ACCUMULATION OF RADIOACTIVE CALCIUM IN LARVAE OF THE SEA URCHIN PSEUDOCENTROTUS DEPRESSUS

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The prominent role of calcium in the sea urchin egg, especially with reference to physiological events at the time of fertilization, has been repeatedly emphasized. But comparatively little is known of the accumulation of calcium and its utilization in the formation of the spicule during the course of development. Using radioactive calcium, Rudenberg (1953) reported that the *Arbacia* egg did not show any uptake of calcium up to the time of the swimming blastula stage, while the jelly coat accumulated calcium to a considerable extent. On the other hand, Hsiao and Boroughs (1958) reported that the unfertilized eggs of the Hawaiian sea urchin, *Tripneustes gratilla*, took up radioactive calcium even though the jelly coat was removed. From the observation that total calcium of the egg did not change after incubation with Ca^{45}, they concluded that Ca^{45} entered the egg cytoplasm by exchange with Ca^{40} of the egg. Örström and Örström (1942) found that the total calcium content of the *Paracentrotus* egg remained unchanged for a period of 15 hours after fertilization and then it quickly increased over 10-fold in the next 25 hours. They suggested that this increase was associated with the formation of the spicule. Recently, Yasumasu (1959) measured the calcium content of the spicule during development of the *Hemicentrotus* and *Anthocidaris* larvae and reached the same conclusion. Bevelander and Nakahara (1960) reported that in the *Echinarachnius* egg, the calcium accumulated in the areas of spicules and primary mesenchyme cells, and suggested that the calcium concentrated in the mesenchyme cells was transmitted to the growing spicules.

The present paper supplies some information concerning the uptake of radioactive calcium by sea urchin larvae at different stages of development, and the extent to which accumulated calcium is utilized in the formation of the skeleton. Preliminary experiments were carried out on *Paracentrotus* larvae by one of us (E. N.) at the Institute of Comparative Anatomy, University of Palermo, and the results obtained were briefly reported in Japanese (Nakano, 1960).

Materials and Methods

The sea urchin used in the present experiments was *Pseudocentrotus depressus*, collected mainly at the Sugashima Marine Biological Station. The eggs were obtained by KCl-induced spawning and washed three times with filtered sea water. Dilute suspensions of eggs were fertilized and allowed to develop at room temperature (15–18° C.) under continuous gentle stirring.

In the first series of experiments, larvae were reared in the isotopic medium from the beginning of development at a level of radioactivity of 0.1 μc./ml. The

concentration of added calcium (0.5 mM) was so low that no effect was found on the spicule formation, as reported by Okazaki (1956). At appropriate intervals, larvae were withdrawn from the culture and washed with sea water five times. They were then homogenized with 0.54 M KCl at pH 7.2 in a Teflon homogenizer of the Potter type. The homogenate (called "total homogenate") was centrifuged in the cold at 800 g for 10 minutes and the sediment was discarded, while the supernatant ("supernatant") was used for analysis. A 0.2-ml.-sample was placed on a stainless steel planchette, dried and counted with a thin end-window gas flow counter and an ordinary scaler. The samples for the determination of total nitrogen were combusted and the nitrogen was estimated by direct Nesslerization.

In the second series of experiments, larvae were reared in normal sea water until desired stages. They were then removed from the culture and incubated in the isotopic medium for two hours at room temperature. As the culture medium, artificial sea water containing Ca^{45} (1 μc./ml.) was used. After incubation, larvae were washed repeatedly with sea water and divided into two parts. One part was homogenized with 0.54 M KCl and used for the counting of radioactivity as described above, while the remaining part was used for the examination of autoradiographs *in toto* and in section. For making whole mount preparations, larvae were flattened and fixed with methanol between the slide and coverglass. After

