Digestion of Egg Envelopes and their Chemical Properties of the Lamprey's Egg, *Lampetra japonica*[1)]

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(*2 Text-figures*)

To the nature of the egg envelope of echinoderms and amphibians, considerable contribution has been made (Sugawara, '43 : Townes, '53, etc.). However only a little has been known about the nature of the chorion of the fish egg, though the hatching enzyme has often been investigated by many authors (cf. Ishida, '44). These things hold true in the case of the lamprey egg. The present investigations to be reported here provide some information on the chemical nature of the egg envelopes of the lamprey as revealed by their reaction to various acids, enzymes and dyes.

Material and method

The lamprey, *Lampetra japonica*, is collected in the neighbourhood of Sapporo. In most experiments the unfertilized eggs of this species, but in a few cases the fertilized ones, are employed. The proteolytic enzymes employed are pepsin (containing saccharide, Kanto Chem. Co.), trypsin (Merck) and papain (Merck). These enzymes are dissolved in Ringer's solution of various concentrations[2)]. After the treatment with these solutions, eggs were returned to fresh water. In the case of unfertilized eggs insemination was done. For the cytochemical studies the eggs were fixed with Bouin's fluid, 10 per cent formalin or 80 per cent alcohol and sectioned at 10 micra in thickness with ordinary paraffin method. The sections were, then, stained with Delafield's hematoxylin and eosin or with other dyes. The experiments were always performed at room temperature (17°-20°C).

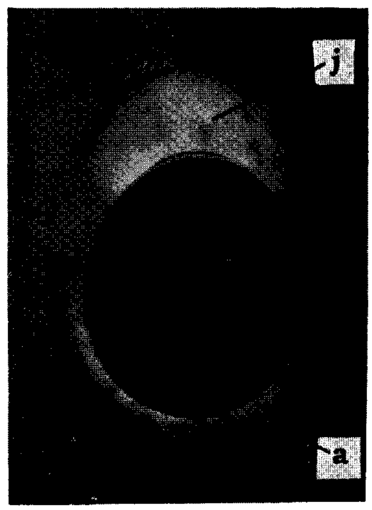

Fig. 1. Unfertilized egg in water containing India ink. a, adhesive layer. j, jelly mass. ×45.

Morphological remarks on the egg[3)] : The unferti-

1) Contribution No.346 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

2) 1 M Ringer's solution = 1 M NaCl, 100 cc + 1 M KCl, 2.1 cc + 2/3 M $CaCl_2$, 2.3 cc.

3) For details, see Herfort ('01), and Kanoh & Akahira (unpublished).

lized egg is oval in shape and opaque with white color in living state and is enclosed with the chorion, which is divided into two layers, the outer and the inner. These two layers of the chorion are almost equal in thickness in the animal pole. The chorion of this region is designated as the polar chorion in this paper. But in the other region the outer layer of the chorion is thicker than the inner layer. As shown in Figure 1, the adhesive layer and the jelly mass are found being attached to the chorion externally. These structures are scarcely to be seen in the living state but become easily observable when the egg is immersed in water containing India ink. The adhesive layer is found only in the vegetative region, while on the other hand, the jelly mass is attached to the polar chorion[1]. As to the egg proper, there is a narrow translucent plasma region, that is, polar protoplasm, beneath the polar chorion, but the other part is entirely opaque.

Upon insemination, spermatozoa passing through the jelly mass reach to the chorion, subsequently one of them penetrates into the egg, then a perivitelline space appears and the further development follows. After spermatozoa have penetrated the jelly mass, relatively clear paths are found in the passing traces. The jelly mass disappears during the cleavage stage. Accompanying with the formation of the perivitelline space, the adhesive layer and the outer layer of the chorion begin to swell and the contour becomes indistinct, resulting in the loss of adhesiveness of the egg. On the other hand, the inner layer of the chorion does not show any remarkable changes, at least in the early cleavage stages.

