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Biologia. — *The stereocilia of the sea urchin embryo, the conditions of their formation and disappearance* (*). Nota di JOHN RUNNSTRÖM (**) e HARRY MANELLI (***), presentata (****) dal Corrisp. P. PASQUINI.

RIASSUNTO. — Durante un certo periodo dello sviluppo embrionale del riccio di mare la regione anteriore dell'acron è coperta da stereociglia dure e lunghe 75 micron. In base alla analisi morfogenetica degli embrioni trattati con actinomicina D, si è dedotto che probabilmente la formazione delle stereociglia dipende, forse indirettamente, dal RNA messaggero embrionale. Ad un certo stadio le stereociglia sono sostituite da ciglia mobili, più corte (circa 25 micron). Viene discusso il meccanismo di questa sostituzione e di altre interazioni che si svolgono all'interno dell'embrione.

The antibiotic actinomycin D reacts with deoxyribonucleic acid (DNA), particularly with its guanylgroups (see Reich and Goldberg, 1964). This does not prevent the replication of DNA but inhibits the DNA dependent RNA–synthesis. Gross and Cousineau (1963) showed that treatment of sea urchin eggs with actinomycin D allowed cleavage and formation of a ciliated blastula but no further differentiation occurred. The conclusion from these results was that certain developmental processes, as, for example, the cell divisions are independent of immediate *m*–RNA–formation. The protein synthesis necessary for the mitotic processes (Hultin, 1961) must occur by a transcription of preformed " maternal " RNA synthesized before fertilization.

Auclair and Meismer (1965) directed the attention to the formation of cilia and their proteins. Actinomycin D does not inhibit the formation of the cilia. Nevertheless the synthesis of ciliary protein demonstrated by incorporation of labeled amino acids is inhibited by actinomycin.

In a late blastula stage the cells of the acron, the most animal embryonic " zone " (see Runnström and Immers, 1966) forms the 70–75 microns long stereocilia.

The rather limited scope of this research is to inquire as to whether the formation of the stereocilia of the animal tuft is inhibited by actinomycin or not, and to discuss the results obtained as far as a morphogenetic analysis would permit.

Another question raised is that of the replacement of the stereocilia by motile cilia, which, in normal development occurs at an early pluteus-stage.

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Material and Methods.

Numerous experiments were carried out on eggs and embryos of *Paracentrotus lividus*. Supplementary experiments were carried out on *Sphaerechinus granularis*. The latter experiments confirmed the results obtained with material of *Paracentrotus*. Owing to the favor of the material, the results on *Sphaerechinus* are more clearcut. The handling of the material was the same as in previous work (Runnström and Manelli, 1964). Actinomycin D was a gift from the Research Laboratories of Merck, Sharp and Dohme. The concentrations used were 10, 12 and 15 μg/ml. According to previous experience, these concentrations give an inhibition of the RNA synthesis (Runnström and Markman, 1966, see also Ellis, 1966).

Results.

We describe here primarily the course of one typical experiment with *Sphaerechinus granularis*.

Ten hours after insemination, embryos at the stage of early blastula, still within the fertilization membrane, were transferred to:

C sea water,
I sea water with 10 μg/ml actinomycin D,
II sea water with 15 μg/ml actinomycin D.

About 15 hours later, *25 hours after insemination*, swimming embryos in test samples I and II were brought into normal sea water. The slightly elongated control embryos were clearly polar. The distance between the animal and the vegetal pole was in a typical case, 162 microns. The largest transversal axis 152 microns. The polarity was recognizable particularly by the truncate form of the vegetal region of the embryo. The vegetal plate had in a typical case a diameter of 187 microns. In the centre of the plate the height of the cells was about 42 microns. The most animal region, the acron, was slightly marked by a cap of rather low columnar epithelium, carrying a sparse tuft of 50 microns long stereocilia. The cap carrying the tuft had, at its base, a diameter of 70 microns. At the border of the cap there was a gradual transition of a low columnar into a squamous epithelium. In embryos of test series I and II, a greater thickness of the vegetal epithelium constituted the only landmark of polarity. In one typical specimen the height of this epitelium was less than 30 microns. The truncate form was only slightly indicated and the not well defined vegetal plate had a diameter of about 85 microns. The whole embryo had often a spherical form, the diameter in one typical case being 152 microns. No acron cap is differentiated and even in the most animal region the cilia are motile and about 25 microns long. The

movements of the actinomycin pretreated embryos are less stable than in the control embryos. They are moving in changing direction around their axis and may even change their axis of movement.

