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Biologia. — *Changes in cytoplasmic organization following fertilization of eggs of the sea urchin* Paracentrotus lividus *L.* (*). Nota di HARRY MANELLI (**) e JOHN RUNNSTRÖM (***), presentata (****) dal Corrisp. P. PASQUINI.

RIASSUNTO. — Come è stato dimostrato in precedenti ricerche, deboli dosi di tripsina (trattamento per 10–15 minuti alla concentrazione di 10^{-4}%) determinano cambiamenti nella struttura del citoplasma delle uova non fecondate di riccio di mare. Quando le uova così trattate sono fecondate, la segmentazione è bloccata. Se però il trattamento avviene dopo la fecondazione, la divisione è normale. Gli effetti della tripsina sono stati studiati dopo vari tempi di trattamento. I risultati inducono a ritenere che il trattamento con la tripsina inizia un processo che porta dapprima ad uno stato di gelificazione. Quando le uova sono fecondate in questo stato, si verifica il sopradetto blocco della segmentazione. Questo blocco, però, viene gradualmente rimosso. I cambiamenti nello strato citoplasmatico delle uova vergini trattate continua anche quando queste siano trasferite nel mezzo normale. L'effetto della tripsina può non essere esercitato direttamente sul sistema interessato alla formazione e alla rimozione del blocco della segmentazione. La tripsina, piuttosto, può attivare certi enzimi che agiscono su questo sistema. Lo stato di gelificazione, che provoca il blocco, sembra localizzato sopratutto in uno strato periferico del citoplasma che contiene precursori dell'apparato mitotico.

The early studies on structural changes in the sea urchin egg upon fertilization was surveyed by Runnström (1949). These studies based on observations with dark field illumination or on polarization microscopy led to the view that certain fibrillar aggregates (koagels) are present in the cytoplasm of the unfertilized eggs. Some of these koagels would grow into light microscopic dimensions and after some dehydration of the egg, also exhibit a birefringence, negative in longitudinal direction of the koagels. Upon fertilization, the koagels would break up and its components rearrange themselves into a more homogenous hydrated fabric.

Sugawara (1943) found that eggs of *Hemicentrotus pulcherrimus*, pretreated with low concentrations of trypsin formed a fertilization membrane adhering to the egg surface. Moreover, the cleavage was often suppressed. If the pretreatment was prolonged, the fertilization membrane became soft and cleavage occurred in a fairly normal way. This study has been extended and

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the results have been further analyzed by Runnström (1961). The present writers have used the mentioned trypsin effect as a tool in the analysis of the changes in structural organization of the egg which follow upon fertilization.

MATERIAL AND METHODS.

The material of *Paracentrotus lividus* was obtained during repeated sojourns of the writers at the " Stazione Zoologica " of Naples. The preparation of the material of egg and sperm was made as previously described (Runnström, 1961). Crystalline trypsin from " Sigma " was used throughout. The different preparations had rather similar ranges of action on the eggs. The strength of the solution is therefore not expressed in usual standard units, but in percentage trypsin in solution. Time of treatment × concentration of trypsin is approximately constant (Minganti, 1953). The dose of trypsin given is thus the decisive factor.

The interruption of the exposure to trypsin occurred by rapid dilution of the test suspension (40 ml) by adding sea water. Dilution to a volume of 500 ml seemed sufficient to bring down the trypsin concentration to non-active values. Often a dilution to 1000 ml was used without changing the effect. The addition of soybean inhibitor to the diluting sea water was tried, but did not seem necessary.

The counts were made on aliquots of the test samples fixed in 4 % neutral formaldehyde. The number of individuals counted from each sample amounted to 500–700.

RESULTS.