Results

1. *Effects of acids* : When the unfertilized eggs are immersed in N/10 nitric acid, N/10 sulfuric acid or N/10 hydrochloric acid respectively, both the jelly mass and the adhesive layer dissolve within a short time as shown in Table 1, whereas in nitric acid and sulfuric acid the outer layer of the chorion does not dissolve though it does swell. However, in hydrochloric acid only the outer layer of the thickened polar chorion is dissolved. Thus, with gentle shaking, the remaining part of the outer layer can be torn away from the inner layer which is not attacked by these acids. In all cases, however, the egg proper undergoes cytolysis sooner or later.

In the fertilized eggs, though the jelly mass and the outer layer of the chorion have somewhat changed as mentioned already, the effects of these acids are similar to those in the unfertilized egg.

Table 1. Effects of acids. Time required for dissolving egg envelopes (17°-20°C)

Acid	Jelly mass	Adhesive layer	Chorion	
			Outer	Inner
N/10 HNO_3	5 min.	3-15 hrs.	(swollen)	(not dissolved)
N/10 H_2SO_4	5 min.	3-15 hrs.	(swollen)	(not dissolved)
N/10-N/400 HCl	5 min.	3 hrs.	18 hrs.*	(not dissolved)

* Only the thickened part of a polar chorion is dissolved.

1) The adhesive layer can be vitally stained with basic dyes such as janus green or toluidine blue. The jelly mass is not vitally stained with ordinary dyes except neutral red.

2. *Effects of proteolytic enzymes* : The effects of proteolytic enzymes dissolved in isotonic Ringer's solution (M/7) on the egg envelopes are summarized in Table 2. Therein, it is seen that 0.2 per cent pepsin dissolved in M/7 Ringer's solution (pH 1.8) digests the entire chorion, that is, not only its outer layer but also the inner one. In this case, however, the egg proper undergoes cytolysis in company with digestion of the entire chorion. The concentrations of Ringer's solution in which pepsin was dissolved, varied from M/5 to M/14, but the results obtained were similar to those of M/7. For removal of the entire chorion, a concentration of more than 0.2 per cent pepsin was required and the pH should be less than 1.8.

The treatment with papain alone or double-treatment of papain and sodium thioglycolate which had been recommended with the aim of removing the vitelline membrane in amphibian eggs (Spiegel, '51), did not give any favorable results on the chorion of the present egg.

Table 2. Effects of proteolytic enzymes dissolved in M/7 Ringer's sol. Time required for digesting egg envelopes (17°-20°C).

Enzyme solution	pH	Jelly mass	Adhesive layer	Chorion	
				Outer	Inner
0.2 % trypsin	7.8-8.4	2 hrs.	2 hrs.	(swollen)	(not digested)
0.2 % pepsin	1.8	5 min.	1 hr.	2-3 hrs.	5 hrs.**
2 % papain*	6.0-7.0	2 hrs.	3 hrs.	(swollen)	(not digested)

* Activated with 0.25% KCN. ** Egg proper undergoes cytolysis.

Concerning the digestion of the fertilization membrane of sea urchin eggs by proteolytic enzyme, Sugawara ('43) has reported that the dissolution of the fertilization membrane is influenced by the salt concentration of the medium in which the enzyme is dissolved and that in a medium of high salt concentration the enzymatic activity is considerably inhibited. The present author, therefore, performed experiments dissolving the enzymes in hypotonic Ringer's solution (M/200). The results are presented in Table 3. From these results, it is concluded that the digestion of egg envelopes is much accelerated with the decline of salinity of the medium as in the case of sea urchin egg. The pepsin in M/200 Ringer's solution dissolves the entire egg envelopes by the following process.

Time after immersion in min.	
2	Jelly mass and adhesive layer are dissolved. Egg becomes wrinkled.
4	The outer layer of the polar chorion begins to dissolve.
7	The outer layer of the chorion disappears almost completely : the inner layer of the vegetative half begins to dissolve.
10	The inner layer of the chorion disappears entirely and the naked egg is obtained.

