OBSERVATIONS ON THE UPTAKE OF TRITIATED THYMIDINE IN THE PRONUCLEI OF FERTILIZED SAND DOLLAR EMBRYOS

EVA B. SIMMEL and DAVID A. KARNOFSKY

From the Divisions of Biophysics and Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, and Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

ABSTRACT

Following fertilization of the egg of the sand dollar Echinarachnius parma, tritiated thymidine (H\textsubscript{3}TDR) was taken up independently by the male and female pronuclei beginning within about 15 to 20 minutes, and the labeled pronuclei fused at about 30 to 40 minutes. At cleavage 90 minutes later the labeled nuclear material was distributed to both daughter cells. Unfertilized eggs and sperm exposed to H\textsubscript{3}TDR did not show nuclear localization of thymidine. DNA replication, thus, is initiated in the haploid pronuclei shortly after fertilization and prior to fusion. The major portion of DNA synthesis, as evidenced by thymidine uptake, appears to be during a 20 to 30 minute period after fertilization. Fertilization is associated with the activation of a mechanism which initiates early and independent replication of DNA in both the male and female pronuclei.

The immediate changes in the echinoderm egg following fertilization have been described in detail (1–3). One of the aspects that has apparently escaped careful analysis, however, is the time relationships in the replication of DNA following fertilization. This important matter is described, for example, in a recent review by Runnstrom et al. (2) as follows: “A more or less intimate fusion of the gamete nuclei takes place (karyogamy). In this way the diploid synkaryon is formed.” Another standard reference source also implies that DNA replication follows the formation of a diploid synkaryon (4). Recently Bucher and Mazia (5) have stated that in the sea urchin (Strongylocentrotus purpuratus) “DNA synthesis is normally preceded by pronuclear fusion.” On the other hand, evidence that DNA replication precedes karyogamy has come from various workers who have used quantitative microspectrophotometric measurements of Feulgen-stained material. Alfert (6) reported doubling of the DNA content of pronuclei preceding the first cleavage prophase of the mouse embryo, and Pasteels and his coworkers (7, 8) have confirmed this observation in rodents (7) and in Sabellaria (8). The exact time of DNA synthesis, however, could not be determined. The clearest statement concerning this point is provided by Swift and Kleinfeld (9), working with the grasshopper egg (Melanoplus differentialis, Bodine strain). They state: “It appears that DNA synthesis occurs in the sperm nuclei shortly after entry and in the egg nucleus right after the second meiotic division, so that the amount is doubled by the time the pronuclei come together. The two adjacent pronuclei thus together contain four times the haploid amount, the value characteristic of the normal diploid somatic prophase.”

It therefore was of interest to examine the uptake of tritiated thymidine (H\textsubscript{3}TDR) in an
echinoderm egg at short intervals following fertilization, on the assumption that the selective uptake of H\textsuperscript{3}TDR by nuclear structures represents an event in DNA synthesis (10, 11). Our observations demonstrate that DNA replication begins prior to the fusion of the pronuclei.

**MATERIALS AND METHODS**

Sand dollars, *Echinarchnus parma*, were collected in Salisbury Cove, Maine, during July and August, 1960. Eggs and sperm were obtained from the mature sand dollars by the potassium chloride injection method (12), and were washed in filtered sea water.

The eggs were transferred to a fingerbowl containing 100 cc. of filtered sea water to which 100 µc. of H\textsuperscript{3}TDR\textsuperscript{1} (activity 3 mc./mM) were added. The eggs then were stored in a constant temperature refrigerator at 15°C. for 1 hour and then fertilized with 5 drops of dilute sperm suspension. A sample of 50 eggs was removed immediately and fixed in 10 per cent neutral formalin; samples were removed and treated in a similar manner at 5, 10, 15, 20, 30, 40, 50, 60, 70, 90, and 105 minutes after the addition of the sperm.

Control samples used were: (a) smears of sperm cells exposed to 1 µc./cc. of tritiated thymidine for 1 hour; (b) unfertilized eggs stored in 1 µc./cc. of tritiated thymidine for 1 and for 5 hours; and (c) untreated fertilized eggs, fixed at 15 and 40 minutes, for radioautographic control.