We begin by comparing the effect of 1×10^{-4} % ($\sim 4 \times 10^{-8}$ M) trypsin applied to unfertilized and fertilized eggs for 15 min. As follows from the curves of fig. 1 and its legend, unfertilized eggs exposed to trypsin were inseminated after washing with sea water. The fertilized eggs were subjected to treatment for 15 min. with trypsin 0.5 min. and 16 min. after insemination. As soon as some two-cell stages were observed in the control, fixations in formalin were made with intervals of three min. At the time of the fourth fixation, 73 min. after insemination, 83 percent of the control eggs had attained the two-cell stage. Conversely, in the eggs pretreated before insemination with trypsin, no cleavage had occurred within 72 min. after insemination. Only later, 2–3 % of the eggs showed a superficial furrowing. Almost all of these eggs exhibited a rich formation of the earlier described protrusions (see Runnström, 1961, figg. 4–9). After 21 hours, a rather strong disaggregation had occurred in the eggs treated before fertilization.

In contradistinction, the curves 3 and 4 compared with the control curve 2, show that treatment of the fertilized eggs with 10^{-4} % trypsin for 15 min. had no significant influence on the rate of division. Irrespective of the time of trans-

fer of the eggs to the trypsin solution, 0.5 min. or 16 min. after insemination the 50 % division time was around 4 min. After 21 hours, both control eggs (C2) and the eggs treated with trypsin after fertilization had developed into vigorous prism stages. This experiment confirms the great sensitivity of unfertilized eggs to a low dose of trypsin, but shows, moreover, the lack of response found even when the eggs are brought into the trypsin solution as early as 30 sec. after fertilization.

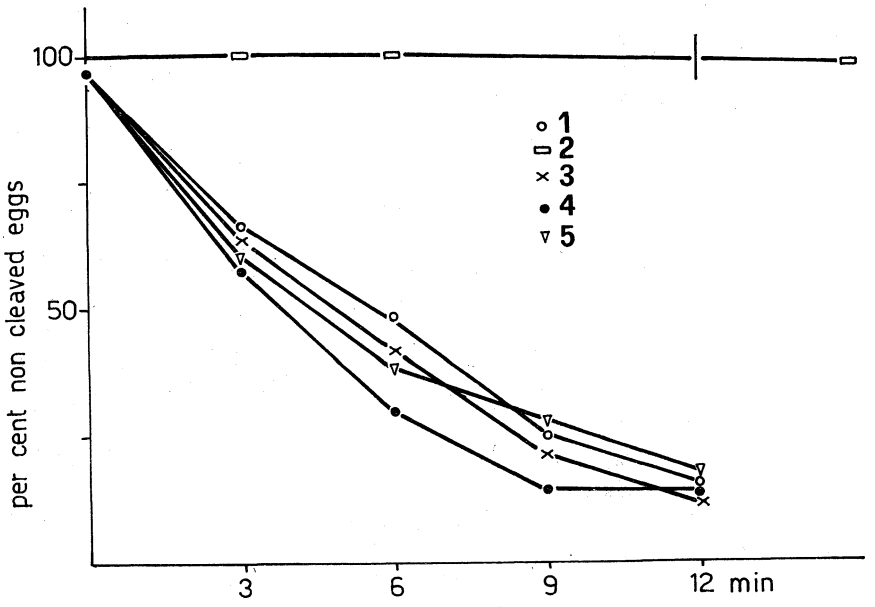

Fig. 1. – Comparison of the effect of exposure for 15 min. to 10^{-4} % trypsin of unfertilized and fertilized eggs of *Paracentrotus lividus*. The percentages of non cleaved eggs have been plotted against time.

Curve 1 refers to a control in sea water (C_1). — *Curve 2* refers to a test sample in which unfertilized eggs were exposed for 15 min. to trypsin. The exposure was interrupted by dilution with pure sea water; 36 min. later, the eggs were inseminated in parallel with curve C1 and after further 63, 66, 69 and 72 min. portions were fixed in 4 % formaldehyde. — *Curves 3, 4, and 5* refer to parallel tests of which *3* represents the control (C2). — *Curve 4* refers to eggs which, 30 sec. after insemination, were exposed to 10^{-4} % trypsin for 15 min. The first fixation in 4 % formaldehyde was made 48 min. after interruption of treatment, i.e. 63 min. after insemination, followed by three fixations at 3-min. intervals. — *Curve 5* refers to a test in which the eggs were transferred to trypsin only 16 min. after insemination and exposed to trypsin for 15 min. The first fixation was made 33 min. later, i.e. 63 min. after insemination, followed by three fixations at 3-min. intervals. The figures allow the calculation of the 50 % division time for the eggs in the different test samples.