Naked unfertilized eggs thus obtained by the action of the pepsin in M/200 Ringer's solution still retain their fertilizability but they are so brittle as to be easily broken by a slight agitation of the medium.

Table 3. Effects of proteolytic enzymes dissolved in M/200 Ringer's sol. Time required for digesting egg envelopes (17°–20°C).

Enzyme solution	pH	Jelly mass	Adhesive layer	Chorion	
				Outer	Inner
0.2 % trypsin	7.8-8.4	30 min.	30 min.	2 hrs.	(not digested)
0.2 % pepsin	1.8	2 min.	2 min.	6 min.	10 min.

In pepsin-M/7 Ringer's solution, however, the egg undergoes cytolysis as mentioned above if it is in unfertilized state. On the contrary, the fertilized egg does not undergo cytolysis in the same solution though its envelopes dissolve in the same manner as those of unfertilized egg. When the naked egg obtained by the treatment with pepsin solution is transferred to the fresh water, cleavage occurs subsequently as will be described below. Thus it can be said that the unfertilized and fertilized egg have different susceptibilities to the proteolytic enzymes, but so far as the present investigation goes, the reaction of the chorion to the proteolytic enzymes is similar before and after fertilization.

3. *Cytochemical reactions and chemo-histological properties of fixed material:* Using sectioned material provided from eggs which had been fixed with Bouin's fluid, with 10 per cent formalin or with 80 per cent alcohol respectively, cytochemical reactions for polysaccharides and protein were investigated. Jelly mass of the unfertilized egg is easily dissolved in the acidulated fixatives but is kept safe in 80 per cent alcohol. As shown in Table 4, the results indicate that the egg envelopes including jelly mass contain polysaccharides and protein, however, it must be pointed out that in Millon's reaction, the outer layer of the chorion differs slightly from its inner layer.

Table 4. Reactions of egg envelopes to cytochemical tests.

Method employed	Jelly mass	Adhesive layer	Chorion	
			Outer	Inner
for polysaccharides				
Bauer's	+	++	++	++
Langhans'	−	−	−	−
for protein				
Millon's	−	−	+	++
xanthoprotein	+	±	++	++
Romieu's	−	−	−	−
Biuret's	−	−	−	−
Salzar's		++	++	++

The jelly mass and the adhesive layer are stained with basic dyes (Table 5). The adhesive layer reveals particularly a characteristic metachromasia with safranin. The chorion is stained with both acid and basic dyes, however, its capacity for binding basic dyes is, in general, so weak that the staining color is easily faded with alcohol. Weigert's resorcin-fuchsin which is known as a dye staining the elastic fiber, stains also the chorion, but faintly. Between the outer layer and the inner layer of chorion, there is a difference in staining intensity to methylene blue and azan stain.

Table 5. Dye-binding capacity of egg envelopes to various dyes.

Dye sol.	Fixative	Jelly mass	Adhesive layer	Chorion	
				Outer	Inner
1% toluidine blue	alcohol	+	++	++	++
1% gentian violet	〃		−	±	+
0.5% Nile blue	〃		−	+	+
0.1% safranin	〃		++	+++	+++
0.5% Bismark brown	〃		−	++	++
0.1% methylene blue	Bouin		−	+++	++
0.5% malachite green	〃		−	−	−
Delafield's H-E	formalin		++(H), −(E)	−(H), +(E)	−(H), +(E)
Heidenhain's azan	Bouin	++(AN)	+++(AN)	++(AN)	+(AN)
Weigert's resorcin-fuchsin	〃		−	±	±
Mucicarmine	alcohol	+	−	−	−

H : hematoxylin. E : eosin. AN : aniline blue.

