Cytochemical Studies on Hatching Gland Cells in Two Fresh-water Telests, *Odontobutis obscura* and *Zacco platypus*

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(*With 1 Text-figure*)

There are several reports on the morphological study of hatching gland cells of teleosts. It has been shown that the hatching glands cells of teleosts contain secretory granules which can be stained strongly with eosin and picro-blue-black, and that those granules contain a hatching enzyme which acts on the chorion at the hatching stage. According to Ishida ('48), the hatching enzyme seems to be a sort of proteolytic substances like trypsin. The present author has attempted to learn the cytochemical nature of the hatching gland cells in two teleosts, *Odontobutis obscura* and *Zacco platypus*.

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Materials and Methods : The embryos of *Odontobutis obscura* and *Zacco platypus* at the hatching stage were subjected to the following experiments.

(1) *Cytochemical procedures*

Test for protein : Millon's original solution consisting of mercury and nitric acid (1 : 9 in quantity) was used for protein test. The specimens were treated for 30 to 60 minutes in it and mounted with glycerin.

Test for nucleic acids : Use was made of Unna-Pappenheim's solution consisting of methyl-green and pyronin according to Brachet's original formula. Further, nucleic acids were examined metachromatically as follows : slides were treated in 10 per cent chromic acid for 1.5 hours, and stained with 1/1000 per cent toluidine blue solution at pH 7.0.

Test for lipids : The demonstration of lipid was made after Ciaccio's method, using Sudan black B. Slides after staining were mounted with distilled water, after having been washed in 50 per cent alcohol and distilled water.

Test for polysaccharides : In order to discriminate polysaccharaide with nitrogen from glycogen, Bauer's technique was adopted in the specimens previously treated in saliva for 30 minutes at 37°C. To verify the result, some specimens were stained with Best's carmine.

Solubility in 2 per cent NaCl aq. or in 60 per cent ethyl alcohol was examined to discriminate mucoprotein, glycoprotein and mucopolysaccharides. Acidity of the dissolving medium was prepared at pH 7.0. Slides were treated with this medium for 45 minutes at 19°–20°C in room temperature or 75°–85°C on the water bath, and afterward they were stained with toluidine blue at pH 7.0.

To discriminate acid mucopolysaccharide from neutral mucopolysaccharide, the metachromatic colouration in 1/1000 per cent toluidine blue (pH 7.0) was examined. To verify the result, the same treatment was performed on slides which were previously treated with 10 per cent chromic acid for 30 and 60 minutes.

Staining tests were performed with solution of various pH values in order to distinguish simple and complex acid mucopolysaccharides: 1/1000 per cent toluidine blue solutions at pH 2.4, 3.0, 3.6, 4.2, 4.6, 5.4, 7.0 and 8.0 were used. The results were verified by the following test: the change in metachromatic colouration caused by 1/1000 per cent toluidine blue (pH 7.0) was examined with the specimens which were previously treated with human seminal fluid for 16 hours and washed for 3 to 4 hours. Toluidine blue solutions (1/1000 per cent at pH 2.4 and pH 7.0) were applied on the slides which were treated in 0.3 M trichloracetic acid for 15 to 20 minutes at 75°–85°C on the water bath. Also, the change in metachromatic colouration caused by 1/1000 per cent toluidine blue solution containing 1 per cent NaCl (pH 7.0) was examined. In every test, the metachromatic colouration was fixed with the solution consisting of 5 per cent ammonium molybdate and 1 per cent potassium ferricyanide (1 : 1 in quantity).

(2) *Test through paper chromatography*

To demonstrate amino acid and protein, the perivitelline fluid, 0.1 ml. in amount was taken out of about twenty eggs of *Zacco platypus* at 94, 64, 34, 22, 17, 12 and 3 hours before hatching. The fluid was divided into two parts: one part was hydrolysed and the other not hydrolysed. The hydrolysed fluid was treated with 1 N-HCl for 5 hours at 75°–85°C. Both were developed by the one-dimension method with the developer which contained n-butyl alcohol, glacial acetic acid and distilled water (4 : 1 : 2 in quantity), and the results were compared between the two groups. For the development of amino acid, 0.2 per cent ninhydrin was used.

For the demonstration of saccharides in the hatching gland cells, embryos of *Odontobutis obscura* at just before the hatching stage were used; after the removal of blanchial cartilages, the ventral part of the operculum where the hatching gland cells occur in masses was also removed. As a control material, the epidermis covering the eyes was taken. These materials were hydrolysed in 1 N-HCl for 5 hours at 75°–85°C, and developed one-dimensionally with the developer above described. Ammonio-silver nitrate solution was used for the development of saccharides.

Results

1. The application of Millon's test indicated that the hatching gland cells were positive in reaction. The epidermal cells also showed positive reaction to the test. But the hatching gland cells were distinguishable from other cells in their staining reaction, in that the hatching granules showed especially intense reaction.