Embryos of the same material were observed *45–46 hours after insemination*. In the control test, the size of the acron had increased and the tuft of the ca. 75 micron long stereocilia was denser. The motile cilia outside the acron were 25 microns long. Mesenchyme formation and gastrulation had progressed in normal way. In the actinomycin treated embryos from test samples I and II, the embryos were still rather spherical. Mesenchyme cells had entered the blastocoel but their distribution was random; gastrulation was no more than just beginning (cf. Markman, 1963; Barros, Hand and Monroy, 1966). In general, the epithelium of the animal region is squamous and its cilia are motile.

In the test sample I one remarkable exception was observed. The embryo was smaller than the other ones. As a compensation, the epithelium was columnar. An archenteron had invaginated into the rather narrow blastocoel. A tuft of stereocilia had developed covering an area even greater than in the control embryos.

About *70 hours after insemination* the specimens in the control test had attained the first pluteus stage with four arms and corresponding skeleton rods. The stereocilia were now replaced by motile cilia. As a derivative of the oral ectoderm, a normal stomodeum surrounded by a ciliary band and a pharynx had appeared.

The embryos of test sample I had no arms. The acron often formed a protruding bulge consisting of rather low columnar cells carrying a sparse tuft of stereocilia. The bilateral symmetry of the embryos was marked by the oblique direction of the invaginating archenteron. It attained contact with a region of the ectoderm which was the presumptive oral field. This is composed of low columnar cells which include intercellular spaces which are well distinguishable in phase contrast. This presumptive oral field was not separated from the surrounding epithelium by a ciliary band but it was somewhat concave. In this way the bilateral symmetry of the embryo was further accentuated. However, acron, tuft, and oral field were absent in about 40 % of the embryos. This held also for the archenteron which could still be rudimentary. In this latter case the pigment cells were present in the vegetal region of the embryo. When the archenteron is growing out the pigment cells spread out below the ectoderm.

The cells of the primary mesenchyme had a normal appearance. In some embryos there seemed to be a certain tendency to formation of groups of these cells. However, a normal bilateral arrangement was never established, and only rarely some rudimentary skeleton fragments appeared. In another experiment with *Sphaerechinus* embryos, in which the time of exposure to actinomycin D (10 μg/ml) was only 11 hours, a rudimentary skeleton could finally appear. However about 40 % of the embryos had no skeleton even after 90 hours. If the primary mesenchyme cells were not organized, they

were phagocytized by secondary mesenchyme cells (see also Runnström and Immers, 1966).

In embryos of *Paracentrotus lividus*, the interactions between primary mesenchyme cells and the attachment zone were also very sensitive to actinomycin, although the bilateral symmetry of the ectoderm was manifested by a dorso-ventral elongation and the formation of an oral field, which first is rudimentary but may extend to normal proportions (Markman, 1963).

The embryos of test sample II of the current *Sphaerechinus* experiment underwent a pathological development with increasing opacity of the cells, particularly in the vegetal region.

At 95 hours after insemination, the control larvae had continued their differentiation as plutei. In the test sample I, two types of larvae could be distinguished. In one, the archenteron was not, in the other it was subdivided into its compartments. Fig. 1 shows an embryo in which a straight still undifferentiated archenteron extended in oblique direction from the anus toward a point in the oral ectoderm adjacent to the acron which carries a tuft of stereocilia.

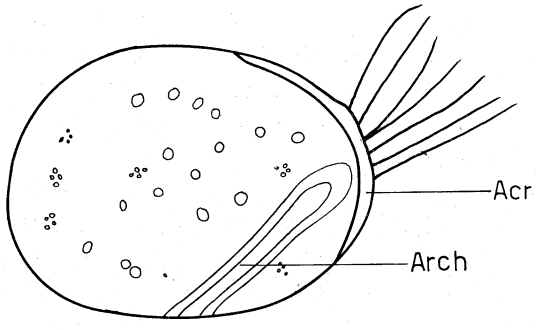

Fig. 1. – Embryo subjected to 10 μg/ml actinomycin D from 10 to 25 hours after fertilization, and kept in sea water until 95 hours after fertilization.

