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Biochemical Property of the Membrane of the Herring Egg, with Special Reference to the Role of the Micropyle in Fertilization¹⁾

By

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(With 1 Plate and 5 Tables)

As has long been well known, the egg of teleostean fish is clothed in a tough membrane. Recent investigations made by Kanoh ('57) have made clear the important role of the egg membrane in the monospermic fertilization of the fish egg, but only a little has been known about the biochemical nature of the membrane of fish egg. Working on the chemistry of egg membrane, Steudel and Osato ('23) reported that the egg membrane of a herring was not dissolved in NaOH and that it was composed of ichthulin, a sort of protein.

In the present paper, the author wishes to report a biochemical character of the membrane of the herring egg with some additional remarks of the fertilization and development of the naked egg derived from the dissolution of the membrane by means of enzymatic proteolysis.

Material and method

Eggs used in this investigation were all taken from fully matured females of the Pacific herring, *Clupea pallasii*. Most of the experiments were carried out with the use of unfertilized egg. In the case of fertilized egg, use was made of eggs in the stage of bipolar differentiation which had been kept in isotonic (M/4.5) Ringer's solution²⁾ for 30 or 60 minutes after insemination (Kanoh '52).

Investigation was made of the effects (A) of acidulated salt solutions (pH 1.8, with HCl) of various concentrations and those (B) of single treatment with proteolytic enzyme, pepsin, trypsin, pancreatin or papain, and (C) of double treatment with acidulated Ringer's solution and with trypsin-Ringer's solution. The double treatment was performed in the following manner; eggs were treated with acidulated solution (pH 1.8, with HCl) for a certain length of time, then washed with isotonic (M/4.5) Ringer's solution (pH 7.6) and

1) Contribution No. 423 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

2) In the present paper the author designates following solution as 1 M Ringer's solution: 1 M NaCl 100 ml + 1 M KCl 3.5 ml + 2/3 M CaCl₂ 1.5 ml + 2/3 M MgCl₂ 2.4 ml (pH 7.6). Ringer's solutions of various concentrations used in this study were made with diluting this 1 M Ringer's solution with buffered dist. water (pH 7.6). For details, see Yanagimachi ('53).

transferred into 0.2% trypsin dissolved in Ringer's solution (pH 9.0) (cf. Kanoh and Yamamoto '57). For the study of fertilization and development of the naked egg treated with the double method mentioned above, eggs were inseminated after thorough washing with isotonic Ringer's solution and put into isotonic Ringer's solution at 4°-6°C. Internal structure was studied by sectioning with ordinary paraffin method after fixation in Bouin's fluid. Staining of sections was carried out by use of Delafield's hematoxylin. Except cultivation of embryos, experiments were all performed at room temperature (17°-20°C).

*Morphological remarks on the egg*¹⁾: The unfertilized egg is spherical in shape, having translucent slightly yellow color in living state. The egg membrane which is about 50 μ in thickness consists of an adhesive layer and a chorion which is divided into two layers, outer and inner layers. Next to the chorion, there is a cortical protoplasmic layer having cortical alveoli. After fertilization there appears a perivitellin space between the plasma surface and the chorion in accordance with the breakdown of the cortical alveoli. Though the egg membrane hardens after fertilization, no conspicuous change is observed morphologically.

Experiments

1. *Effects of acidulated salt solutions.* The unfertilized egg was immersed in Ringer's solutions of various concentrations acidulated to pH 1.8 with N/5 HCl. The observations are summarized in Table 1. It is found that the membrane of unfertilized eggs is dissolved only in acidulated hypotonic Ringer's

Table 1. Effects of acidulated Ringer's solution. Time required for dissolution of the egg membrane.

Concentration of Ringer's sol.	pH	Adhesive layer	Chorion	
			Inner	Outer
M/4.5 (isotonic)	1.8	—	—	—
M/8		4.5 hrs.	(Swollen)	—
M/16		2 hrs.	(Swollen)	(Swollen)
M/32		2 hrs.	2 hrs.	5.5 hrs.
M/32	7.6	—	—	—

solution, and low salt concentration is necessary to dissolve the egg membrane with acidulated solution. When the eggs were immersed in isotonic (M/4.5) acidulated Ringer's solution, they became opaque and remained without dissolution or swelling of the membrane for 24 hours. On the other hand, when the eggs were immersed in acidulated hypotonic Ringer's solutions, they became at first opaque as in the case of acidulated isotonic Ringer's solution but soon after they again became translucent. In this case, the adhesive layer swelled, then jellied and finally disappeared resulting in the loss of its adhesiveness. Swelling occurred also in the inner and outer layers of the chorion except in the

1) For details, see Kanoh ('49) and Yamamoto ('57a).

micropylar region where the membrane did not swell at all. Probably because of this character of the membrane, cytoplasm of the animal pole just under the micropylar structure was projected as seen in Fig. 1. Presumably this indicates the peculiarity of the micropylar region of the egg.