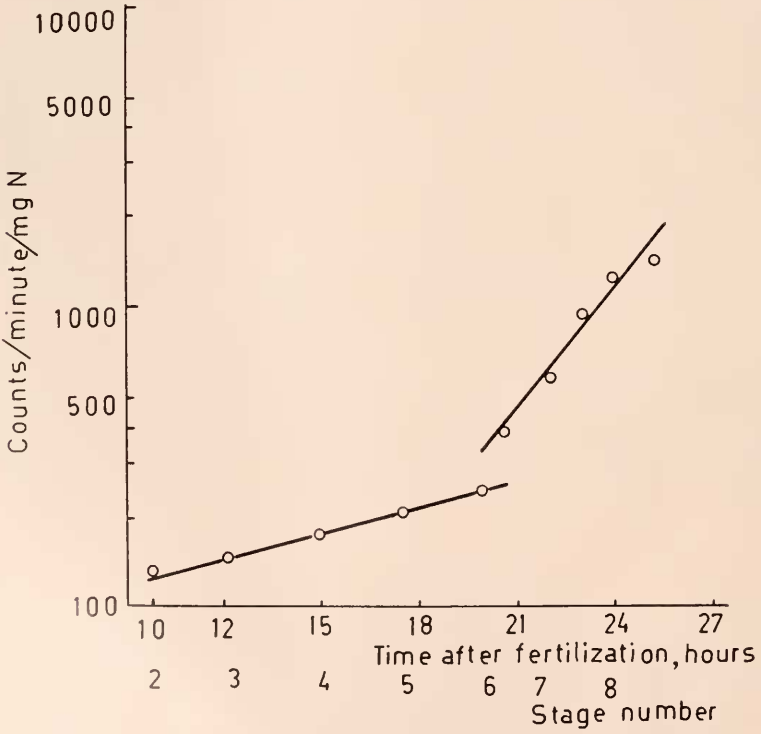

Figure 1. Total accumulation of Ca^{45} in *Pseudocentrotus* larvae during development. For the stage number see Table I.

TABLE I

Accumulation of Ca^{45} in Pseudocentrotus larvae during development. The values represent counts per minute per mg. nitrogen. Larvae were incubated at 18–19°C.

Stage number	Stage	Experiment 1		Experiment 2	
		Total	Supernatant	Total	Supernatant
1	4-cell	65	33	—	—
2	Blastula before hatching	133	131	—	—
3	Swimming blastula without mesenchyme cells	152	140	143	47
4	Early mesenchyme blastula	164	176	183	154
5	Mesenchyme blastula	205	195	241	177
6	Late mesenchyme blastula*	246	216	394	192
7	Early gastrula	596	201	987	257
8	Gastrula	1,290	208	1,985	305
9	Prism	15,530	1,082	11,280	1,036
10	Early pluteus	55,100	3,640	52,000	5,360
11	Pluteus	148,000	8,420	—	—

* Appearance of the spicular rudiment.

standing at least one hour, the coverglass was removed in water containing a trace of calcium (about 10 m*M* $CaCl_2$) and the slide was dried quickly after passage through graded ethanol. For making sections, larvae were fixed with methanol, and sections 10 microns thick were mounted on slides from which paraffin was removed by xylene. The slide was dried after passage through ethanol. All preparations were coated with a thin layer of celloidin without staining. Autoradiographs were made by the stripping emulsion technique, using Fuji autoradiographic plates. Exposure was completed in the light- and moisture-proof box at 2° C. for 2 to 10 weeks and developed photographically. Examination of the autoradiographs was made with both light and phase contrast microscopes.

RESULTS

When fertilized eggs were reared in sea water containing Ca^{45}, there was practically no accumulation of Ca^{45} during the early stages of development. At the early gastrula stage, however, the radioactivity of the total homogenate began to increase, showing the onset of calcium accumulation (Fig. 1). Thereafter, this accumulation increased rapidly and high radioactivity was observed at the pluteus stage (Table I). On the other hand, the radioactivity of the supernatant fraction remained relatively constant during gastrulation. Since the spicule is removed from the supernatant by centrifuging, the radioactivity found in the supernatant is not attributable to the spicule. It is highly probable that the supernatant contains bound calcium besides its free ions.

A rapid and abrupt accumulation of Ca^{45} in total homogenate at advanced stages of development suggests that the rate of calcium uptake may change during development. Figure 2 gives the time course of calcium uptake by *Pseudocentrotus* larvae at different stages. In these experiments, larvae were incubated in the iso-

