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**Radioautographic studies on uptake of calcium in
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Embriologia sperimentale. — *Radioautographic studies on uptake of calcium in embryos of the sea urchin.* Nota (*) di J. IMMERS (**) e L. CONTOLI-AMANTE (***), presentata dal Corrisp. P. PASQUINI.

RIASSUNTO. — Nelle blastule mesenchimatiche, il Ca non si accumula, ma rimane ad un livello costante e moderato nel mesenchima primario; vi è invece un forte ricambio di Ca nell'ectoderma e nello strato ialino ed un particolare accumulo nel gel blastocelico.

Nelle gastrule si ha pure un forte accumulo nella regione posteriore dell'archenteron, soprattutto alla superficie delle cellule e probabilmente anche negli spazi intercellulari. Le spicole, pur presenti, non risultano marcate, probabilmente per motivi tecnici ancora non chiari; in esse, o nello ialoplasma circostante, sono presenti gruppi alfa-glicol. Con varie colorazioni, si possono evidenziare differenze, rispetto al Ca ed ai suoi tipi di legame, tra le cellule del mesenchima primario e secondario.

È discussa la funzione che il Ca, reagendo con componenti acidi, può avere nella adesione reciproca delle cellule epiteliali in questi stadi di sviluppo.

Four decades ago Prenant (1926 a, b) described "un système d'anastomoses" among the primary mesenchyme cells in living sea urchin embryos. The system consists of a circular tract of cells surrounding the base of the archenteron with a pair of longitudinal protrusions of pseudopodial character extending in animal direction. He emphasizes that the formation of the tract preceded skeletal development and that the skeletal growth invariably followed the course of the tract.

Gustafson and Kinnander (1956), Runnström (1957) and Gustafson (1964) have shown that an organic material surrounds the spicule.

Recently, Nakano (1960) has found that a rapid increase in incorporation of ^{45}Ca occurs in the mesenchyme blastula. The process and pattern of the initial steps of skeleton formation was investigated by Bevelander et al. (1960) and particularly by Okazaki (1960, 1965). The calcium salts are deposited within vacuoles of the hyaloplasm. The calcareous granules which first appear merge to form the spicular rudiment. The process of skeleton formation is very sensitive to a lowering of the pH of the medium. Boxin (1926) observed that an envelope having the same shape as the skeleton remains after dissolution of the calcareous material of the skeleton. According to Okazaki (1960) this may even occur at pH 5.0–5.2 without deformation of the envelope. Conversely, the envelope was destroyed when embryos were treated for 30 min at 100°C in a solution of M/5 NaOH at pH approximately 8.0 (Okazaki, 1960).

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The present note will give some radioautographic and histochemical data about the calcium metabolism in early embryos of *Paracentrotus lividus* (*Pa. lividus*).

MATERIAL AND METHODS.

The gametes and embryos of *Pa. lividus* were obtained and handled as earlier described (Immers and Runnström, 1965). In the incorporation experiments the embryos were incubated for one to three hours in $0.5 \mu\text{C } ^{45}\text{Ca/ml}$ in normal sea water. The ^{45}Ca (CES2, Calcium-45) was received from Radiochemical Centre, Amersham. The embryos were fixed in Carnoy's fluid for one hour or in abs. ethanol-chloroform (6:3) for 2 hours. The radioautographs were processed as earlier described (Immers and Runnström, 1965).

RESULTS.

Incorporation experiments with ^{45}Ca carried out on mesenchyme blastulae showed that the primary mesenchyme cells are able to take up calcium, although no strong accumulation occurs. The ^{45}Ca was incorporated also into the cells of the ectoderm and into the hyaline layer (fig. 1). The grains of the ectodermic cells were located in the outer region. The ^{45}Ca incorporation was found besides in the blastocoelic gel. If the time of incubation was extended from one hour (17-18 hours after fertilization) to three hours (17-20 hours after fertilization) there was no evidence of an increased uptake of ^{45}Ca into the primary mesenchyme cells. Conversely, both the ectodermic cells and the hyaline layer showed a considerably increased incorporation (cf. fig. 2). The increase of incorporation was particularly strong in the blastocoelic gel after prolonged incubation. During the development of the mesenchyme blastula the blastocoelic gel seems thus to become richer in calcium (fig. 2).

