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## On the hatching of ascidian larvae

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# SEZIONE III

**(Botanica, zoologia, fisiologia e patologia)**

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**Embriologia sperimentale.** — *On the hatching of ascidian larvae* (*). Nota di SERENA CAGGEGI, ANNA FLUGY, EGIDIO PUCCIA e GIUSEPPE REVERBERI, presentata (** dal Corrisp. G. REVERBERI.

RIASSUNTO. — È stato studiato l'enzima della schiusa preparato dall'acqua di coltura di embrioni di *Ciona intestinalis*.

L'acqua di larve, saggiata su opportuni substrati, ha rivelato duplice attività enzimatica: proteolitica e glicolitica; l'analisi elettroforetica ha corrispondentemente dato due bande di assorbimento.

L'incorporazione di $^{3}H$-leucina ha mostrato che la sintesi di tale enzima avviene in un'epoca precoce dello sviluppo embrionale.

In opportune condizioni ne è stata possibile la cristallizzazione.

## INTRODUCTION

1. In many animals the eggs are protected by a few membranes, within which they perform their embryonic development. At a given stage the embryo or larva hatches, and becomes free. The mechanism of hatching is various, the most interesting being the chemical one: in this case the embryo produces one or more enzymes, which dissolve the membranes.

These enzymes are generally called " hatching enzymes ". In only a few cases the enzymes have been isolated and purified: as in *Anthocidaris crassispina* (Yasumasu [1]); in *Strongylocentrotus purpuratus* (Barrett *et al.* [2]), and in *Oryzias latipes* (Yamagami [3]).

2. The egg of the Ascidians is also protected by membranes: that of *Ciona intestinalis* has only one membrane, the chorion, from which numerous, long, estremely modified, follicular cells depart; however, between the chorion and the egg's surface one notices also a sheet of free cells, the " test cells " whose function is still unknown. All the embryonic development up to the complete larva, occurs inside the chorion which, when the larva emerges, remains free floating in sea-water.

3. The hatching, as shown by Berrill [4], is motivated by an enzyme, a protease, which according to the Author seems to be secreted by the epidermic cells of the larva. The enzyme does not seem to be specific, and is inactivated by heat. The hypothesis of Knaben [5] that the enzime is secreted by the " test cells " is not confirmed by the research of Osti [6] who showed

(*) Lavoro eseguito nell'Istituto di Zoologia dell'Università di Palermo.
(**) Nella seduta del 28 maggio 1974.

that the larvae which originate from eggs precociously deprived of their membranes dissolve the chorion of unfertilized eggs situated in their proximity.

4. Apart from the research of Berrill [4] already mentioned, no other investigations have been carried out on the "hatching enzyme" of the Ascidians; thus it seemed useful to enter that field. The present research is preliminary. The enzyme was isolated in cristalline form; some of its properties were determined and the time of its synthesis throughout the embryonic development was established.

## Material and methods

*a*) The "larval water".

Larvae of *Ciona intestinalis* after hatching were removed from the culture medium by filtration or centrifugation, and the water thus obtained was dyalized against distillated water and concentrated 5–10 times by evaporation under vacuum 37° C. Its biological activity was tested on the membranes of the unfertilized eggs.

*b*) Isolation of the enzyme.

The enzyme contained in the water was precipitated by ammonium sulfate (75% of saturation). The precipitate was collected, dissolved in distillated water and dyalized. A sample of enzyme solution was analyzed by electrophoresis on polyacrialamide gel (7 %, pH 8.9; 90 min; 3 m Ampers-tube) as described by Davis [7].

*c*) Proteolytic activity.

Samples of "larval water", at different degrees of concentration, were incubated for one hour with an equal volume of 1 % casein solution. The insoluble fraction was precipitated by adding trichloric acetic acid (TCA) up to 0.2 N of final concentration and the free aromatic aminoacids obtained by the enzymatic hydrolisis were determined by the pholin reagent, reading at 750 nm.

Blanks of casein plus sea water and blanks of "larval water" were run simultaneously.

*Glycolytic activity.*

The glycolytic activity of the "larval water" was tested by a slight modification of the method described by P. Bernfeld [8] for the amylase test.