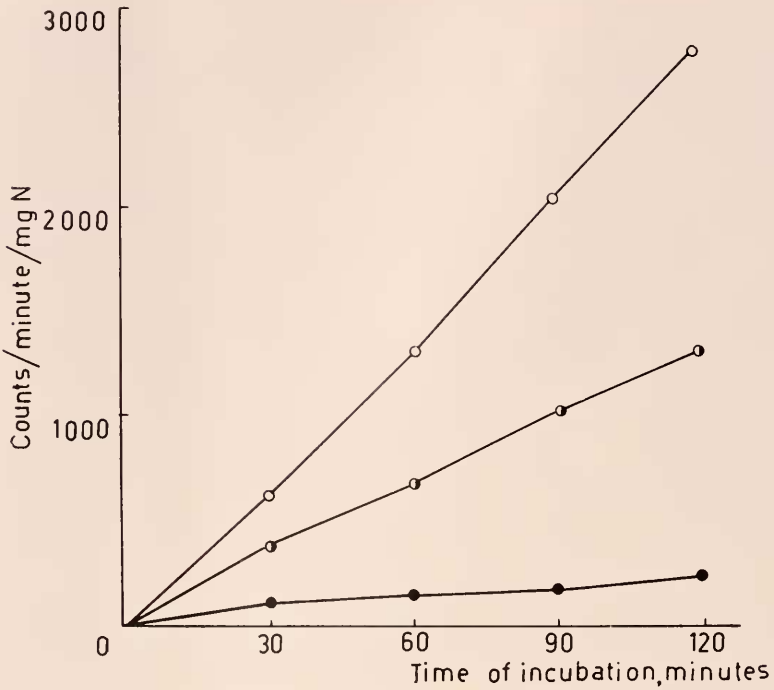

FIGURE 2. Time course of uptake of Ca^{45} at different stages of *Pseudocentrotus* larvae. Solid circles, blastula without mesenchyme cells; semi-solid circles, early gastrula; open circles, late gastrula.

topic medium at different intervals for a period of two hours and the radioactivity of total homogenate was counted. During gastrulation, the rate of calcium uptake increases rapidly and at the late gastrula stage, it becomes over 10 times greater than that of blastula without mesenchyme cells. These changes of calcium uptake were confirmed by autoradiographic techniques which ran in parallel with the above experiments. In the autoradiograph of the early gastrula, dark grains were found to be located in the two small areas, suggesting the onset of accumulation of Ca^{45} at the spicular rudiments (Figs. 3A and a). When the spicular rudiment develops into a triradiate spicule, its radioactivity increases markedly and this increase continues to the pluteus stage (Figs. 3B–E and b–e). Since these photographs clearly demonstrate that Ca^{45} is concentrated in the spicule, it seems likely that the rapid increase of calcium uptake observed by counting directly reflects the formation of the spicule.

Preliminary experiments on *Paracentrotus* larvae showed, however, that calcium uptake increases at the mesenchyme blastula stage before spicule formation. A detailed investigation of this point was carried out on *Pseudocentrotus* larvae. As shown in Figure 4, the rate of calcium uptake increases preceding appearance of the spicular rudiment. In accord with this finding, it was found in the autoradiograph of sections that dark grains were evenly distributed in the blastocoel before spicule formation (Fig. 5B). At the early stages of development, accumu-