The inhibitory effect of bivalent cations in the action of acidulated solution was a conspicuous one. The results of experiment are shown in Table 2. It is found that even in acidulated M/54 CaCl_2 solution¹⁾ no swelling was observed.

Table 2. Effects of acidulated single salt solution (pH 1.8). Time required for dissolution of the egg membrane.

Concentration	Adhesive layer				Chorion			
	NaCl	KCl	CaCl_2	MgCl_2	NaCl	KCl	CaCl_2	MgCl_2
1/1 S.	—	—	—	—	—	—	—	—
1/2 S.	4 hrs.	4 hrs.	—	—	—	—	—	—
1/4 S.	2 hrs.	1 hr.	(Swollen)	(Swollen)	(Swollen)	(Swollen)	—	—
1/8 S.	2 hrs.	1 hr.	(Swollen)	(Swollen)	5 hrs.	4 hrs.	—	—

1/1 S. means M/4.5 in the case of NaCl and KCl, or M/6.75 in the case of CaCl_2 and MgCl_2 .

The eggs immersed in acidulated isotonic Ringer's, NaCl, KCl or MgCl_2 solutions underwent cytolysis without showing any indication of activation; those in acidulated isotonic CaCl_2 solution, prior to cytolysis, showed the breakdown of the cortical alveoli and the bipolar differentiation.

From the observations mentioned above, it is evident that the high salt concentration inhibits the swelling or dissolution of the egg membrane in the acidulated solution, and the bivalent cations effect inhibitory to the same.

In the case of the fertilized egg, the action of acidulated Ringer's solution on the egg membrane was identical with that in the case of the unfertilized egg. All of the fertilized eggs underwent, however, cytolysis in the acidulated M/32 Ringer's solution during the course of the swelling of the membrane.

2. *Effects of enzymes.* Although the fertilized egg underwent cytolysis as a result of the action of enzymes, no remarkable difference of the susceptibility of the membrane was observed between the unfertilized and the fertilized eggs.

i) Pepsin: One per cent pepsin solutions were made with Ringer's solutions of various concentrations (pH 1.8). When the egg was immersed in these solutions, the membrane was dissolved in a relatively short range of time as seen in Table 3.

In the case of unfertilized egg, most of the naked eggs thus obtained underwent cytolysis but a few showed normal appearance and reacted to pricking

1) Egg is isotonic to M/6.75 CaCl_2 .

with a fine glass needle as does an intact egg; that is to say, the breakdown of the cortical alveoli was occurred after the pricking of the egg. However the formation of the blastodisc was considerably retarded.

Table 3. Effects of pepsin (pH 1.8). Time required for dissolution of the egg membrane.

1 % pepsin dissolved in	Adhesive layer	Chorion	
		Outer	Inner
M/4.5 Ringer's sol.	21 hrs.	24 hrs.	(Swollen)
M/8	0.5 hr.	21 hrs.	(Swollen)
M/16	0.5 hr.	2 hrs.	4.5 hrs.
M/32	0.5 hr.	0.5 hr.	1 hr.

ii) Trypsin and pancreatin: One per cent trypsin or 2% pancreatin solutions were made with Ringer's solutions of various concentrations. With the addition of NaOH, pH of trypsin solution was adjusted to 9.0 and that of pancreatin solution to 9.0 or 11.0. In cases of both enzymes the results obtained were almost identical. The adhesive layer was hardly dissolved with these enzymes but the chorion was swollen (Fig. 2). The observations are summarized in Table 4. As will be seen, the concentration of Ringer's solution has less inhibitory effect on the enzymatic action as compared with the case of pepsin. The egg deprived of its membrane by these treatments showed multiform shape and in most cases the egg did not react to pricking.

Table 4. Effects of trypsin (pH 9.0). Time required for dissolution of the egg membrane.