After three hours incubation with ^{45}Ca the embryos of mesenchyme blastula were transferred in normal sea water and left there for five hours, then the embryos (gastrula of 25 hours) were fixed and subjected to radioautography. The radioautogram (fig. 3) shows the presence of grains in some mesenchyme cells, in ectodermic cells particularly at their outer margin and in the hyaline layer. Conversely, the labeled material of the blastocoel had disappeared. Radioactivity could be demonstrated neither in spicule nor its envelope (cf. fig. 3).

Figs. 4-6 refer to embryos in the gastrula stage which were incubated in ^{45}Ca for one hour (24-25 hours after fertilization). The obvious result is that a heavy incorporation occurs in a posterior (animal) region of the archenteron, corresponding to the hindgut and a part of the midgut. Fig. 4 represents longitudinal section whereas figs. 5 and 6 are cross sections of the region with strong accumulation of grains. Fig. 5 is a section at the level of the

midgut. It tends to show that the ^{45}Ca was accumulated particularly in the surface of the cells and probably also in the intercellular spaces between them. Fig. 6 is an oblique section at the level of the heavily labeled hindgut from which a number of labeled radial strands extend toward the ectoderm. The character of the labeled strands could not be determined. They may line or cover filopodia of mesenchyme cells.

The skeleton spicules left no imprint on the radioautograms although spicules were unmistakably present in the section referred in figs. 3-6. Fig. 7 is a photograph of a section through a gastrula before it was covered with film. It shows the triradiate spicule clearly. The spicule disappeared fully during the development and fixing of the film. The same occurred also after Hale staining and to a considerable extent after PAS staining. At the site of the spicule there was no trace of Hale reaction. PAS staining, on the other hand, gave weak indications of the presence of α -glycol groups, which, however, may rather belong to the hyaloplasmic mass surrounding the skeleton. Naphthol yellow did not stain the skeleton spicules. The Giemsa method was used throughout in preparations which were subjected to radioautography. This staining lets the skeleton stand out very clearly in the preparations as evident in fig. 7.

Alizarin red S (sodium alizarin sulfonate stain) is a very sensitive reagent for demonstrating the presence of calcium in tissues (Dahl, 1952; Pearse, 1960). It fails, however, to demonstrate the presence of calcium in the primary mesenchyme cells, ectoderm, hyaline layer or blastocoelic gels and in the posterior region of the archenteron. Conversely, alizarin red S stains the spicules and their envelopes (figs. 8 and 9). Furthermore certain granules in the cells of the secondary mesenchyme become stained (fig. 9). The stain was crimson or scarlet.

The method proposed by von Kósa (see Gurr, 1958; Pearse 1960) for demonstration of calcium phosphate or carbonate gave no positive reaction at the level of the spicules and their envelopes. On the other hand, the secondary mesenchyme cells gave a clearcut reaction when the sections were treated according to von Kósa method (fig. 10 and 11). The reaction seems to be bound to certain granules which were traced also radioautographically. As figs. 10 and 11 indicate the primary mesenchyme cells show no von Kósa reaction.

DISCUSSION.

As mentioned above, Nakano (1960) reported a rapid increase in incorporation of ^{45}Ca into sea urchin embryos in the mesenchyme blastula stage. This increase begins before the formation of the skeleton and should therefore involve an accumulation of calcium in the mesenchyme cells. Bevelander and Nakahara (1960) likewise assumed an accumulation of calcium in the primary mesenchyme cells of sand dollar embryo. However, the results presented above rather indicate that calcium taken up from the medium does

not accumulate but remains on a rather constant but moderate level in the primary mesenchyme cells.

On the other hand, there is evidence presented above of a considerable uptake of calcium into the outer region of the ectoderm cells and into the hyaline layer. Moreover an accumulation of calcium was found to occur in the blastocoelic gel.