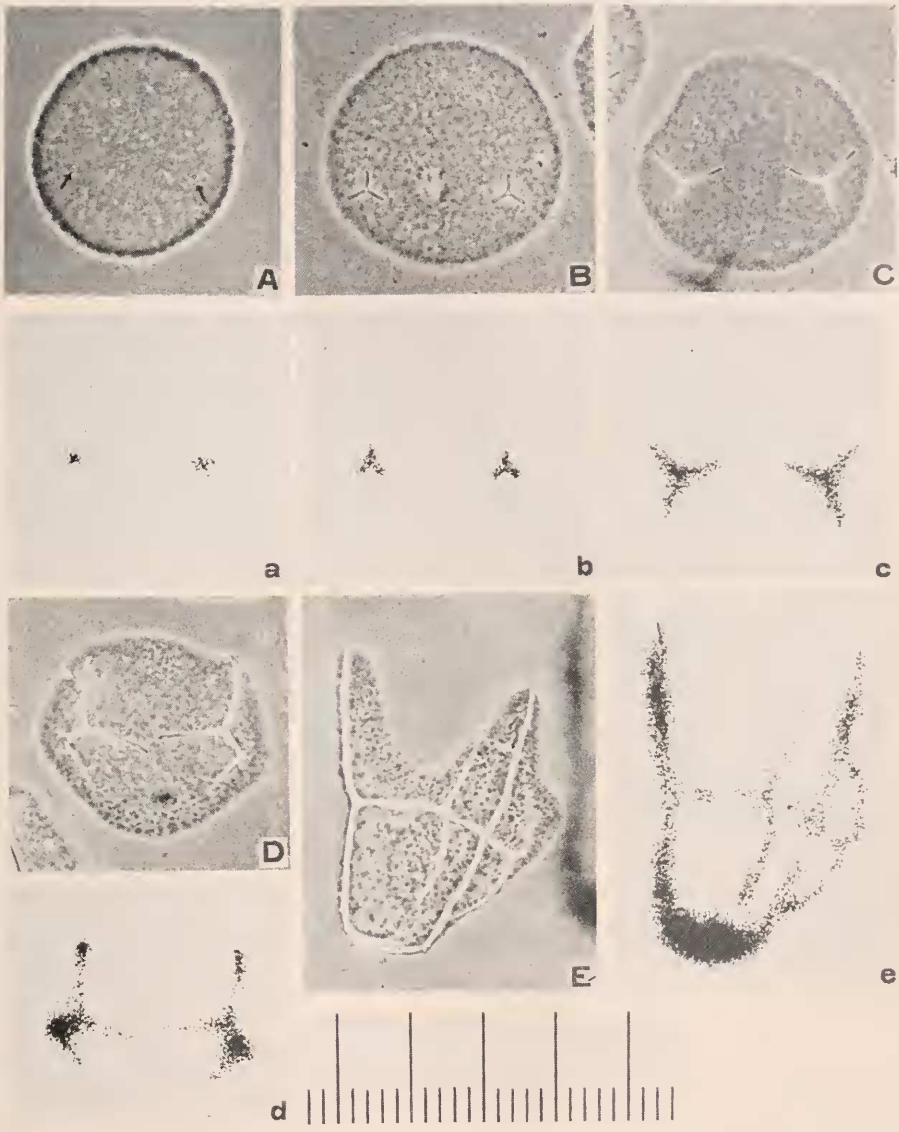

FIGURE 3. A–E, photographs of whole mount preparations of *Pseudocentrotus* larvae incubated in Ca^{45}-containing sea water for two hours, photographed by phase contrast optics. Arrows in A indicate the spicular rudiments. a–e, autoradiographs of the same specimens as in A–E, photographed by ordinary microscope. Autoradiographic emulsion was shifted after exposure to radiation so that dark grains were not superimposed over larvae.

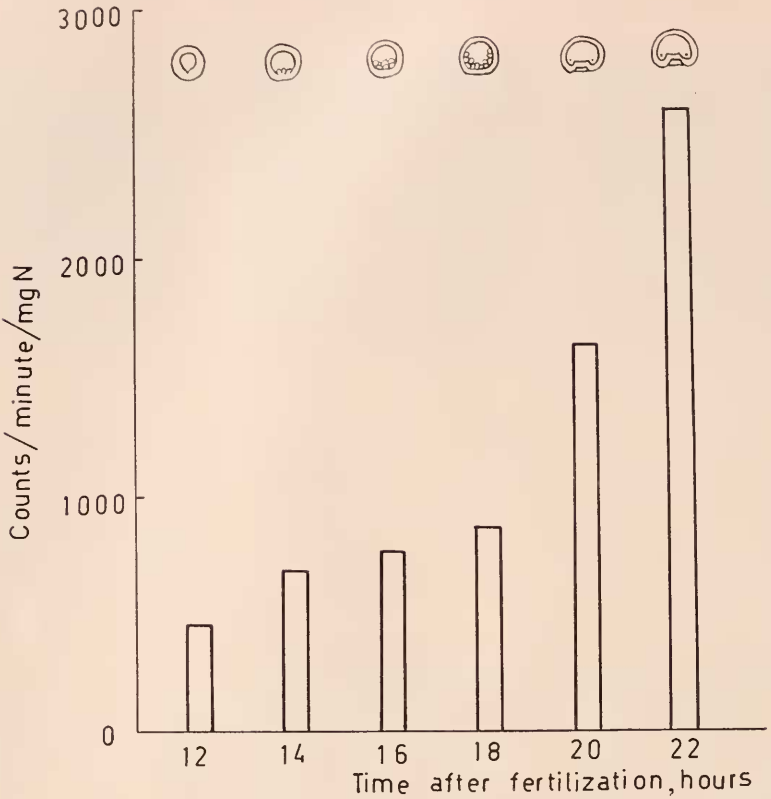

FIGURE 4. Rate of uptake of Ca^{45} by *Pseudocentrotus* larvae at different stages of development. Larvae were incubated in Ca^{45}-containing sea water for two hours. Upper diagrams show the stage at the end of incubation.

lation of Ca^{45} is insufficiently recognized in the blastocoel, as shown in Figure 5A. It may be assumed that permeability of larvae to calcium changes at the mesenchyme blastula stage and that the penetrated calcium is held as a Ca complex in the blastocoel before deposition on the spicule. There is no accumulation of Ca^{45} in mesenchyme cells.

