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Instructions for use

On the Fertilizability of the Frog Egg, III. Removal of Egg Envelopes from Unfertilized Egg[1][2]

By

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(*With 7 Text-figures and 2 Tables*)

Chemical analysis of the amphibian egg envelopes has revealed that the envelopes are primarily composed of mucoprotein (Folkes *et al.* '50, Minganti '55). The works of Bliss ('40), Spiegel ('51) and Townes ('53) have shown that the rather higher concentrations of proteolytic enzymes digest the egg envelopes without hindering the cleavage and the subsequent development. Furthermore there are demonstrations of a hatching enzyme which is secreted during the stages from the early tail-bud up to the hatching of some amphibian species (Cooper '36, Ishida '47, Yanai *et al.* '53). The above cited works, however, are concerned only with the developing embryos, and the effect of the enzymes on the unfertilized egg is still unknown.

It has recently been found that with frog eggs trypsin treatment before insemination, which affects the jelly envelopes in some manner without inducing their complete digestion, gives a significantly higher percentage of fertilization (Barch & Shaver '59, Katagiri '62). Thus, in connection with the analysis of fertilization, it is of interest to try to remove the frog egg envelopes. The present paper deals with the attempt to do this without imparing fertilizability.

Material and method

The material used was the grass frog, *Rana temporaria*, collected in the neighborhood of Sapporo. Most experiments were performed on unfertilized eggs. As the occasion demanded, fertilized eggs were also employed. The egg of the present material is, in addition to the vitelline membrane, firmly enclosed by dense jelly envelopes. The latter are composed of 4 layers, designated from inner to outer J_1, J_2, J_3 and the adhesive layer (*cf.* Katagiri '61). To study the digestion of the egg envelopes, proteolytic enzymes such as pancreatin (Merck), papain (Merck), pepsin (Kanto Chem. Co.) and trypsin (Merck) were

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employed. They were dissolved in De Boer's solution or its 1/20 dilution (1/20 De Boer's solution) which had been adjusted to the optimal pH of each enzyme. The natural hatching enzyme was prepared from the developing embryos. Other particular procedures employed for digesting the egg envelopes will be described in each case. The effect of different treatments on the egg proper was examined both in the fresh and the sectioned materials. For the latter case, eggs fixed with Bensley's fluid were sectioned through the methylbenzoate-paraffin method, followed by staining with Feulgen's nuclear reaction or Delafield's hematoxylin. For the study of cortical granules, the sections were stained with Delafield's hematoxylin after bleaching of pigment granules. All the experiments were carried out at room temperature, 16–20°C.

Results

Effect of alkali. In the first place, the effect of pH of the surrounding medium on the egg envelopes was studied. Unfertilized eggs were immersed in solutions of various pH from 12.6 through a graded series down to 1.8. In alkaline solutions at pH above 11.0, the jelly envelopes become translucent and rather soft, whereas in acid solutions at pH below 3.0 the envelopes soon become milky-white and rather hard, with smooth contours. In all cases, however, the envelopes do not dissolve. As the jelly envelopes become translucent in higher pH solutions, the effect of strong alkaline solutions was next examined. The media used were 1N and 1/10N solutions of NaOH, KOH and KCN. As summarized in Table 1, the jelly envelopes and the vitelline membrane swell and dissolve in

Table 1. Effect of alkali on egg envelopes; time required for dissolution of egg envelopes.

	Jelly envelopes	Vitelline membrane	Remarks
1N NaOH & KOH	50 min.	50 min. (swollen)	Egg cytolyzes in 15 min.
N/10 NaOH & KOH	11/4 hrs.	11/4 hrs. (swollen)	Egg cytolyzes in 15 min.
1N KCN	11/2 hrs.	3 hrs. (unswollen)	Egg rotates.
N/10 KCN	4 hrs.	20 hrs. (unswollen)	Egg rotates.

NaOH and KOH, though the egg proper undergoes cytolysis within a short time. In KCN solutions, however, the dissolution takes place only in the jelly envelopes. The vitelline membrane does not swell in this solution, but ruptures at some point after prolonged immersion. That the vitelline membrane is highly resistant to KCN seems to be due to some change of the membrane in the solution owing to egg activation, because egg rotation occurs soon after immersion.

Effect of proteolytic enzymes. Preliminary tests employing several proteolytic enzymes proved that enzymes such as pancreatin (1.0%), papain (2.0%; activated

with 0.05% cysteine-HCl), pepsin (0.5%) and trypsin (1.0%) are not effective for the complete digestion of the egg envelopes of the present material. Partial digestion is obtained, however, when papain is employed, *viz.* the outer layers of jelly envelopes J_2 and J_3 are digested after 6 hours' treatment, but the innermost layer, J_1, and the vitelline membrane fail to undergo dissolution even after 24 hours' treatment.