The reaction mixture consisted in 0.55 % soluble starch in 0.02 M Na phosphate buffer pH 7.0 as substrate; and "larval water" at different concentrations as enzyme.

Incubation was carried out for 5 minutes at 25° C; colour reagent (1 %, 3,5 dinitrosalicylic acid, 30 % K–Na tartrate, in 0,4 N Na OH) was added, followed by 5 minutes incubation in a boiling bath. The enzymatic hydrolisis of the starch in maltose was determined, reading at 540 nm.

Blanks of starch plus sea water, and blanks of " larval water " were analyzed simultaneously.

*Crystals preparation.*

Drops of " larval water " were poured into an agar-well, on a microscopic slide. The preparations were left for several days in a vacuum dessicator over $CaCl_2$.

*Embryonal development in* $^3$H-*Leucine medium.*

Embryos at different stages of development were separately incubated for 3 hours in sea water containing 40 μc/ml of 3 H-leucine (specific activity 785 mc/mmole) followed by several washings in 0.25 mM (cold) leucine, and cultured in free leucine medium up to hatching stage.

" Larval water " samples were then collected and the enzyme preparations so obtained were analyzed for the $^3$H leucine incorporation in a liquid scintillation counting.

## RESULTS

The " larval water " shows peculiar qualities when compared with normal sea water; unfertilized eggs with their membranes, if introduced into such water, after some time lose their membranes and become " naked "; however, the " larval water " does not completely dissolve the chorial membrane. It is not species specific, as the " larval water " of Ciona also acts on the chorial membranes of the eggs of *Ascidia malaca*. That agrees with the investigations of Berrill [4].

The hatching enzyme is not produced from the " test-cells ", as claimed by Knaben [5]; we have many times repeated the experiment of Osti [6] always confirming her results. The larval water has proteolitic activity when tested on casein substrate (Table I).

TABLE I

*Proteolitic activity of larval water on casein substrate*

| Larval water (proteic content in mg/ml) | Optical density at 750 nm |
|---|---|
| 0.4 | 0.120 |
| 0.2 | 0.062 |
| 0.1 | 0.035 |

Comparatively a trypsin (Sigma, type III) solution, at a concentration of 0.2 mg/ml, acts on the casein substrate, giving at the end of the enzymatic reaction 0.280 optical density. Thus the proteolytic activity of the larval water, if compared to that of an equal concentration of trypsin, is 22.10 %.

Besides the proteolytic activity, the " larval water " has a glycolytic activity when tested on soluble starch (Table II).

TABLE II

*Glycolytic activity of the larval water on soluble starch substrate*

| Larval water (proteic content in mg/ml) | Optical density at 540 nm |
|---|---|
| 2.00 | 0.144 |
| 1.00 | 0.070 |
| 0.50 | 0.037 |

Comparatively a α amylase (Sigma type IIA) solution at concentration of 1 mg/ml acts on the soluble starch, giving at the end of the enzymatic reaction 0.350 optical density. Thus the glycolytic activity referred to that of an equal concentration of α amylase is 20 %. Both activities are linear functions of proteic content.

This double activity is confirmed by the results obtained when unfertilized eggs with their membranes are treated with trypsin or amylase, or using both. A 0.4 mg/ml trypsin solution destroys the egg membranes in 3 hours: at the same concentration α amylase acts to a lesser extent.

The enzyme preparation from the larval water shows two distinct electrophoretic bands (Plate I, fig. 1).

Crystals were obtained from larval water: under phase contrast microscope they appear needle-shaped and often they are aggregated so as to present a radiated form (Plate I, fig. 2).

The results of the embryonal development in the $^{3}$H-leucine medium are reported in Table III.

TABLE III

*$^{3}$H-leucine incorporation in the hatching enzymes*

| Stage of incubation | CPM/mg protein |
|---|---|
| From fertilization up to gastrula . . . . . | 2000 |
| From tail bud up to swimming larva . . . . . | 2700 |
| From swimming larva up to 2 hours later . . . . . | 200 |

As can be seen the highest rate of synthesis is reached after the tail bud stage. There is no appreciable incorporation after the hatching.

