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On the reception and activation system of the sea urchin (Paracentrotus lividus)

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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Biologia. — *On the reception and activation system of the sea urchin (Paracentrotus lividus)*. Nota di JOHN RUNNSTRÖM e C. NUZZOLO, presentata (*) dal Corrisp. P. PASQUINI.

RIASSUNTO. — Si tratta di nuovi esperimenti eseguiti al fine di distinguere nel processo di fecondazione dell'uovo di riccio di mare, due diversi sistemi di reazione: il sistema di ricezione dello spermio ed il sistema di attivazione dell'uovo. Il primo si è potuto bloccare con il pretrattamento dell'uovo con tripsina o pronasi. Le uova così pretrattate possono ciononostante essere attivate con il timolo. Il sistema di ricezione è anche bloccato, per esempio, dall'uretano e dal laurilsulfato; ma è sempre possibile l'attivazione con timolo in presenza di questi inibitori della fecondazione.

La velocità di attivazione dell'uovo è stata misurata seguendo la formazione di acido che interviene dopo la fecondazione o l'attivazione sperimentale dell'uovo per aggiunta di timolo. Questa formazione di acido si considera segnale dell'attivazione. Si dimostra che la formazione di acido è più lenta della fecondazione normale che nell'attivazione sperimentale Questo indica che la fase di ricezione dello spermio ha un valore limitante nella fecondazione. Tenuto conto della sensibilità del sistema di ricezione agli enzimi proteolitici, si può concludere che le proteine possono costituire una parte essenziale del sistema di ricezione dello spermio che gli attuali esperimenti mettono in evidenza.

Hagström (1958) found that pretreatment of unfertilized eggs of several sea urchins with 10^{-3} % cryst. trypsin for 10 min. caused a considerable decrease in the rate of fertilization. Runnström and Kriszat (1960) observed that upon pretreatment of unfertilized eggs of *Psammechinus miliaris* with cryst. trypsin a number of the eggs became non fertilizable. They showed moreover that these non fertilizable eggs could be activated by exposure for 5 min. to 10^{-4} M sodium periodate in sea water. The eggs went through the early stages of the mitotic cycle and some of them underwent a more or less regular cleavage into two cells. It was inferred from these data that in the fertilization of the sea urchin two systems are operating, a reception system and an activation system. Using a somewhat different technique, Aketa (1967) has confirmed the previous results in work on the sea urchin *Hemicentrotus pulcherrimus*. He pretreated the eggs with solution of e.g. 0.4% pancreatine for 20 min. and found that 80% of the eggs failed to respond to sperm addition. It was shown, however, that the non responding eggs could be activated by treatment with an urea solution.

(*) Nella seduta del 10 gennaio 1970.

The following paper reports about new experiments pertaining to the sea urchin *Paracentrotus lividus* and to a lesser extent to *Spaerechinus granularis* with the aim of studying the reception and activation system in the eggs of these species.

METHODS.

The gametes of the sea urchins were obtained by removing the ovaries or testicles from the animals. The eggs released from the ovaries were filtered through bolting silk and washed thoroughly with off shore sea water. The dry sperm was collected in tubes and kept in the refrigerator until use. Cryst. trypsin and pronase from Sigma Chemical Co. were used. In order to activate the eggs parthenogenetically they were exposed to 20–30% saturated solution of thymol for different numbers of seconds (Ishikawa, 1962).

RESULTS.

The effect of proteolytic enzymes on the reception and activating system.

In a number of experiments the eggs have been exposed to 50 μg/ml trypsin or 10 μg/ml pronase. As was determined by several trials these concentrations seemed to be appropriate.

One experiment with Paracentrotus will be quoted here (N. 68, 2/16).

C is a sample of non pretreated control eggs.

A is a parallel sample where the eggs were subjected for 15 min. to 50 μg/ml trypsin in sea water. After the pretreatment, the samples were concentrated from 30 ml to about 10 ml, and then washed with 1000 ml of pure sea water. The control was washed at the same time but only with 500 ml. The washed samples were brought to a volume of 20 ml and inseminated in this (ca. 15,000 eggs and 1 million spermatozoa per ml). Eighty minutes after insemination the number of fertilized eggs was determined.