1 % trypsin dissolved in	Adhesive layer	Chorion	
		Outer	Inner
M/4.5 Ringer's sol.	5 hrs.	5 hrs.	21 hrs.
M/8	2.5 hrs.	2.5 hrs.	5 hrs.
M/16	2.5 hrs.	2.5 hrs.	5 hrs.
M/32	1.5 hrs.	1.5 hrs.	2.5 hrs.

iii) Papain: Two per cent papain solutions were used (pH 7.0, activated with KCN). Results were all negative and no change was observed for 24 hours.

iv) Double treatment: From the results obtained in the salmon egg (Kano and Yamamoto '57), it was expected that double treatment would yield a result better than the single treatment for the purpose of the removal of the herring egg membrane without inducing cytolysis. In view of the results of the single treatment mentioned above, the first step of treatment was carried out with the use of hypotonic solution, namely acidulated M/32 Ringer's solution (pH 1.8),

and in the second step use was made of 0.2% trypsin dissolved in M/4.5 Ringer's solution (pH 9.0). The results are shown in Table 5, from which it is evident that by 60 minutes' immersion in acidulated Ringer's solution and subsequent treatment with trypsin, a number of naked eggs are obtained (Fig. 3). The eggs thus obtained showed normal fertilization reaction as will be described in the next section. Shorter immersion in the acidulated Ringer's solution induced cytolysis of the egg in the trypsin solution.

Table 5. Effects of double treatment.

Duration of pretreatment in acid. Ringer's sol. (M/32, pH 1.8)	Time required for dissolution of the egg membrane in 0.2% trypsin-Ringer's sol. (M/4.5, pH 9.0)			Cytolysed egg
	Adhesive layer	Chorion		
		Outer	Inner	
10 min.	140 min.	140 min.	140 min.	100%
20	10	130	130	100
40	0	110	110	70
60	0	60	90	20
80	0	70	70	20
100	0	20	50	20

3. *Fertilization and development of the naked egg.* Developmental process of the naked egg obtained by the double treatment described above was observed in isotonic Ringer's solution (pH 7.6) after thorough washing with the same. Upon insemination, breakdown of the cortical alveoli in these eggs occurred as in the intact egg (Fig. 4). Though the spermatozoa did not show active movement in isotonic Ringer's solution as reported previously by Yanagimachi and Kanoh ('53) and Yanagimachi ('57a), fertilization was completed by the agitation of the medium. Judging from the cleavage pattern, the fertilization was apparently polyspermic (Fig. 5). In the most of these cases, however, cleavage furrows suddenly disappeared in the early developmental stage; thus the egg showed the appearance of that in one cell stage (Fig 6). In a few eggs, regulation of the arrangement of the blastomeres occurred at morula stage and the embryo developed through the normal blastula and gastrula stages (Fig. 7).

In the sectioned material provided from these eggs at morula, blastula, gastrula and neurula stages, particular cells were observed. Namely, among the normal blastomeres there were large cells having conspicuous monaster and indistinct nucleus (Figs. 8, 9). These cells were found in the blastocoel at the blastula stage, but degenerated in the blastocoel at the gastrula stage (Fig. 10) and disappeared in later stages. However, most of the blastomeres of the egg which showed sudden disappearance of the cleavage furrows in late stage may be regarded as belonging to this particular type (Fig. 11).

Discussion

Kanoh ('51) has reported that the adhesive layer of the herring egg swells in the medium having low salt concentration or having monovalent cations, and that high salt concentration inhibits the swelling as well as bivalent cations. Aoki ('41) has also reported similar phenomena in his "swelling layer" of the chorion of the salmon egg. These findings are entirely in agreement with the present results.

The membrane of the herring egg was completely dissolved by the single treatment with pepsin dissolved in hypotonic Ringer's solution. This is quite similar to the case of the membrane of the lamprey egg (Yamamoto '56), but different from the case of the membrane of the salmon egg (Kanoh and Yamamoto '57) in which pretreatment with acidulated solution is indispensable for the enzymatic dissolution of the membrane.

