a fluctuation has been seen in the decrease of SH groups at meta-anaphase in the calcium insoluble fraction.
of the mitotic apparatus isolated by Sakai (1965). A
decrease in the number of SH groups means a proportional
increase of S-S linkages, which would be used to link to-
gether the subunits of the mitotic apparatus. The avail-
ability of SH groups was seen to be more pronounced at
cleavage when the mitotic apparatus is disintegrating.

Thus, the intracellular calcium levels may be falling
off at metaphase because of substitution by disulfide
linkages, the fully constructed apparatus in its compacted
and microtubular form being primarily sustained by S-S
bonds.

At the same time that the calcium levels have dropped
to the low point (mid-metaphase), the magnesium levels
have again risen to near the top of their second peak, the
period of increase having extended from late prophase to
early anaphase.

The magnesium uptake can be related to a structural
phenomenon, especially since the high magnesium level car-
rries into mid-anaphase and early cleavage. It was seen in
the first rise and fall of calcium and magnesium that the
magnesium fluctuation preceded that of calcium. This
occurs again in the second cycle. Thus, when the magnesium
curve begins to fall off at 100 minutes, the calcium level
is rising and reaches its peak only at 100 minutes. By
the time of mid-cleavage, the magnesium low point has al-
ready been reached while the calcium level has just
begun to decline.

The mitotic events most prominent during the second calcium and magnesium cycles are spindle elongation and cytokinesis. Rustad (1959) measured the changes in mass of the mitotic apparatus of dividing sea urchin eggs using interference microscopy. He found the interzonal area of the anaphase apparatus to be a region of high density. This would appear to represent a large increase in the amount of protein present in the interzonal spindle. Structurally, this region shows the same material components as the rest of the mitotic apparatus. The second peaks of calcium and magnesium may consequently be related in part to the formation of a gelating precursor of the interzonal spindle, similar to the precursor proposed for the earlier constituents of the achromatic figure.

Probably the largest accumulation and investment of divalent ions would be associated with cytokinesis. Although many theories have been proposed concerning the mechanism of cytokinesis, the most fully documented explanation is that of Marsland and Landau (1954). Marsland (1939) was first to note that the region of the cleavage furrow in dividing Arbacia eggs was highly resistant to the liquifying effects of hydrostatic pressure. Later experiments by Marsland and Landau (1954) led to the formulation of the gel contraction theory of cytokinesis, according to which a solation of the polar regions of the
blastomeres together with the contraction of a cortical gel in the furrow (contractile ring) characterizes the cleavage mechanism. It seems likely that the large uptake of calcium and magnesium seen just prior to cleavage may be referred, in part, to the formation and contraction of the cortical gel of the cleavage furrow. It may be noted that the role of these ions in the gel contraction of amoeboid movement is well established (Pantin, 1926a,b; Jahn and Bovee, 1969). Mercer and Wolpert (1958), moreover, found numerous microtubular structures in the cortex of dividing sea urchin eggs. Very recently, Szollosi (1970) was able to demonstrate in the furrow region of dividing marine eggs a sheath made up of filaments 50-70 Å in diameter. Thus a fibrous structural component of the contractile ring has been identified.

The process of cytokinesis also requires the synthesis and assembly of new cell membrane. According to Wolpert (1960), the first two blastomeres contain about 28% more surface area than the original undivided egg. The construction and assembly of new membrane very likely requires the addition of a divalent ion component, though quantitatively this might be small.

Divalent ions may be involved in enzymatic reactions associated with spindle function and with cleavage. Miki (1963a) found an ATPase present in the isolated mitotic apparatus of sea urchin eggs. This enzyme had an
activity three times as high as the ATPase of the cytoplasmic fraction (the supernatant of the mitotic apparatus isolation). She later showed cyclic ATPase activity in the 0.6M KCl soluble fraction of the dividing egg cortex, with activity doubled at metaphase and decreased at cleavage (Miki, 1963b). Weisenberg and Taylor (1968) isolated a 13 S magnesium and calcium dependent ATPase from the mitotic apparatus which they believed showed enough activity to account for the energy output of the spindle. However, it is not likely that the large uptake of calcium and magnesium described in the present study can be accounted for simply by its role as activator in an enzymatic reaction.