	Non Fertilized	2-cell stages	number counted
C	3%	97%	403
A	97%	3%	483

In the few fertilized eggs the expulsion of the lamellae (Endo, 1961) took place. The lamellae were fixed at the outer border of a gel protruding from the egg. The gel could swell and disappear under the release of almost sphaerical lamellae which spread in the outer medium, or the lamellae were converted into rods or plates, often of triangular form. The spermatozoa seemed to have a certain affinity even to the eggs which did not become fertilized. They could be attached to the surface and carry out pendling or circular movements with the acrosome as the fixed point.

There is a considerable variation in the number of eggs which are refractory against fertilization following pretreatment with trypsin or pronase.

After treatment with the enzymes under the conditions mentioned, the rate of fertilization is reduced, even if the number of completely refractive eggs is low. In the non pretreated control eggs, usually close to 100% are fertilized after 1 minute. After insemination of trypsin or pronase pretreated eggs, the inhibition may for example, be 50% at 60 sec. but only 10% 90–120 min. after insemination. If, however, the insemination gives no, or a very low fertilization during the first minute, only a very low number of eggs are fertilized during a consecutive period of about two hours. There are many intermediate degrees between these extreme cases, which creates the great variations observed also in the experiments of Bohus-Jensen (1953) devised in a different way.

When the washings after trypsin treatment were accomplished, the volume of the test samples was brought to 30 ml and 7.5 or 12 ml of a saturated solution of thymol in sea water (20–30% thymol) were added under rapid mixing. After different times (50, 80, 160 and 180 sec.) portions of 6 ml of the mixture was transferred into 500 ml sea water in order to interrupt the action of thymol. It could be seen that an activation of the thymol treated eggs had occurred in the whole series of exposure to thymol. Elevated membranes were observed in the non pretreated control samples, whereas in the trypsin pretreated samples the activation was recognized by the migration of the nucleus to the centre of the eggs, its growth and final dissolution. Radially directed fibre systems appear in the cytoplasm, these were later converted into mono- or diasters. Only few divisions into two or more cells were observed. Thirty minutes after transfer to normal medium, eggs treated with thymol for 80 sec. were exposed to hypertonic sea water; to a test sample of 10 ml, 1.5 ml 2.5 M NaCl were added under rapid mixing. After 30 minutes the egg suspension was concentrated and 120 ml normal sea water was added. The examination of the test samples occurred 2 hours, 20 min after the transfer from the hypertonic medium to normal sea water. The counts gave the following result where figures indicate the frequency of the different catogories of eggs expressed in percent of the total number of eggs.

	Cytolysis dark, granular	Non activated	Activated non divided		Divided	Number counted
Control non pretreated with trypsin	18.2	—	7.5	79.8	72.3	333
Pretreated with trypsin	28.5	—	26.6	69.6	43.0	439

The eggs which have been pretreated with trypsin are activated to almost the same degree as the non pretreated eggs. In any case the number of activated pretreated eggs belong to a different range as compared with the

number of eggs which underwent fertilization after trypsin treatment. The cytolyzed eggs passed through a stage in which the interior cytoplasm seemed granular. They are in fact activated eggs where the radiation in the interior has coarsened. The cytolyzed non pretreated eggs have a fertilization membrane. The number of cytolyzed eggs is somewhat higher in the trypsin preteated than in the non pretreated eggs. Likewise, the number of non divided eggs is higher in the former than in the latter category. This shows that the trypsin pretreated eggs have undergone a certain damage. This can