2. With Unna-Pappenheim's solution, the hatching gland cells were stained rosy reddish. In the material taken 27 hours before the hatching stage of *Zacco platypus* and at the lens-formative stage of *Odontobutis obscura*, the boundary of each hatching gland cell, as well as the included hatching granules, showed an especially intense colouration.

Just before the hatching stage of the latter form, the hatching granules were stained faintly with vague outline. The cartilagenous matrix and mucous gland cells were stained metachromatically, being bluish-red in colour: they are distingu-

ished from the hatching gland cells by thier colouration.

3. With Sudan black B, the inner part of the hatching granules was stained bright purplish-red : this seems to indicate the presence of lipids. The membrane-like element lying along the inner side of each cell and the surrounding secretory granules is clearly visible : the element shows a chemical reaction similar to that of granules. The outer part of the granules was stained green. The lipid reaction in the granules has remained unchanged even at just before the hatching stage in *Odontobutis obscura*.

4. The test for Bauer's reaction resulted in showing that the hatching gland cells were positive in reaction, and the granules remained positive even after treating with saliva. Especially, the inner part of each granule and the membrane-like element lying along the inner side of each cell were also positive to Bauer's reaction. The hatching gland cells showed no affinity to Best's carmine.

The hatching granules were stainable with Bauer's stain even after having been treated with 2 per cent NaCl solution or 60 per cent ethyl alcohol, irrespective of the temperature of the medium.

The inner part of each granule was stained metachromatically following Bauer's reaction test, and the membrane-like element was coloured bright purplish-red. The outer part of each granule was green in colouration through Bauer's reaction. The metachromatic reaction of the hatching granules was lost following treatment with chromic acid. The prolonged treatment with the latter agent induced a change in colouration of the granules from reddish-blue to green.

5. Colour change in an experiment with toluïdine blue at various pH values was examined : with toluidine blue at pH 2.4, the hatching gland cells were uniformly stained slightly green. With the adjustment of pH from 3.0 to 3.6, granules showed more rich colouration into green. At pH 4.2, the inner part of each granule was stained slightly reddish-blue. As the pH of the stain was raised from 4.6 to 8.0, the inner part of each granule took more intensive reddish-blue colouration, while the outer part remained green. The membrane-like element lying along the inner side of each cell was stainable with toludine blue even at pH 2.4.

After having been treated with human seminal fluid, the hatching granules were negative to toluidine blue staining; they were slightly green in colour. When treated previously with trichloracetic acid, the hatching gland cells were not stained with toluidine blue at pH 4.2, but at pH 7.0 they were stained uniformly dark purplish-blue the hatching granules being indistinguishable in them. With toluidine blue containing 1 per cent NaCl at pH 7.0, the hatching granules were negative in reaction. The membrane-like element lying along the inner side of each cell remained unchanged in colouration; it was stained purplish-red.

6. By means of the application of the paper chromatography technique, the contents of amino acid and protein were examined in the perivitelline fluid of *Zacco platypus* : the results obtained are summarized in Figure 1.

As seen in that Figure, the content of amino acid showed a gradual increase in

the non-hydrolsyed perivitelline fluid during the period before hatching, while that in the hydrolysed perivitelline fluid showed a rapid increase during the period from 34 hours to 3 hours before hatching. As shown in Table 1, the epidermis contain-

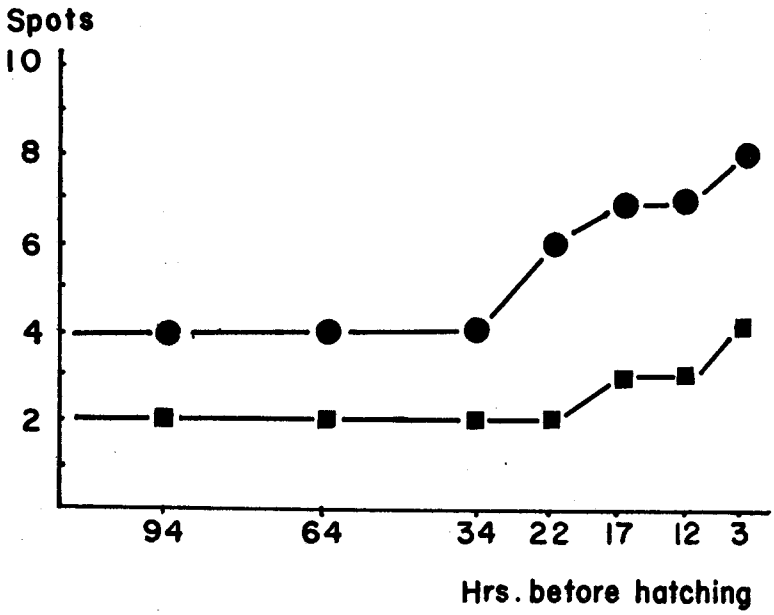

Fig. 1. Diagram showing amino acid in the perivitelline fluid of *Zacco platypus*. -●- Amino acid in hydrolysed perivitelline fluid. -■- Amino acid in non-hydrolysed perivitelline fluid.