In the gastrula stage a surprisingly strong accumulation takes place in the posterior region of the archenteron. It may be underlined here that the incorporation into the ectoderm and the hyaline layer is as strong in the gastrula as in the mesenchyme blastula stage, although fig. 4 does not seem to substantiate this (owing to shorter exposure to the covering film in order to give relief to the strong incorporation into the archenteron). The pictures presented in Plate I suggest the possibility that accumulation of calcium occurs in ectoderm and particularly in the posterior region of the archenteron rather than in the primary mesenchyme. Continued work is necessary to decide whether the accumulated calcium provides material for the skeleton formation.

The calcium accumulation in ectoderm, hyaline layer and posterior part of the archenteron may also have a special function. By reacting with acid components, i.e. sulfated glycoproteins, calcium may have a solidifying effect that could increase the adhesion within the epithelia. The effect of lack of calcium in preventing the adhesion is well known, particularly for the sea urchin egg (Herbst, 1900).

Histochemical reagents for calcium revealed rather interesting differences between primary and secondary mesenchyme cells. Although both incorporated ^{45}Ca , the primary mesenchyme cells were negative to staining with alizarin red S or to the silver nitrate method introduced by von Kósa. Conversely, certain granules within the secondary mesenchyme cells gave a positive reaction with both the mentioned methods.

The cause of this difference is obscure but it is indicative of a cytochemical segregation of the two kinds of mesenchyme cells. Hörstadius (1928) showed that primary mesenchyme cells may arise from secondary ones in embryos which in the 16-cell stage had been deprived of their micromeres. In later stages, however, pronounced differences in behaviour, survival, etc. of the two kinds of cells were recently demonstrated by Runnström and Immers (1966).

It is intriguing that we were not able to demonstrate any incorporation of ^{45}Ca into the skeleton. This failure may have technical reasons which will be subject to further investigation.