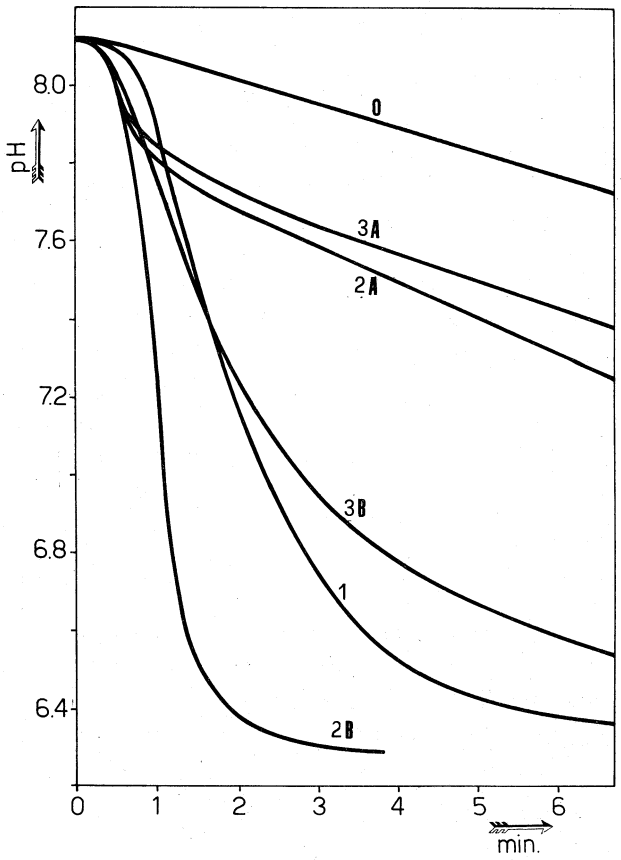

Fig. 1. – Curve 1 represents acid formation in a fertilized control sample. Curves 2 A and 2B refer to eggs which have been pretreated with 50 μg/ml trypsin, inseminated and then exposed to thymol of 30% saturation (curve 2 B). Curves 3 A and 3 B are a similar pair but the concentration of thymol was 20% saturation (3 B). O is a control curve referring to titration of pure sea water.

be avoided, however, if lower doses of trypsin are used but the difference in number of non-fertilized and artificially activated eggs becomes then less striking but may still be convincing. The primary structural changes observed in the activated eggs will be described elsewhere. The cleavage was rather irregular and resulted only in a minor number of filled blastulae in the non pretreated eggs, whereas in the pretreated ones a strong dissociation of the cells occurred. No attempts have been made to adjust the periods of exposure so as to obtain more successful results with respect to development.

As known from the manometric determinations by Runnström (1933) an acid is formed upon fertilization of sea urchin egg. In newer experiments by e.g. Mehl and Swann (1961), Ohnishi and Sugyiama (1963) and Aketa (1961a), the acid formation has been determined by titration in non buffered artificial sea water. This method has been used by us in the present work. Fig. 1 shows some of the curves obtained by automatic registration of the *p*H–values of the egg suspension different times after fertilization or addition of thymol. The acid formation is low upon insemination after trypsin treatment (curve 3 A). This is due to the small number of fertilized eggs. A part of the decrease in *p*H may be due to carbon dioxide produced by respiration and to uptake of carbon dioxide from the atmosphere, curve O. If now thymol of 30% saturation is added to the inseminated eggs, a very rapid acid formation occurs (curve 2 B). The steepness of the curve is greater than in the control (curve 1). If, on the other hand, the thymol concentration is lowered to 20% saturation (curves 3 A and 3 B), the steepness of the curve is lowered. Quite corresponding results were obtained when the eggs were pretreated with 10 μg/ml pronase for 15 min. and thereafter were subjected to 30% saturated thymol (fig. 2).