In another series of experiments the unfertilized eggs were pretreated for a varying number of minutes with 8×10^{-5}–10^{-4} % of trypsin in sea water. The eggs were either inseminated one min. before the end of the intended period of exposure, or the eggs were washed at the end of this period and fertilized only after this procedure had been accomplished. In this way it was possible to compare the immediate effect of exposure to trypsin and the effect obtained when a period of washing (lasting for 35–45 min.) was inserted between the period of treatment and the fertilization. Fig. 2, curves 1 and 2, gives the

results of one such experiment. Curve 1 gives the percentage of unfertilized eggs in suspensions which have been inseminated 9, 14, 19 and 24 min. after the start of the exposure of the eggs to trypsin and washed by dilution 1 min. later. The counts were made on eggs fixed 120 min. after insemination. After shorter times of exposure as, for example. 10 and 15 min. (*a* and *b*), the number of eggs with inhibited cleavage was rather low, whereas those exposed for 20 or 25 min. (*c*, *d*) showed about 80 per cent non divided eggs. A process seems to advance continuously, which leads to a gradually increasing inhibition of cleavage. The eggs which were inseminated after the washing (curve 2) became on the average, fertilized 40 min. (*a'* 45, *b'* 41, *c'* 38 and *d'* 35) later than those inseminated while still exposed to trypsin. Curve 2 shows the percentage of non cleaved eggs, 120 min. after fertilization. The eggs which had undergone shorter exposure (*a'*, *b'*) to trypsin were more inhibited than those which had been exposed for a longer period (*c'*, *d'*).

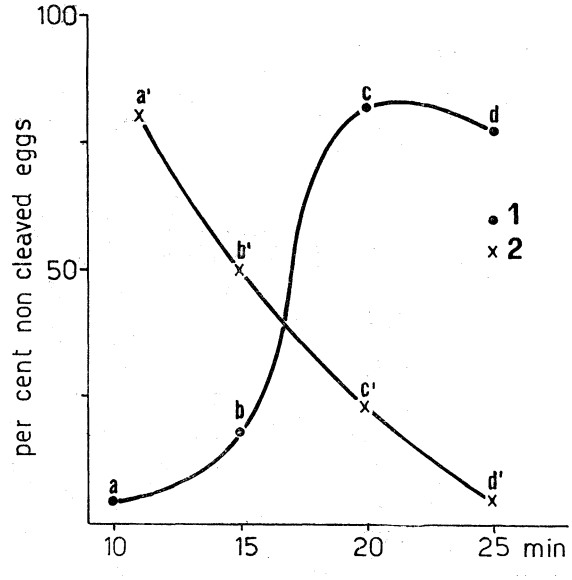

Fig. 2. – The same egg material was used in two parallel series of tests. Exposures were made to 1×10^{-4} % trypsin in sea water for 10, 15, 20 and 25 min.

In one series (curve 1, *a–d*) the eggs were inseminated one minute before the exposure was interrupted by dilution with pure sea water. In the second series (curve 2, *a'–d'*) the insemination occurred only at the end of the washing. The time difference between the fertilization of *a'* and *a* was 45, of *b'* and *b* 41, of *c'* and *c* 38, of *d'* and *d* 35 min. In every case the fixation in 4% formaldehyde occurred 120 min. after insemination. The figures give percentages of non cleaved fertilized eggs and thus express the degree of cleavage inhibition.

Fertilization of the eggs during exposure seems to stop the progress of the process started by the treatment with trypsin. This is in keeping with the results of the previous experiment, according to which the fertilized eggs are not sensitive to the trypsin doses, applied here. The percentages of non cleaved eggs recorded in fig. 2, curve 1, reflect thus approximately the state of inhibition attained at a certain time of treatment. If, however, the eggs are washed without being fertilized, the inhibitory process must continue in

the eggs, as follows from the high values of cleavage inhibition, recorded in points a' and b' (pretreatment for 10 and 15 min., respectively). The value of inhibition found after a pretreatment for 15 min. (b') is, however, lower than that found after pretreatment for 9 min. (a'). With prolonged pretreatment the inhibition decreases rapidly.