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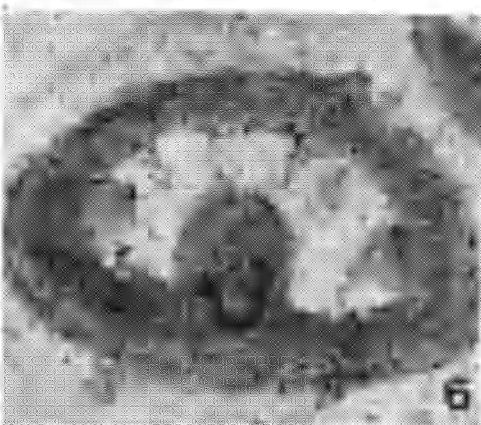
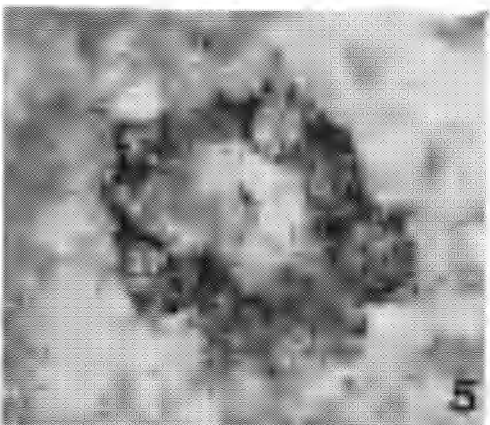
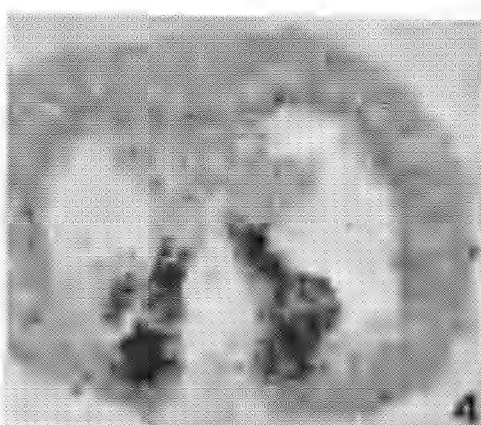
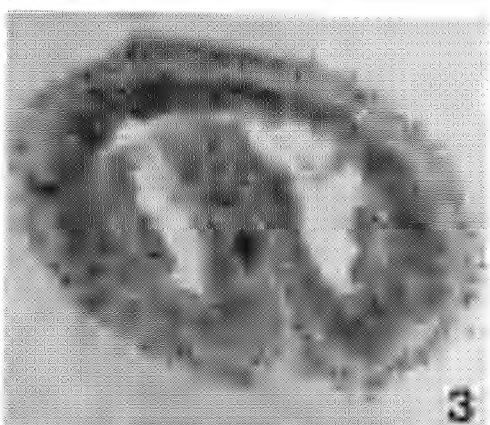
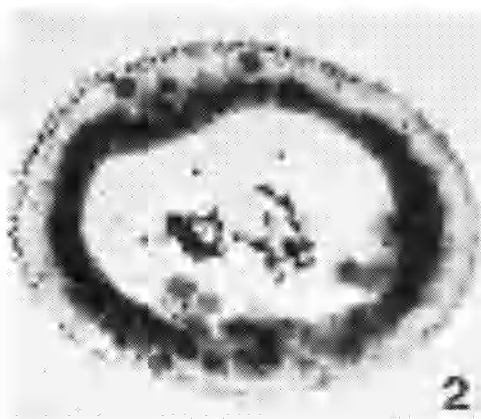
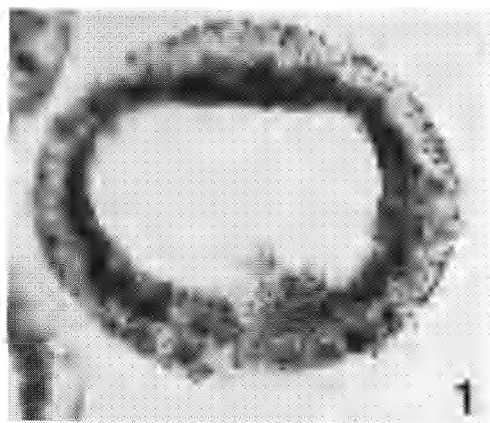
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EXPLANATION OF PLATES I-II.

Micro-radioautograms of *Pa. lividus* embryos exposed to $0.5 \mu\text{C } ^{45}\text{Ca}/\text{ml}$ in sea water at 20°C . Sections were cut 5μ thick, deparaffined and covered with Kodak autoradiographic stripping film AR 10 also 5μ thick. Sections counterstained with Giemsa mixture through covered film. Linear magn.: figs. 1, 2, 3, 4, 6, 8, 9 and 10 - $700\times$; figs. 5 - $200\times$; fig. 7 - $1400\times$ and fig. 11 - $1200\times$.

PLATE I (figs. 1-6).

- Fig. 1. - Mesenchyme blastula with primary mesenchyme cells 18 h after fertilization. Incubated for one hour (between 17-18 h a.f.) with ^{45}Ca .
 Fig. 2. - Mesenchyme blastula 20 h after fertilization. Incubated for 3 h (between 17-20 h a.f.) with ^{45}Ca .
 Fig. 3. - Gastrula 25 h after fertilization. ^{45}Ca was incorporated in the mesenchyme blastula stage as in the embryo of fig. 2. The incorporation was interrupted at 20 h after fertilization. The ^{45}Ca was washed out by centrifugation ($3\times$) and embryos were raised in normal sea water for five hours.
 Fig. 4. - A longitudinal section of 25 h gastrula after fertilization. ^{45}Ca incubated for one hour (between 24-25 h a.f.).
 Fig. 5. - A cross section of 25 h gastrula through midgut. Incorporation conditions as in embryo of fig. 4.
 Fig. 6. - An oblique cross section of 25 h gastrula through hindgut. Incorporation conditions as in embryo of fig. 4.