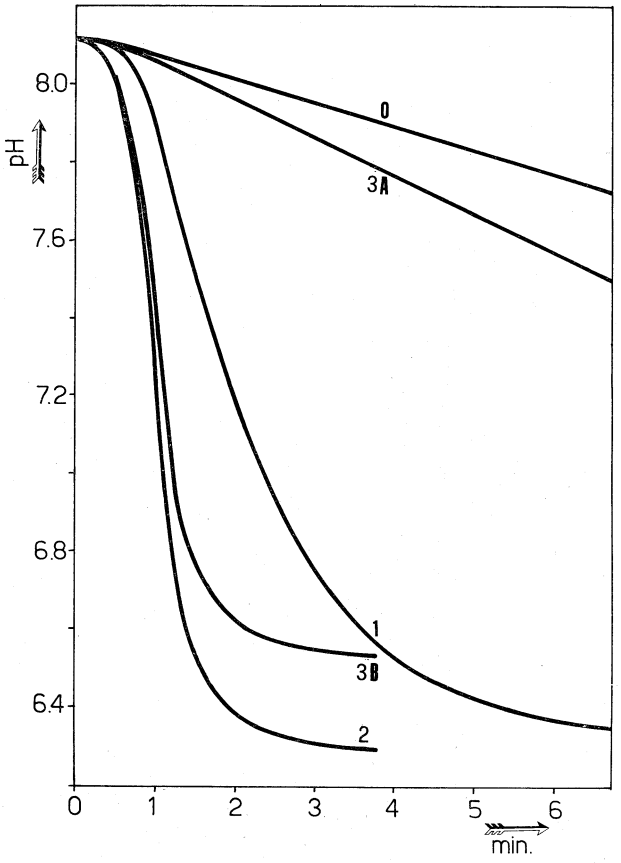

Fig. 2. – Curve 1 as in fig. 1. Curve 2 refers to the acid formation after adding thymol 30% saturation in sea water in non pretreated eggs. Curves 3 A and 3 B refer to a material pretreated with 10 μg/ml pronase and inseminated (3A) and afterwards exposed to thymol, 30% saturation in sea water (3 B). O as in fig. 1.

The effect of urethane on the reception and activation system.

Aketa (1961 b) made the interesting discovery that the acid formation following upon insemination of the eggs of the sea urchins *Pseudocentrotus depressus* or *Hemicentrotus pulcherrimus* is kept down or abolished by addition of urethane. The extent of this effect is dependent on the concentration of urethane. Aketa interprets his results as being due to an action on the cortical particles. This action should inhibit the opening of the cortical particles.

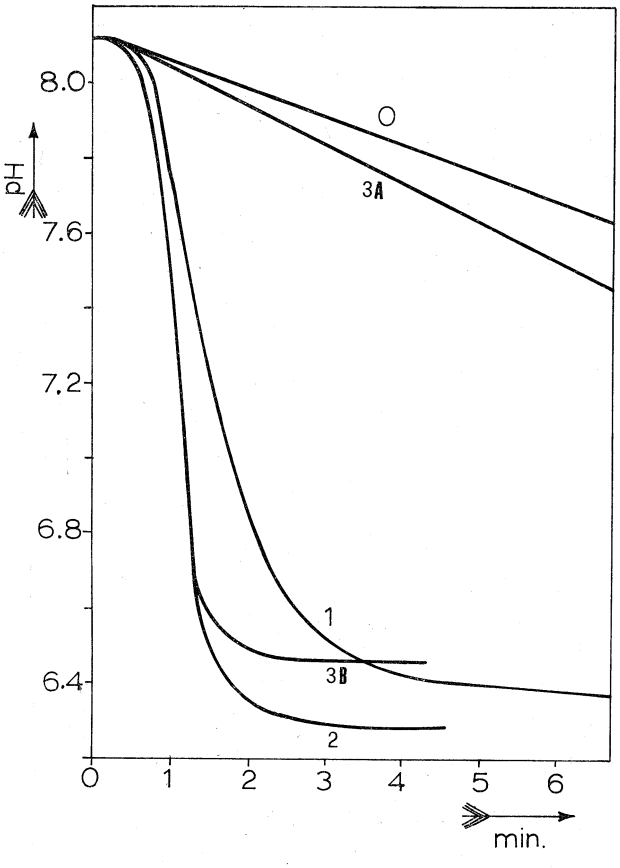

Fig. 3. – Curve 1 as in fig. 1. Curve 2 corresponds to eggs treated with thymol alone. After pretreatment with 0.5 M urethane the eggs were inseminated (curve 3 A) and afterwards exposed to thymol 30% saturation in sea water (3 B). O as in fig. 1.

An experiment is here presented which possibly points to a conclusion congruous with the results of the preceding section of this paper. In experiments with *Sphaerechinus granularis*, the inhibition of the acid formation by 0.5 M urethane was confirmed; on subsequent addition of thymol of 30% saturation in sea water, a very rapid acid formation occurred. The eggs were subjected to 0.5 M urethane in sea water for 5 or 8 minutes. The urethane was then removed by 5 times washing with sea water, only some few eggs with elevated membrane were observed. Thirty minutes or even as late as 43 minutes after beginning of washing, sperm was added, causing no fertilization. The

narcotizing effect of urethane remains thus for a certain period after removal of the urethane. When then thymol is added the acid formation is observed (fig. 3, curve 3B). The eggs treated for 5 minutes in urethane and then washed 5 times with water, cytolyzed after about 80 minutes after transfer to sea water.