Curves 1 and 2 of fig. 2 are almost reversed, relative to each other. When one reports a high, the other reports a low degree of cleavage inhibition for the same time of exposure. This could mean that two consecutive processes are at work. One building up an inhibitory state, the other relaxing this state.

Character of the states of inhibition and of release of cleavage.

Runnström (1961) considered that the inhibition of cleavage and accompanying processes (lack of membrane elevation, formation of protrusions) are due to a gelation, caused by the release of a proteolytic enzyme(s) subsequent to treatment with a low trypsin dose. Data like those presented in fig. 2 show that, at short duration of the treatment, the gelation may not occur during the exposure proper, but rather in the course of washing the eggs, even if any trace of trypsin was removed by short treatment with soybean inhibitor (2×10^{-4} %). A further study of fig. 2, curve 1, shows that at prolonged treatment with trypsin (for 19 and 24 min.) a gelation occurs while the eggs are still exposed to trypsin. After washing, however, no, or only a low degree of inhibition of cleavage occurs. This is the reversal referred to above and may mean a shift in enzyme activity.

Lundblad (1954) demonstrated the presence of two groups of proteolytic enzymes which are activated upon fertilization, the trypsin-like E2 and the SH–enzymes E1 and E3. According to Lundblad, E2 initiates the process and is of importance in the early cortical changes. E2 soon undergoes a decrease in activity but, at the same time, the activity of E1 and E3 is enhanced. The shift shown in curves 1 and 2 of fig. 2 may involve similar changes in activity of concatenated proteolytic enzymes, E2 possibly exerting the gelating, E1 and E3 the dispersing effect on the cytoplasm of the unfertilized egg. The gelation in the unfertilized eggs following treatment with a low dose of trypsin is completely removed by subjecting the eggs after the treatment to glutathione (Runnström and Kriszat, 1962). In the course of the present work, it was shown that simultaneous exposure to 10^{-4} % trypsin and 2.5×10^{-3} M reduced glutathione for 15 min. gave no inhibition of cleavage, whereas in a parallel test sample the trypsin treatment alone gave 98 % cleavage inhibition. This is again parallel to results obtained by Lundblad: E2 is rather inhibited but E1 and E3 enhanced by the presence of cysteine which, in this respect, is equivalent to glutathione.

Why should low doses of trypsin induce the inhibited state in the unfertilized, but not in the fertilized egg? Observations still in course have confirmed that tangentially oriented fibrous structures are present in the periphery of the unfertilized eggs. Upon activation of the egg, these structures assume gra-

dually a radial orientation. They are now visible as the rays of the « spermaster ». Exposure of the eggs to 10^{-4}% trypsin may cause an increased bonding between the fibrous tangential components, which are discernible even in the light microscope. These may, however, not be the primary elements in the reorientation process. Submicroscopic fibres may play this role. Their orientation may be reflected also in that of elongated microscopic fibres. How can an increased bonding in a peripheral layer of the unfertilized egg impede cleavage? It can be shown by centrifugation that the peripheral layer of the eggs, exposed to trypsin in low doses, has a higher apparent viscosity than that of control eggs. Moreover, the fibrous structure shows more continuity in centrifuged pretreated eggs than in control eggs. This is, however, not the only cause of the inhibition of cleavage. One essential circumstance is that the mitotic apparatus is reduced in size in the pretreated eggs. The astral rays may be in continuity with a peripheral tangential layer. This favors the view that material which participates in the formation of the mitotic apparatus has, before activation, a position in a peripheral subcortical layer.

The reason why exposure to trypsin in low doses induces cleavage inhibition in unfertilized but not in fertilized eggs seems thus clear. In the former, the treatment provokes an increased bonding between peripherally arranged components. After fertilization, however, these components change both their position, orientation and properties in such a way that they can no longer undergo the bonding. Treatment with histone may produce effects similar to those obtained upon exposure to trypsin of low dose.

The relaxation of the state leading to cleavage inhibition which is expressed in the slope of curve 2, Fig. 2, has been discussed above. This may be a process of great significance in the initiation of development.

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