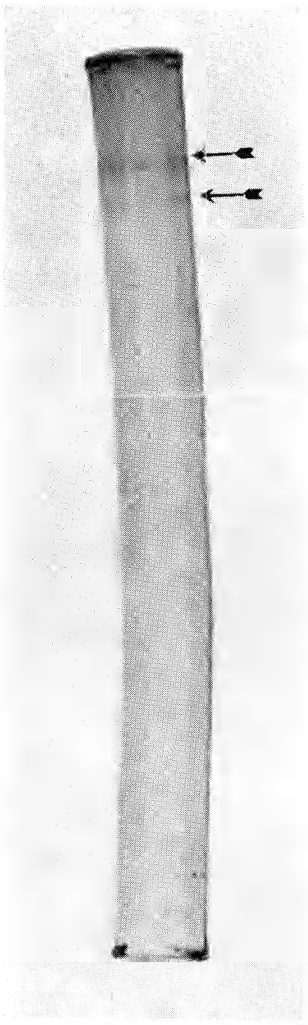

Fig. 1. – Electrophoresis on polyacrylamide gel of the hatching enzyme. Two distinct bands are visible.

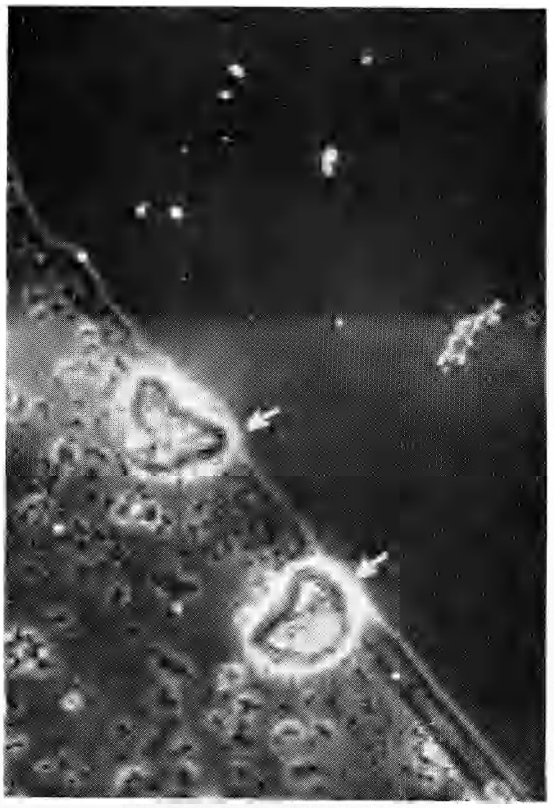

Fig. 2. – Crystals preparations of the hatching enzyme (×375).

## DISCUSSION

The results of the present research can be summarized as follows:

*a*) The water in which larvae have hatched shows proteolytic as well as glycolytic properties which seem dependent on the presence of two distinct enzymes. This hypothesis is supported by the results of the electrophoretic analysis (two distinct bands), and from the treatment of the eggs with trypsine and amylase. Following treatment the membranes are dissolved; the activity of the two enzymes together is also stronger than that of the separate enzymes. The heterogeneous nature of the hatching enzymes has also been demonstrated in *Oryzias* (Yamagami [3]), and *Strongylocentrotus* (Barrett *et al.* [2]).

*b*) The enzyme (*s*) was obtained in crystalline state: the biological activity of the crystals however was not tested since they were too small to be used for that purpose.

*c*) Incubation of the eggs at different developmental stages with tritiated leucine shows that the enzyme (*s*) are released into the culture medium at coiling larva stage; their synthesis starts much before, probably at gastrula stage, reaching, at tail bud stage, the highest rate.

Experiments with actinomycine (Librera [9]) showed that this antibiotic does not block the hatching of the larvae. For this reason we maintain that the information for the synthesis of the enzyme is released during the oogenetic development and is mainly translated from the tailbud stage ownards. This conclusion agrees with the results of Barrett and Angelo [10], not however with those of Yasumasu [1] (*).

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