It was an advantage of the experiments with urethane that the vitelline membrane is not damaged as after trypsin treatment, so that a membrane is elevated after the addition of thymol. The registration of the effect of the addition of thymol can thus be done immediately without waiting for the inner changes.

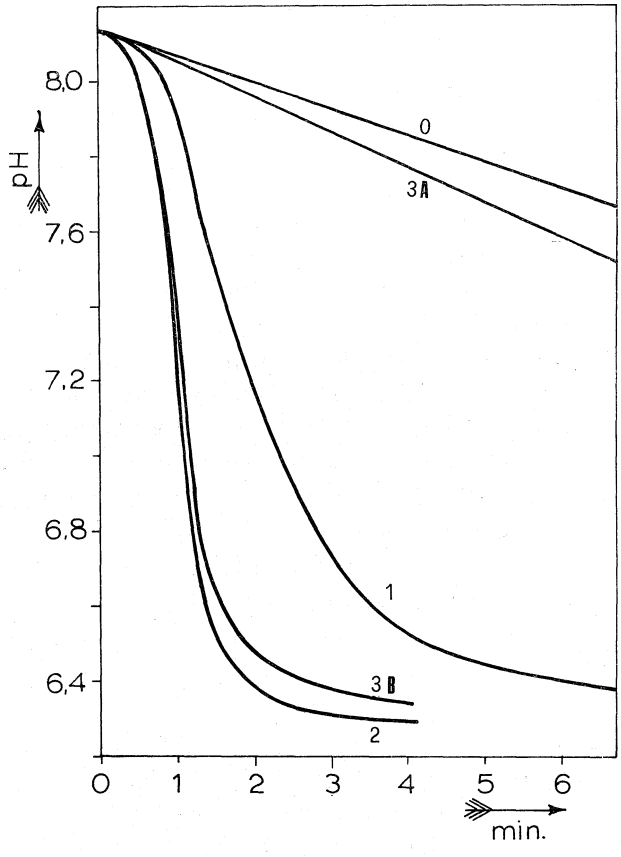

Fig. 4. – Curve 1 as in fig. 1. Curve 2 shows the acid formation in non pretreated eggs after addition of thymol, 30% saturation in sea water. Curves 3 A and 3 B show the acid formation of laurylsulfate pretreated eggs after insemination (curve 3 A) and after subsequent addition of thymol 30% saturation in sea water (curve 3 B). O as in fig. 1.

The effect of laurylsulfate on the reception and activating system.

Runnström *et al.* (1945) found that a low concentration of detergents including a detergent isolated from sperm (A 3) have an inhibitory effect on fertilization which is not only due to a disturbance of the motility of the spermatozoa (loc. cit.), even if this latter effect predominates at the concentration of 10 μg/ml laurylsulfate used in the experiments of Hagström and Hagström (1954). Fig. 4 shows that laurylsulfate does not prevent the activation of eggs upon addition of 30% saturated thymol.

DISCUSSION.

The results reported above confirm the previous conclusion regarding the presence in the sea urchin egg of a reception and of an activation system. The separation of the two systems has thus been demonstrated for four different species, *Psammechinus miliaris* (Runnström and Kriszat, 1960), *Hemicentrotus pulcherrimus* (Aketa, 1967), *Paracentrotus lividus* and *Spaerichenus ganularis* (Runnström and Nuzzolo, present paper). The reception system has been removed by treatment with trypsin and pronase (above) and pancreatine (Aketa, 1967). The activation was brought about by treatment with periodate (Runnström and Kriszat, 1960) urea (Aketa, 1967) and thymol (above). The receptor system is not affected by a hyaluronidase from bull testicle (Runnström *et al.*, 1943) or by neuramidase from vibrio cholerae (Runnström and Kriszat, 1960).