Ishida ('44) has reported in developing *Oryzias* egg that the membrane is dissolved by tryptic enzyme as well as cathepsin. It is held true in the salmon egg (Kanoh and Yamamoto '57). Unlike to these facts, the egg membrane of the herring was not dissolved with a cathepsin such as papain (activated with KCN) so far as the present study is concerned. Thus the results obtained in the herring egg indicate that the egg membrane consists predominantly of protein which is easily attacked by a tryptic enzyme. Histochemical study of the membrane of the herring egg revealed also the presence of protein, SS bridges and polysaccharides (Yamamoto '57a, b). Although the membrane of the herring egg was dissolved by a single treatment with enzyme, healthy naked egg was obtained at more adequate rate by means of a double treatment which was adopted by Kanoh and Yamamoto ('57) for the purpose of the removal of the membrane in the salmon egg.

Susceptibility of the egg membrane to acid or enzyme was quite identical both in unfertilized and fertilized eggs as mentioned already. In this respect, the herring egg is similar to the lamprey egg (Yamamoto '56). The nature of the *Oryzias* egg membrane coincides with that of the present material, because the membrane of the developing egg is easily dissolved by the enzymatic treatment (Ishida '44). The membrane of fish egg may be, therefore, divided into two groups in respect to the susceptibility to the enzymatic treatment. One is found in the lamprey, herring and *Oryzias* eggs, of which the membrane is dissolved with enzymatic treatment even after the fertilization or activation of the egg. The other is found in the salmon egg, in which the membrane is not digested by enzymatic treatment after egg activation (Yamamoto '57c). In this connection it is interesting to note that, differing from the case of first noted group, the membrane of unactivated salmon egg is not dissolved with a single treatment of an enzyme (Kanoh and Yamamoto '57).

Occurrence of polyspermic fertilization observed in the naked egg coincides in the case of the salmon egg (Kanoh and Yamamoto '57), and suggests a possibility that the micropylar canal of the chorion has an important role in the blocking of polyspermy. Kanoh ('57) has discussed the mechanism of monospermic

fertilization in fish egg and pointed out the peculiarity of the micropylar structure of the chorion. In his earlier paper, Kanoh ('49) has described that the staining reaction of the micropylar region of the herring egg differs from that of the other parts of the chorion. Peculiarity of the micropylar region of the chorion was also noticed in the present study. In an acidulated hypotonic Ringer's solution, the chorion except micropylar region swelled easily and dissolved as stated above. On the other hand, the chorion of the micropylar region did not swell.

Polyspermic fertilization has been observed also in the egg of which membrane was removed mechanically by gentle treatment with fine scissors and needle (Kanoh '57, Yanagimachi '57b). It may be considered, therefore, that polyspermic fertilization observed in the present study is not caused by the agent used, but takes place in the absence of the egg membrane.

As to the origin of the particular cells observed in the developing egg of which cleavage pattern was polyspermic, there is no available datum, but the nucleus of this particular cell may be derived from the excess sperm. It is of interest to note that these particular cells undergo degeneration in the blastocoel during the development, a condition which is quite similar to that observed in the amphibian interspecific cross by Baltzer ('52).

Summary

The membrane of the herring egg is dissolved by a single treatment of acidulated Ringer's solution or enzymatic solution but healthy naked egg is obtained by a double treatment with acidulated Ringer's solution (M/32, pH 1.8) and 0.2% trypsin-Ringer's solution (M/4.5, pH 9.0) at more adequate rate. The fertilization of the naked egg is apparently polyspermic. Based on these results, the importance of the chorion of the micropylar region in the monospermic fertilization of the fish egg was discussed.