DISCUSSION

The results of the present experiments show that the rate of uptake of Ca^{45} by sea urchin larvae is extremely low at the beginning of development. It rises at the mesenchyme blastula stage, which is followed by the second rise at the gastrula stage. Low uptake of Ca^{45} at the early stage may be attributable to an exchange of Ca^{45} with Ca^{40} of cytoplasm, as was pointed out by Hsiao and Boroughs (1958). Of the two steps of the increase, the latter one at the gastrula stage can be explained by deposition of Ca^{45} on the spicule, as assumed also from the data of previous workers (Örström and Örström, 1942; Yasumasu, 1959). The former increase at the mesenchyme blastula stage, however, seems to be additional infor-

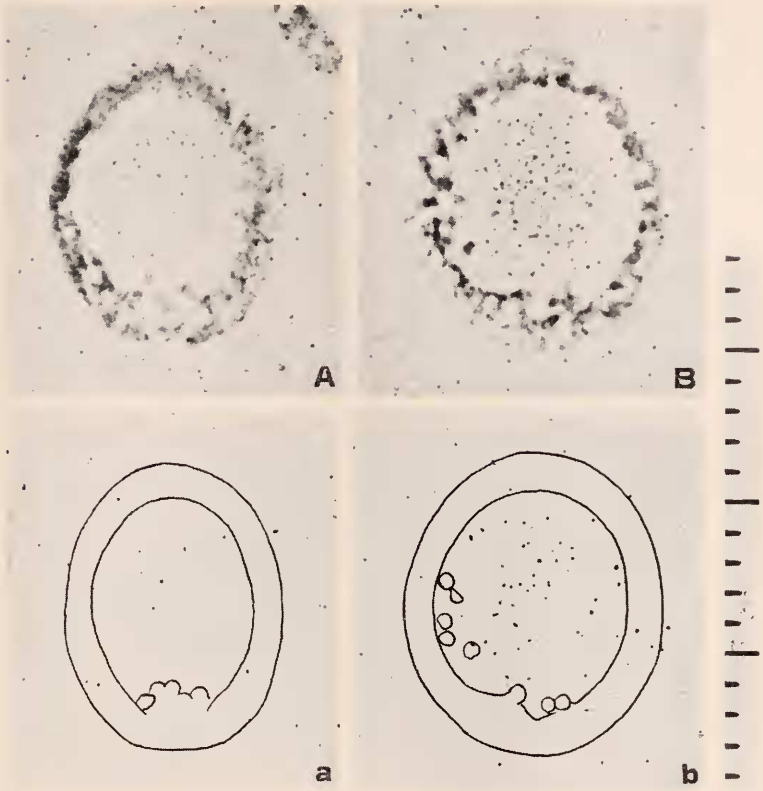

FIGURE 5. A and B, photographs of sections of *Pseudocentrotus* larvae incubated in Ca^{45}-containing sea water for two hours, photographed by phase contrast microscope. A, early mesenchyme blastula. B, late mesenchyme blastula before the formation of the spicular rudiment. a and b, autoradiographs of the same specimens as in A and B, photographed by ordinary microscope.

mation concerning the calcium metabolism in sea urchin larvae. By the autoradiographic analysis, Ca^{45} taken up by the mesenchyme blastula was found in the blastocoel. There is a possibility that Ca^{45} in the blastocoel may be bound with protein or mucopolysaccharide. In fact, blastocoelic gel was reported to be composed of mucopolysaccharides (Monné and Hårde, 1950; Monné and Slautterback, 1950, 1952; Immers, 1956, 1961). Bevelander and Nakahara (1960) supposed that calcium mobilized in the primary mesenchyme cells was transmitted to the growing spicules. But whether or not calcium is accumulated in these cells, as they claimed, is not clear from the present data. The investigation of this point will be a subject of future research.

From the fact that the rate of uptake of Ca^{45} begins to increase at the mesenchyme blastula stage, it seems likely that permeability of the larvae to calcium may change at this stage. In experiments with *Strongylocentrotus purpuratus*, Bolst and Whiteley (1957) reported that uptake of inorganic phosphate by the larvae increases rapidly at the early stage of development and then decreases with the

onset of gastrulation. This pattern is entirely different from that of calcium uptake. It is of interest to note in this connection that the spicule of sea urchin larvae consists of calcium carbonate, not of calcium phosphate (Yasumasu, 1959).

Summary

The uptake of radioactive calcium by sea urchin larvae was investigated. Up to the blastula stage, calcium uptake proceeds at an extremely low rate, while it increases greatly during gastrulation. Autoradiographs of whole larvae show the increase of calcium uptake in parallel with the formation of the spicule. Before appearance of the spicular rudiment, however, calcium uptake increases at the mesenchyme blastula stage. Autoradiographs of sections show that penetrated calcium is distributed in the blastocoel. It is assumed that calcium is concentrated in the blastocoel before deposition on the spicule.

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