Table 1. Saccharides found in the hatching gland cells of *Odontobutis obscura*

	Epidermis containing hatching gland cells	Epidermis without hatching gland cells
Glucuronic acid	++	++
n-acetyl glucosamin	++	++
Ribose	++	−
Glucid	++	++

The marks (+, −) show a comparative degree in reaction.

ing hatching gland cells of *Odontobutis obscura* showed the existence of glucronic acid, n-acetyl glucosamin, ribose and glucid, while there was no ribose in the epidermis excepting in the hatching gland cells.

Discussion

It has been found by Millon's test that protein showed a remarkable increase in the perivitelline fluid with the approach of hatching. Further, the existence of

RNA was demonstrated in the granules of the hatching gland cells by the application of the Unna-Pappenheim method.

The negative reaction to the solubility test also indicates that there is neither mucoprotein nor glycoprotein in the hatching granules. Judging from the fact that so far as the staining reaction is concerned, RNA reaction is not clearly distinguishable from protein-reaction, one is led to believe it most probable that RNA and protein exist in the form of ribonucleic protein.

The test with Ciaccio's reaction resulted in finding that the hatching granules contain lipids in their inner part. The outer part of the hatching granule was proven to contain ribonucleic protein, since it was stained with Sudan black B, showed a rosy red colouration with Unna-Pappenheim's solution, and exhibited a metachromatic colouration with toluidine blue. Such a structual difference found in the hatching granule seems to indicate that lipids occurring in the hatching granules does not exist in a form of lipoprotein, but in the form of a metabolized lipids as found in some endocrine glands, e.g., the cortical adrenal gland and the corpus luteum.

According to Lison ('53), Bauer's reaction is confined to the demonstration of polysaccharides. Since the hatching granules are positive to Bauer's reaction, it is apparent that they contain polysaccharides in their inner parts. The fact that the granules were positive to the reaction even after treatment with saliva seems to indicate that the polysaccharides are not glycogen but polysaccharides with nitrogen. A similar situation was shown both by the non-affinity to Best's carmine and also by the decreasing metachromasia after treating with chromic acid. Bauer's test after the treatment with chromic acid, and the examinations with 2 per cent NaCl aq., or 60 per cent ethyl alcohol resulted in an indication that the hatching granules contain neither mucoprotein nor glycoprotein.

The hatching granules and the membrane-like elemlent lying along the inner side of each hatching gland cell were stained metachromatically with toluidine blue, while after the treatment with chromic acid, their metachromaticity was reduced to a great extent. This seems to indicate that they contain acid mucopolysaccharides.

The hatching gland cells were stained with toludine blue at pH 7.0 but not at pH 2.4 after treatment with trichloracetic acid: this indicates the presence of hyaluronic acid in them. From the facts that the hatching gland cells were stained with toluidine blue at pH 7.0, while the hatching granules were not stained after treatment with trichloracetic acid, it can be supposed that hyaluronic acid occurs in the cells which are free from nucleic protein.

The results of paper chromatography analysis accord with those produced by the cytochemical analysis: there is present more glucronic acid in the epidermis containing the hatching gland cells than in the epidermis containing no hatching gland cells. It was found by this analysis that the hatching gland cells contain at least the following substances: hyaluronic acid, glucronic acid, RNA and n-acetyl glucosamin, as shown in Table 1.

Evidence was presented to show that the hatching granules are of double structure, consisting of an outer part and an inner part which differ chemically. Each granule is surrounded by a membrane-like substance which is composed of lipids and complex mucopolysaccharides, probably mucoitin sulphate or heparin monosulphate. At the onset of hatching, the ectoplasmic layer of the hatching gland cells and the membrane-like elelment surrounding the granules are broken down and the substances from the hatching granules flow out into the perivitelline space : this may result in disolution of the chorion surrounding the egg.

Summary

The cytochemical structure of the hatching gland cells was investigated in two fresh-water teleosts, *Odontobutis obscura* and *Zacco platypus*, by the use of cytochemical methods.

Each fully grown hatching gland cell contains hatching granules which are surrounded by a membrane-like element of lipids nature which shows a reaction indicating the presence of complex acid mucopolysaccharides, chondroitin sulphate and heparin monosulphate.

The hatching granules are of double structure, the outer part consisting of ribonucleic protein, and the inner part consisting of ribonucleic protein, lipids and hyaluronic acid.

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