No stomodeum formed; non subdivided archenteron (Arch); Acr, acron. 300 × ca.

The vicinity of the top of the archenteron had evidently no effect on the tuft of stereocilia.

In other specimens, the archenteron had been differentiated into the three compartments of the intestine and the coelom rudiment. In a number of these specimens a stomodeum had been formed as an ectodermic invagination which was in communication with the oesophagus. In general, the stomodeum had an often laterally elongated exterior opening of subnormal size. The opening was surrounded by a band of motile cilia. In some cases, the stomodeum had larger dimensions, but its anterior portion could be lacking. It had thus the form of a horse-shoe with its open side directed anteriorly. The connection with the oesophagus (pharynx) was at the bottom of the posterior part of the shallow invagination. Finally, the stomodeum formation

could have been completed so as to attain a normal or almost normal form and relative size, as represented in fig. 2. It was evident that the number of cells in the acron was reduced in the specimen represented in fig. 2. The stereocilia seemed to be partly replaced by the smaller motile cilia. The stereocilia seemed also more slender that in earlier stages. Only in one case out of 30 could it be verified on living material that the stereocilia were fully replaced by shorter cilia in an embryo of the type of fig. 2. The gained impression was that an inverse correlation exists between differentiation of the stomodeum and partial or complete replacement of the stereocilia.

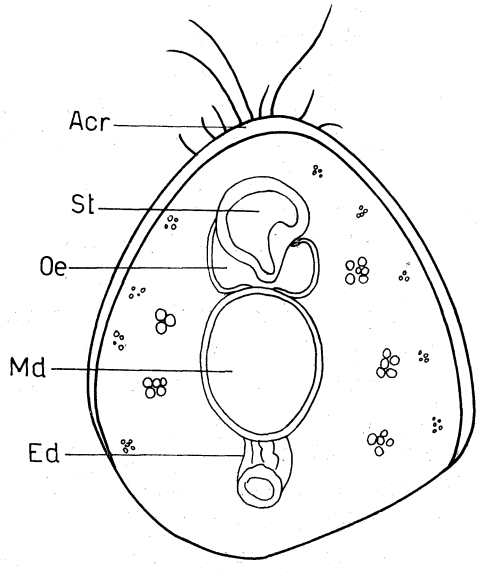

Fig. 2. – Treatment with actinomycin etc. as in the case of fig. 1.
Stomodeum formed; intestine divided into three compartments.
Acr, acron; St, stomodeum; Oe, oesophage; Md, midgut; Ed, endgut. 330 × ca.

The regulative differentiation of the stomodeum after previous treatment with actinomycin D was studied in fairly great detail in *Paracentrotus*. It seems evident that the interaction is so close that acron cells may even migrate in ventral direction and participate in the formation of the anterior part of the stomodeum (see also Runnström, 1957). There is no correlation between the size of the oesophagus and that of the stomodeum. If they joined, the fit was not always at normal level. The outgrowth and differentiation of the archenteron seemed to be favored by a contact with the ventral ectoderm in the embryos pretreated with actinomycin D. The retarded archenteron is often moving close to the ventral epithelium, both in *Sphaerechinus* and *Paracentrotus*. When, in the latter species, the archenteron was growing and differentiating without contact with the ectoderm, the oesophageal part often remained rudimentary as was previously shown by Markman (1963).

Discussion.

The data presented show that treatment with actinomycin D suppresses the formation of the stereocilia, although it does not in any way suppress the formation of the motile cilia. Some considerations are necessary before making an attempt at interpretation of the results. Actinomycin D could exert some general non specific injurious action. After exposure to the lower concentration of actinomycin, 10 μg/ml, the cytoplasm of the sensitive, primary mesenchyme cells offered, even 70 hours after insemination, a clear homogeneous appearance. About 90–100 hours after insemination, the primary mesenchyme cells became phagocytized by the secondary ones. At the same time, however, other tissues were able to proliferate. In the oral ectoderm a ciliary band and a stomodeum may be formed. The conclusion may thus be allowed that the primary mesenchyme cells finally degenerate because they have not interacted with the attachment zone in the ectoderm (Runnström and Immers, 1966). In the experiments with *Paracentrotus* a delayed formation of the attachment zone caused a revival of the skeleton forming activity of the primary mesenchyme.