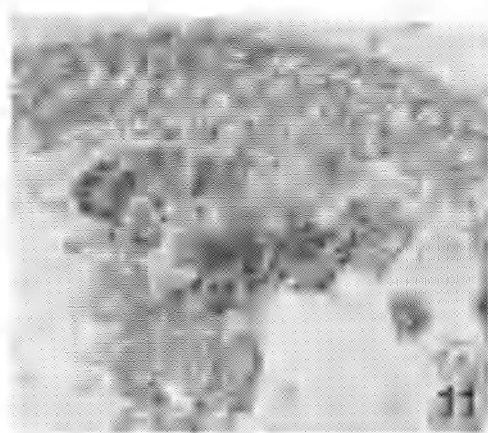
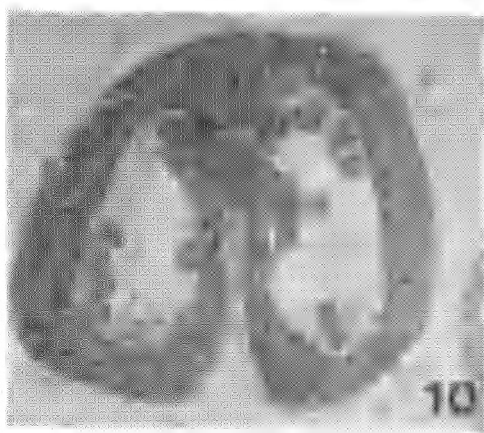
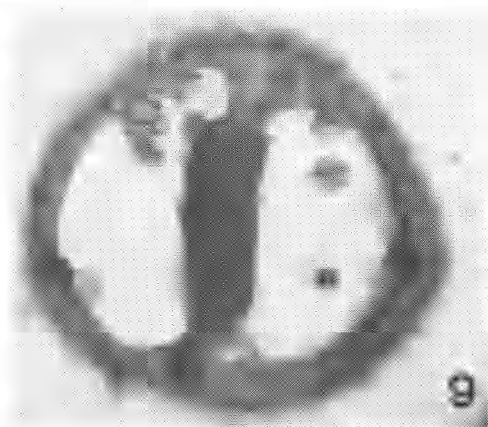
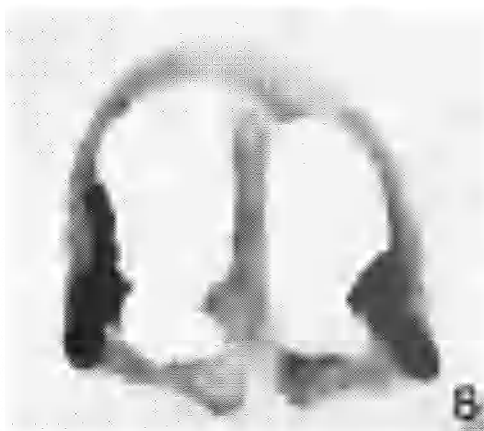
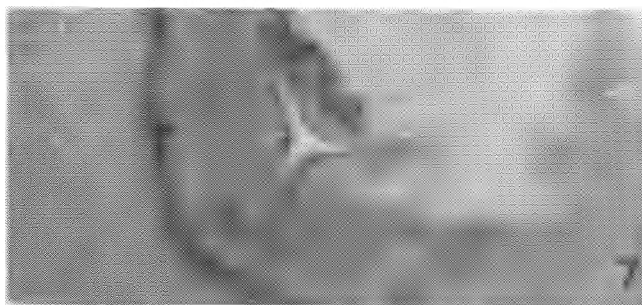


PLATE II (figs. 7-11).

- Fig. 7. - A longitudinal section 25 h gastrula from embryo raised under conditions as mentioned below. Figs. 4-6. It was stained according to Giemsa before covering with film.
- Fig. 8 and 9. - The sections of 25 h gastrula from embryos raised under conditions as mentioned in the legend of figs. 4-6 and stained with alizarine red S.
- Figs. 10 and 11. - A longitudinal and a cross section of 25 h gastrula raised as mentioned in the legend of figs. 4-6, respectively. The sections were treated according to the von Kósa silver nitrate method with counterstaining by naphthol yellow.