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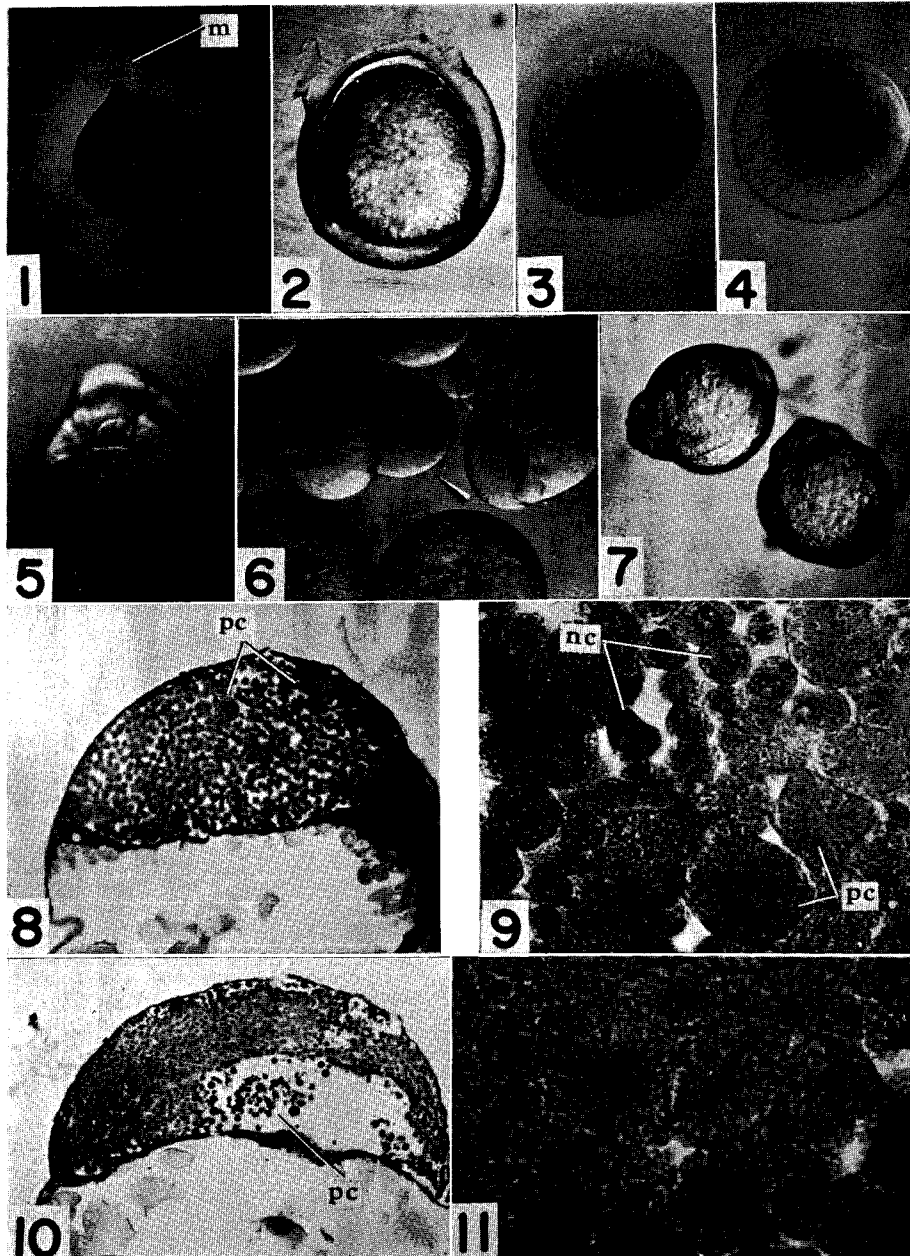
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Explanation of Plate I

- Fig. 1. Photomicrograph of the egg immersed in acidulated M/32 Ringer's solution for 50 minutes. Adhesive layer is almost entirely jellied and chorion is swollen considerably. Note the projection of the protoplasm just under the micropyle (m). ca. $\times 3$.
- Fig. 2. Photomicrograph of the egg treated with 0.2% trypsin dissolved in M/4.5 Ringer's solution for 4.5 hours. ca. $\times 20$.
- Fig. 3. Naked unfertilized egg obtained by the double treatment. ca. $\times 20$.
- Fig. 4. Fertilized naked egg. Its cortical alveoli are broken down. ca. $\times 20$.
- Fig. 5. Polyspermic cleavage in the naked egg. Note the irregular arrangement of the blastomeres. ca. $\times 20$.
- Fig. 6. Fertilized egg showing disappearance of its cleavage furrows (indicated by an arrow). Its appearance is quite like to that of one cell stage. ca. $\times 20$.
- Fig. 7. Embryos formed in the naked eggs. The appearance is normal. ca. $\times 20$.
- Figs. 8 and 9. Section of the naked egg at blastula stage. Note the particular cells (pc) among the normal blastomeres (nc). Fig. 8, ca. $\times 80$; Fig. 9, ca. $\times 420$.
- Fig. 10. Section of the naked egg at gastrula stage showing the degeneration of the particular cells (pc) in the blastocoel. ca. $\times 80$.
- Fig. 11. Section provided from an egg which showed irregular cleavage. Its blastomere has a conspicuous monaster and indistinct nucleus. ca. $\times 420$.



T. S. Yamamoto : Biochemical Property of Egg Membrane