The exposure to the higher concentration of actinomycin D (15 μg/ml) provoked a degeneration of the cells of the vegetal plate. This may, however, be the consequence of a more persistent suppression of transcriptions within the vegetal region of the egg [1].

There is another more important consideration to make. The suppressing action of actinomycin D on the stereocilia may be an indirect one, affecting directly some process which is necessary for the differentiation of the stereocilia. Weight was laid on the study of the polar organization of the treated and non treated egg. No stereocilia appeared in the animal region as long as the polarity was not manifest in the ectoderm. Only when an acron consisting of columnar cells was formed by an aggregation of cells in animal direction, the stereocilia appeared. It may thus be that the formation of the stereocilia are not directly inhibited by actinomycin D but it inhibits primarily the transcriptions leading to the state of cells, necessary for their aggregation to an acron. By means of autoradiography, Markman (1966) showed that aggregation of acron cells parallels an increased transcription. During this state of increased synthetic activity the formation of stereocilia may be induced.

This may still occur on basis of preformed material as in the case of the motile cilia or new material would be formed by immediate gene action or by translation of preformed *m*–RNA. The present experiments do not allow a

(1) Professor M. De Vincentiis (private communication) demonstrated that 17 μg/ml actinomycin C3 (Müller, 1962) had no toxic effect on the early development of *Paracentrotus lividus*. This substance, although not reacting with DNA (specific effect) should have about the same *general* toxic effect as actinomycin D. This effect evidently does not play any role in the low concentrations used in the present research.

decision between these possibilities. Nevertheless, it can be concluded that the formation of stereocilia is *controlled* by immediately gene-dependent processes.

There seems to be no essential difference between the ultrastructure of motile cilia and that of stereocilia. Ingredient sub units may be identical in both types of cilia. In the stereocilia, however, some new element may be adjoined, the formation of which could depend on embryonic *m*–RNA. This would then be formed only in an acron with adjacent columnar cells.

The tuft of stereocilia is not only formed at a certain stage, at which it probably serves as a stabilizer of the movement. It disappears also at a certain stage, when the role of stabilizer is taken over by the outgrowing arms. A certain slenderness and weak motility of the stereocilia is a sign of the start of their fading which may be due to depletion of the stiffening component. This may also control the length of the stereocilia.

Some data presented above indicate that the fading of the stereocilia is due to a negative inducing action of the differentiation of the oral field. The anterior part of the stomodeum seems to be particularly important in this respect. The interactions between acron and stomodeum are analogous to those prevailing at earlier stages (see Runnström, 1966, fig. 4) but at the more advanced stage the range of interaction is shorter and their character more specified (see also Czihak, 1961).

In a previous study (Runnström, 1966, pp. 254–255) highly animalized embryos were produced under the effect of 0.005–0.01 % trypsin. Even 40–50 hours after insemination, a strongly enlarged acron could carry stereocilia. At this time quite atypical ridge-like structures appeared outside the acron. This seemed to give a signal to the replacement of the stereocilia by motile cilia. The addition of 10 μg/ml actinomycin D prevented the formation of the ridges. Under these conditions, the stereocilia of the acron were not replaced even if many of the acron cells left the epithelial connection and accumulated in the blastoecel. The tight adhesion of the acron cells is thus more important for the formation of the stereocilia than for their maintenance. The studies on the animalized embryos are thus in keeping with the tentative view based on the data presented above. During the development of animal fragments, isolated at the 16-cell stage, first an extended tuft of stereocilia appeared, but about 42–48 hours after insemination the stereocilia are replaced by motile cilia (see Hörstadius, 1935). This may due to an enhancement of the oral or ventral character in connection with directed cell migration. A support for this contention was given by Runnström et al. (1964). They showed that the tuft of stereocilia is more extended and disappears later in animal halves, raised in sulphate-free than in those raised in normal sea water. The lack of sulphate interferes with the cellular movements (see for further discussion e.g. Runnström, 1966, p. 261).

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