The difference between the outer layer and the inner layer of the chorion is also demonstrated by means of the method introduced by Dempsey et al. ('47). The procedure in applying this method consists of progressive staining in buffered solution of methylene blue. The solutions of 0.1 per cent methylene blue were adjusted to different pH values with McIlvaine's citric-phosphate buffers and the sections were immersed in these solutions, then, transferred into 5 per cent

Table 6. Dye-binding capacity of chorion to 0.1% methylene blue at various pH values.

Fixative	Formalin		Bouin		Alcohol	
Staining duration	48 hrs. (36.5°C)		24 hrs. (36.5°C)		30 min. (20°C)	
pH \ Chorion	Outer	Inner	Outer	Inner	Outer	Inner
4.6	−	−	−	−	−	−
5.0	±	−	−	−	−	−
5.6	++	+	±	−	±	−
6.0	++	+	+	±	±	−
6.6	++	++	+	±	+	−
7.0	+++	++	++	+	+	±
8.0	+++	++	+++	++	++	+
8.4	+++	++	+++	++	++	+

ammonium molybdate for fixation of the staining, thereafter rapidly dehydrated with alcohol and covered as permanent preparations. Under this method the outer layer of the chorion fixed with Bouin's fluid becomes stained at pH 6.0, but the inner one at pH 7.0 (Table 6).

4. *Fertilization and cleavage of the naked egg :* As mentioned above, naked unfertilized egg obtained by dissolving the chorion with pepsin-M/200 Ringer's solution, is easily broken but can be fertilized with careful treatment. Such naked fertilized egg reveals nearly normal reaction to fertilization and begins to cleave after about 6 hours without any indication of polyspermy. In this case, however, the blastomeres are separated from each other and after the third or the fourth division, cleaving proceeds no further (Fig. 2).

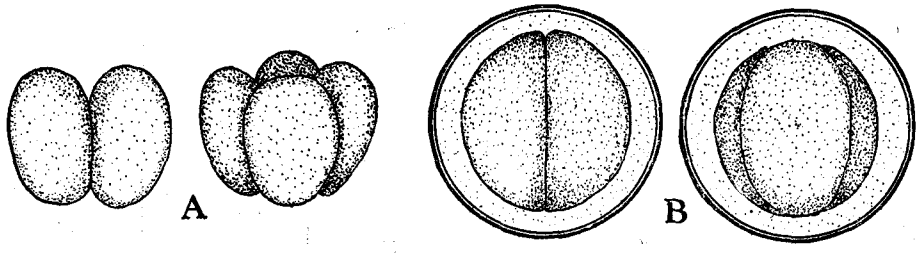

Fig. 2. Cleavage of the naked egg, of which chorion was removed with pepsin-M/200 Ringer's solution. A, Blastomeres are separated from each other. B, Untreated egg (control).

In the egg treated with pepsin or trypsin after fertilization, cleaving proceeds similarly to that in the egg deprived of the chorion prior to insemination.

Discussion

The jelly mass, adhesive layer and the chorion of the lamprey egg are entirely digested with pepsin. It is indicated that these structures are, as in the egg of amphibians (Townes, '53) and echinoderms (Hultin, '48), predominantly composed of protein. Furthermore, considering the specificity of pepsin and trypsin (Bergmann and Fruton, '41), the peptide linkage involving tyrosine or phenylalanine and carboxyl group are suggested to be present in these structures as Townes has stated in the case of amphibian egg. Moreover, from the results obtained by the cytochemical methods, it is obvious that the egg envelopes contain polysaccharides which are neither glycogen nor chitin. The jelly mass of the lamprey egg is easily dissolved in acid and stained vitally with neutral red as is also the jelly coat of the sea urchin egg. Judging from the staining affinities to mucicarmine and aniline blue, it is safe to say that the jelly mass of the lamprey egg is composed of a mucoid substance. The adhesive layer may also contain mucoid substance since it reveals a characteristic metachromasia with safranin.

Similar to the *Ascaris*-egg (Kreuzer, '54), the difference between the outer and the inner layer of the chorion is demonstrated by the enzymatic and cytochemical experiments. Their different susceptibility to the enzymes may be due to the difference of protein. These two layers are also clearly distinguished by means of the method introduced by Dempsey et al.