Following fixation the eggs were pipetted into large depression slides, dehydrated with alcohols of increasing concentrations, cleared in xylol, and embedded in paraffin at 58°C. A trace of cosin was added to the first alcohol to render the embryos more easily visible. After embedding in paraffin, the depression slide was cooled in ice water. The paraffin button containing the embryos was removed and cut into small pieces which were remelted in No. 0 gelatin capsules. This made a convenient block with the embryos concentrated in the apex of the capsule. Sections were cut at 5 µ, and most of the embryos were found to be located in the first 50 to 70 sections.

Radioautographs (RAG's) were prepared with 1H\textsuperscript{3}TDR (3.0 curies/mm) was obtained from Schwarz Bio-Research, Inc., Mount Vernon, New York.

---

Radioautographs (RAG's) of 5 µ sections of sand dollar embryos developing in sea water containing 1 µc./ml. tritiated thymidine (H\textsuperscript{3}TDR). Figs. 1 to 6 are prefusion embryos, Figs. 7 to 11 are postfusion. Formalin fixation, Kodak AR 10 stripping film, hematoxylin and cosin. X 1200.

**FIGURE 1**

Unfertilized egg exposed to H\textsuperscript{3}TDR for 5 hours. There is some diffuse labeling of the cytoplasm, without nuclear concentration. RAG exposure 2 weeks.

**FIGURE 2**

Sperm and egg nucleus 10 minutes after fertilization. No nuclear H\textsuperscript{3}TDR concentration. RAG exposure 4 weeks.

**FIGURE 3**

Prefusion pronuclei 20 minutes after fertilization. Some uptake of H\textsuperscript{3}TDR in female pronucleus, but not in male. RAG exposure 4 weeks.

**FIGURE 4**

As Fig. 3, some uptake of H\textsuperscript{3}TDR in both pronuclei.

**FIGURE 5**

Prefusion pronuclei 30 minutes after fertilization. The pronuclei are still at some distance and show intense uptake of H\textsuperscript{3}TDR. RAG exposure 4 weeks.

**FIGURE 6**

As Fig. 5, pronuclei just before fusion. RAG exposure 2 weeks.
Kodak AR 10 stripping film and were stained with hematoxylin and eosin as previously described (13). Some slides were stained with Feulgen prior to radioautography. Exposure times were kept uniform, for purposes of comparison, but in the heavily labeled later stages the exposure times were reduced.

RESULTS

Simultaneously fixed embryos exhibit some variation in their course following fertilization. Those events which occur generally at each period will be described, although a few exceptions were seen.

The control eggs and sperm nuclei did not concentrate H\textsuperscript{3}TDR. No cytoplasmic uptake of H\textsuperscript{3}TDR was noted in the unfertilized eggs stored in H\textsuperscript{3}TDR for 1 hour, but in those so stored for 5 hours a slight increase in grains over the cytoplasm was found (Fig. 1).

In the first samples taken after fertilization, both egg nucleus and sperm tend to be peripherally located in the egg cytoplasm (Fig. 2). As the sperm approaches the female pronucleus, both move toward the egg center. The male pronucleus increases in size and becomes more round, and there is a consequent diminution in the intensity of its staining reactions. The chromatin of the female pronucleus appears at first as a thin fine ring close to the nuclear membrane (14) and stains weakly with Feulgen and hematoxylin, and the central part of the pronucleus is clear and empty.

<table>
<thead>
<tr>
<th>Time after fertilization</th>
<th>No. female pronuclei or fusion nuclei counted</th>
<th>No. showing uptake of H\textsuperscript{3}TDR</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediately after addition of sperm</td>
<td>51</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>64</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>20</td>
<td>183</td>
<td>124</td>
<td>67</td>
</tr>
<tr>
<td>30</td>
<td>217</td>
<td>206</td>
<td>95</td>
</tr>
<tr>
<td>40</td>
<td>111</td>
<td>110</td>
<td>99</td>
</tr>
</tbody>
</table>

* There was no significant difference between the percentages of H\textsuperscript{3}TDR-labeled nuclei in radioautographs exposed for 2 and for 4 weeks.

\textsuperscript{‡} For this quantitative analysis of the onset of H\textsuperscript{3}TDR labeling, it was more accurate to score only the labeled female pronuclei or fusion nuclei.