The reception system must have a high degree of species specificity which prevents the reception of foreign sperm. Hultin (1948) removed the vitelline membrane from the egg by treatment with a crude preparation of trypsin. As a consequence, the extent of fertilization with foreign sperm increased, i.e. the species specificity was lowered. It is evident that the vitelline membrane is impaired or even removed by the treatment with trypsin. Hultin (1948) inferred that the vitelline membrane is the main site of species specificity in fertilization. Thus the vitelline membrane may be the site of the receptor system. There is a brief period after effective attachment in which the acrosome of the spermatozoon sticks strongly to the vitelline membrane, but its adhesion to the cytoplasmic surface layer is not fully established (Runnström *et al.*, 1959, and for discussion and references, Runnström, 1966, p. 260 and following).

The vitelline membrane should probably not be looked at as a strictly delimited layer. The immuno-electron microscopic studies of Baxandall *et al.* (1964) suggest a more dynamic view of the vitelline membrane. Antibodies against unfertilized jelly-free eggs were prepared in rabbits. A certain fractionation of the antibodies was brought about by absorption experiments (Perlmann, 1959). These allowed the distinction of three different antigens or groups of antigens, 1) heat-stable soluble A–antigens, 2) heat-stable, non soluble F–antigens, 3) heat-labile C–antigens. The total fractionated anti–egg–γ–globulins had been conjugated to ferritin, according to the one-layer or two-layer method before they were applied to the eggs. Thereafter, the site of the antibodies in the egg surface could be demonstrated after fixation and processing of the eggs for electron microscopy. Most of the anti–egg–γ–globulines proved to be present in the vitelline membrane, whereas the plasma membrane was rather poor in labelled anti–egg–γ–globulines. This could be due to lack of penetration of the macromolecular antibodies. However, the removal of the vitelline membrane by pretreatment with trypsin did not increase the deposition of the labellaed antibodies in the plasma surface. Thus it is evident that the vitelline membrane is the main site of the specific anti-

bodies present in the egg surface. As shown by Perlmann, the A–antigen is connected with the activation of the egg; treatment of the eggs with anti–A–γ–globulin may under certain defined conditions induce the first steps in development. The F–antigens, on the other hand, are involved in the fertilizability of the egg, the C–antigens in the spreading of the activation impulses. Baxandall expressed the opinion that the F and C–antigens are the receptors. The egg–antigens present in the vitelline membrane have evidently a certain diffusibility. These antigens are namely often found in the jelly coat, the main substance of which constitutes a thermostable J–antigen. Perlmann (see 1959) observed seasonal variations in immunological properties of the jelly coat which indicate an invasion of substances from the vitelline membrane into the jelly coat.

As the receptors are attacked by trypsin and by pronase they may have protein nature. It seems therefore probable that the C–antigens are the immunobiological counterparts of the receptors. The proteins may, however, cooperate with some thermostable components which are unable to act alone as receptors, possibly the couterparts of the F–antigen.

Aketa (1967) mentions without giving experimental details, that the eggs of *Hemicentrotus* made non fertilizeable by treatment with pancreatine may reestablish their fertilizability when kept for a considerable time in normal sea water. This opens the perspective that the receptors may even normally be subjected to a certain turnover. In a considerable number of experiments with Paracentrotus lividus we have not been able to confirm the findings of Aketa.

The activation system is able to operate even after disorganization or removal of the vitelline membrane, as follows from the work of Perlmann and his collaborators. The initial step of activation corresponds in the sea urchin egg to changes in the plasmalemma and in the membrane of the cortical particles, see Runnström (1966). As a consequence, a fusion of these membranes occurs which must be the expression of inner changes in the mentioned membranes.

The rate of acid formation in the normally fertilized eggs is always lower than in the eggs subjected to 30% saturated thymol, as follows from a comparison of, for example, curves 1 and 2 in fig. 2 and 4, respectively. This tends to show that the reception step is the rate limiting one in fertilization. The narcotizing effect of urethane affects the reception mechanism. A direct action on the spermatozoa is excluded as the narcotized state is lasting for more than 30 minutes after removal of the urethane. It does, however, not affect the activation brought about by brief exposure of the unfertilized eggs to thymol.

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