As shown in the data, the intracellular amount of each ion at the highest level is approximately 3.5 times that of the unfertilized eggs. The base level in the fertilized eggs (experimental series) is approximately twice that in the unfertilized eggs (control series). Clothier's (1961) study also indicated a three- to four-fold uptake of Ca 45 during the mitotic cycle as compared with the level in unfertilized eggs.

Rothschild and Barnes (1953) have shown that the potassium content of unfertilized sea urchin eggs (Paracentrotus lividus) is approximately 6800 mg/liter of eggs. This is 21 times the amount present in the surrounding sea water. Tyler and Monroy (1959) have shown that upon
fertilization three-fourths of the total potassium in the cell becomes exchangeable, which amounts to 5100 mg/liter. Monroy-Oddo and Esposito (1951) showed that the potassium level of dividing eggs drops by 25% forty minutes after fertilization. This means that 1700 mg/liter of potassium leaves the cells, thus producing in the cells a large cation deficiency. The possible exchange for other cations (e.g., calcium, magnesium) has not been studied.

As noted earlier, the Kane and Hersh protein and the Sakai protein are both affected by divalent cations. The Kane and Hersh protein gelated upon exposure to calcium at a minimum concentration of 0.8 g/liter or to magnesium at 2.4 g/liter in vitro. This calcium concentration is approximately twice as high as the maximum calcium concentration recorded in this study. The minimum magnesium concentration for gelation in vitro is approximately three times greater than the highest level recorded in this study. The calcium insoluble protein of Sakai, which is now thought to be the major component of the mitotic apparatus, requires calcium at a concentration of 2.0 g/liter before it precipitates in vitro, which is approximately four times the highest level recorded in dividing eggs in this study. The amounts of calcium and magnesium necessary for gelation of these proteins in vitro are thus quite high when compared to the levels found in this
study. However, the limitations associated with *in vitro* systems not uncommonly necessitate an excess of components or reactants, and the native intracellular processes may be expected to show a higher efficiency.
SUMMARY

This study was initiated to determine the concentration of magnesium and calcium in dividing eggs of the sea urchin Stronglyocentrotus purpuratus and to explore the relationship between these ions and mitotic events.

Samples of developing eggs, maintained at 15°C, were taken at ten-minute intervals from fertilization through the second cleavage, cytologically monitored, and analyzed for magnesium and calcium content. The analytical procedure was spectrophotometric, utilizing a modification of the method of Lamkin and Williams which employs Arsenazo (= o-[(1,8-dihydroxy-3,6-disulfo-2-naphthyl)-azo] benzene arsonic acid) and EGTA (= [ethylene bis (oxyethylenenitrilo)] tetraacetic acid).

Each division cycle was characterized by fluctuations in cellular content of magnesium and calcium which showed for each ion two peaks of uptake and release. The first magnesium and calcium uptakes occurred at very early prophase and mid-prophase, respectively. These peaks were interpreted to be related to the gelation of the 3.5 S soluble protein component of the mitotic apparatus prior to the actual assembly of the apparatus.

The second magnesium and calcium uptakes occurred at meta-anaphase and anaphase-cleavage, respectively. These peaks were interpreted to be related to the gelation of
the proteins associated with cleavage furrowing and the increase of blastomeric cell surface.

At fertilization, the base levels of magnesium and calcium rose to approximately twice the amounts characteristic of the unfertilized egg, i.e., from 11.0 to 22.2 mM for magnesium and 4.0 to 8.0 mM for calcium per Kg of water in the egg. At peak levels during mitosis, the magnesium and calcium concentrations reached 35.0 and 14.0 mM/Kg, respectively, approximately 1.5 times the post fertilization base levels.
BIBLIOGRAPHY