**FIGURE 7**
Fusion nucleus 40 minutes after fertilization. Sperm chromatin is still intact to one side and the female chromatin appears to retain its position, lining the nuclear membrane. RAG exposure 2 weeks.

**FIGURE 8**
As Fig. 7, with chromatin condensing.

**FIGURE 9**
Metaphase nucleus 50 minutes after fertilization. RAG exposure 1 week.

**FIGURE 10**
Anaphase nucleus 70 minutes after fertilization. RAG exposure 1 week.

**FIGURE 11**
Telophase nucleus 90 minutes after fertilization. RAG exposure 1 week.

**FIGURE 12**
Polyspermy found 40 minutes after fertilization. This section contains 4 labeled sperm. RAG exposure 2 weeks.
At 15 and 20 minutes after fertilization the pronuclei are approaching each other (Figs. 3 and 4) and at 20 minutes a few have established contact. Thymidine uptake generally begins in 15 to 20 minutes after fertilization, although a single female pronucleus labeled with H₃TDR was found 5 minutes after fertilization (Table I).

Thymidine incorporation appears to proceed rapidly after initiation; the RAG's generally have relatively few grains in the 20-minute embryos (Fig. 3 and 4) and become very intense in the next 10 to 20 minutes (Figs. 5 to 8).

Fusion of the pronuclei is most likely to occur between 30 and 40 minutes after fertilization. It is not clear from these experiments whether the fusion nucleus continues to pick up H₃TDR, since the sum of the well labeled pronuclei may well account for the intensity of the synkaryon RAG's. Prophase chromosomes, first seen at 40 minutes after fertilization, tend to be obscured by the autographs, but are clearly seen in control sections.

At 50 minutes after fertilization chromosome condensation and metaphase line-up are seen (Fig. 9). Various stages up to anaphase are seen at 60 and 70 minutes (Fig. 10), and telophases and first cleavages are seen at 90 minutes (Fig. 11).

Polyspermic eggs were occasionally seen. Several H₃TDR-labeled sperm were sometimes found in a single egg (Fig. 12).

**DISCUSSION**

The radioautographs of H₃TDR incorporation into sand dollars at various times after fertilization indicate that DNA replication begins in the male and female pronuclei about 15 to 20 minutes after fertilization and proceeds rapidly, and the labeled pronuclei fuse at about 30 to 40 minutes. This observation strongly supports the conclusion of Swift and Kleinfeld (9), working on the grasshopper embryo, that DNA replication precedes karyogamy. H₃TDR incorporation into the nucleus is interpreted to represent net DNA synthesis. Supporting evidence of quantitative measurements of Feulgen-positive material did not seem indicated or feasible. Our observation is consistent with the Feulgen data of Swift and Kleinfeld (9), and McMaster (15) comments on the impossibility of obtaining quantitative Feulgen measurements in the echinoderm embryo (*Lytechinus variegatus*) prior to fusion of the pronuclei.

Bucher and Mazia (5) exposed fertilized sea urchin embryos to β-mercaptoethanol and then H₃TDR, and their first observation at 90 minutes showed that pronuclear fusion was blocked, although the pronuclei incorporated H₃TDR. They did not describe the sequence of H₃TDR uptake in untreated fertilized eggs, but apparently assumed that the uptake was preceded by fusion of the pronuclei.

These data indicate that fertilization is associated with a cytoplasmic change which initiates the prompt onset of DNA synthesis in both the male and female pronuclei; DNA synthesis actually extends to the multiple pronuclei seen in polyspermy. The rate of DNA synthesis is rapid, and a considerable H₃TDR uptake occurs over a 10-minute period, although we have no quantitative data on how quickly DNA is replicated prior to cleavage. The mechanism of the early activation and rapid formation of DNA independently in the male and female pronuclei following fertilization merits detailed study.

This research was supported, in part, by Atomic Energy Commission Contract No. At(30-1)-910, and by grants from the American Cancer Society, Inc. (T-40), the Albert and Mary Lasker Foundation, and the National Institutes of Health, United States Public Health Service.

Received for publication, March 6, 1961.

**REFERENCES**


5. Bucher, N. L. R., and Mazia, D., *Deoxyribonucleic Acid...*