The influence of salinity of the medium upon the proteolytic action is indicated by using pepsin. With pepsin in isotonic Ringer's solution (M/7), the entire chorion is dissolved after about 5 hours but at the same time the egg proper undergoes cytolysis immediately. On the other hand, ten minutes' immersion in pepsin dissolved in hypotonic Ringer's solution (M/200) is enough to obtain the naked egg healthy and fertilizable. The effect of salinity upon the enzymatic proteolysis has been reported by Sugawara ('43) ; he succeeded in the digestion of fertilization membrane of sea urchin eggs in company with less salinity of medium as in the present investigation. Concerning the amphibian egg, hypotonic solutions are also used always in the experiments of the digestion of the egg membrane (for example, 10 per cent Ringer's solution by Bliss, 10 per cent Holtfreter's solution by Spiegel and M/16 Holtfreter's solution by Townes).

Differing from the case of sea urchin and amphibian eggs, the double-treatment of papain and sodium thioglycolate has only the same effect as papain alone on the chorion of the lamprey egg and is of no use in digesting the chorion. The chorion of the lamprey egg, however, may contain S–S or SH group in its component, as is indicated by the fact that when the egg fixed with trichlor acetic acid is immersed in the ferric ferricyanide solution (method of Chévremont et Fréderic) for a prolonged period, the chorion is colored in bluish green.

It is supposed that the chorion of the fish egg undergoes some changes at the time of activation. Recently, Nakano ('54) has reported that the hardening of the chorion of the egg of Medaka, *Oryzias latipes*, after fertilization, may probably be due to the action of the content of cortical alveoli released from the egg cortex into the perivitelline space at fertilization, and that the hardened chorion can be softened again with thioglycolic acid. In *Oncorhynchus*-egg, it has also been recognized that the chorion is digested with double-treatment of acidified Ringer's solution and "Pancreatin", but after the break down of the cortical alveoli followed by activation, the chorion becomes insoluble under the same treatment (unpublished data). At the time of fertilization, there occurs a hardening of the vitelline membrane of sea urchin egg and it is reversibly recovered by the treatment of thioglycolic acid (Monroy and Runnström, '48). Unlike these facts, however, no change concomitant with fertilization occurs in the chorion of the lamprey egg in respect to the cytochemical properties and the susceptibility to the proteolytic enzymes.

The fertilization reaction in the naked egg is nearly identical with that in the untreated egg, and the break down of cortical alveoli which has already been

observed in the egg of the brook lamprey, *L. planeri*, by Yamamoto ('44), begins always near the animal pole and ends at the vegetative pole. The penetration of a spermatozoon into the naked egg may occur at animal pole and the mechanism which blocks polyspermy must be present in the egg proper, because subsequent cleavage does not give any indication of polyspermy. Moreover, it is of interest that the blastomeres of the developing naked egg separated from each other.

Summary

The effects of several acids and enzymes and also some cytochemical reactions on the envelopes of the lamprey egg have been studied. The results obtained are as follows :

1. The jelly mass and the adhesive layer are easily dissolved in the acids used.
2. The jelly mass, the adhesive layer and the outer layer of the chorion are digested with pepsin and trypsin, but the inner layer of the chorion is digested only with pepsin and not with trypsin. In this respect, the outer layer of the chorion differs from the inner layer.
3. The salinity of medium in which the enzyme is dissolved, has a remarkable effect upon the enzymatic proteolysis. Two-tenths per cent pepsin in hypotonic Ringer's solntion (M/200, pH 1.8) digests the entire chorion in a short period and thus fertilizable naked egg is obtained.
4. The egg envelopes are all predominantly composed of protein and polysaccharides.
5. In the naked egg, the fertilization reaction is similar to that of untreated egg and the cleaving naked egg shows no indication of polyspermy though blastomeres are separated from each other.

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