Then the effect was studied of the successive treatment with papain and sodium thioglycolate which was recommended by Spiegel ('51) for the removal of the egg envelopes of *R. pipiens*. It resulted that the denuded egg is obtained by 3 hours' treatment with 2.0% sodium thioglycolate when previously treated with 2.0% papain for 3 hours. The process of digestion of the envelopes is the same both in unfertilized and fertilized eggs.

Besides the above method, successive treatment with sodium thioglycolate and trypsin was also found to be effective for the digestion of the envelopes. In this case, the first treatment was carried out with 2.0% sodium thioglycolate for 30 minutes, and then 0.25% trypsin was applied as the second treatment. In addition it was recognized that the treatment with KCN in place of sodium thioglycolate is also effective for the digestion of the jelly envelopes. When the sodium thioglycolate-trypsin treatment is applied to unfertilized eggs, both the vitelline membrane and the jelly envelopes swell markedly at first and the complete digestion of all the envelopes occurs after 2 hours' immersion in trypsin. Also in fertilized eggs the jelly envelopes are digested sooner or later, but the vitelline membrane does not swell, remaining undissolved even after prolonged immersion in trypsin (Table 2). The failure in both swelling and digestion of the membrane under this dual treatment is also recognized in eggs previously activated by pricking (Fig. 1). Furthermore, it is shown in Table 2 that the innermost jelly layer, J_1, becomes less susceptible to the digesting action if the

Table 2. Effect of successive treatment with Na-thioglycolate and trypsin for digestion of egg envelopes.

Treatment with	Egg used	Time required for digestion of			Remarks
		J_3 & J_2	J_1	Vitelline membrane	
Trypsin after 30 min. treatment with Na-thioglycolate	Unfertilized	2 hrs.	2 hrs.	2 hrs.	Vitelline membrane does not swell.
	Fertilized	2 hrs.	6 hrs.*	—	
	Unfertilized, immersed in tap water	2 hrs.	6 hrs.	6 hrs.	
Na-thioglycolate for 30 min.	Unfertilized	(translucent)		—	
Trypsin	Unfertilized	—	—	—	

* Digested in 2 hrs., if the egg is subjected to the treatment within 30 min. after insemination.

eggs, either fertilized or unfertilized, have been immersed in tap water for over 30 minutes before the dual treatment. J_1 of such eggs shows a rather prominent striated structure in trypsin solution, which persists for 6 hours and finally dissolves away (Fig. 2).

Regarding the state of the egg proper denuded by the above mentioned two kinds of dual treatment, *i.e.* treatment with papain and sodium thioglycolate and with sodium thiogylcolate and trypsin, both exert similar effects. A fairly high percentage of gastrulae subjected to the treatments continue normal development and develop into tadpoles. Denuded unfertilized eggs, however, show a different morphological structure to that of intact eggs. Namely, in the denuded eggs the cortical granules have almost broken down. Furthermore the egg nucleus shows an abnormal feature, either with diminished or irregularly scattered chromosomes instead of the metaphase spindle of the second maturation division, probably owing to some damage of the mitotic apparatus (Fig. 4). Thus the dual treatments described above prove to be favorable only for the removal of the egg envelopes from developing eggs, and not so for denuding unfertilized eggs without harmful effects.

Effect of hatching enzyme. In order to obtain healthy denuded unfertilized eggs, the effect of the natural hatching enzyme was then studied. The method for preparing the enzyme solution is as follows. The jelly envelopes and the vitelline membrane of early tail-bud larvae are removed with microscissors. About 400 of such freed embryos are placed in a small petri-dish containing 5 cc of 1/20 De Boer's solution and are allowed to develop in it. Along with this series, normally enveloped embryos of the same stage serve as an indicator of the close of the hatching period. About 40 hours after that, when the embryos have passed the hatching period, the contents of the experimental lot are lightly centrifuged and the supernatant is employed as a hatching enzyme solution.

When exposed to the enzyme solution, both unfertilized and fertilized eggs are freed from their envelopes after 1 to 4 hours' immersion. The process of dissolution is as follows: At first the jelly layers swell markedly, with J_1 and J_2 losing their striated structure, and are finally digested completely. The vitelline membrane is also affected markedly, either swelling away from the egg proper or actually rupturing, but it does not undergo complete dissolution (Fig. 3). When most of the jelly layers have been dissolved and the swelling of the vitelline membrane is well defined, it is easy to obtain denuded eggs by gentle blowing of the surrounding medium with a pipette.

The rate of digestion of the egg envelopes by the hatching enzyme depends, of course, upon the number of embryos used to prepare the enzyme solution. Usually the envelopes of fertilized eggs are digested in a shorter time than those of unfertilized eggs in the same enzyme solution. Variability of this effectiveness may be attributed to the more pronounced swelling of the jelly envelopes in the former eggs, since the envelopes of unfertilized eggs are digested at the same rate as those
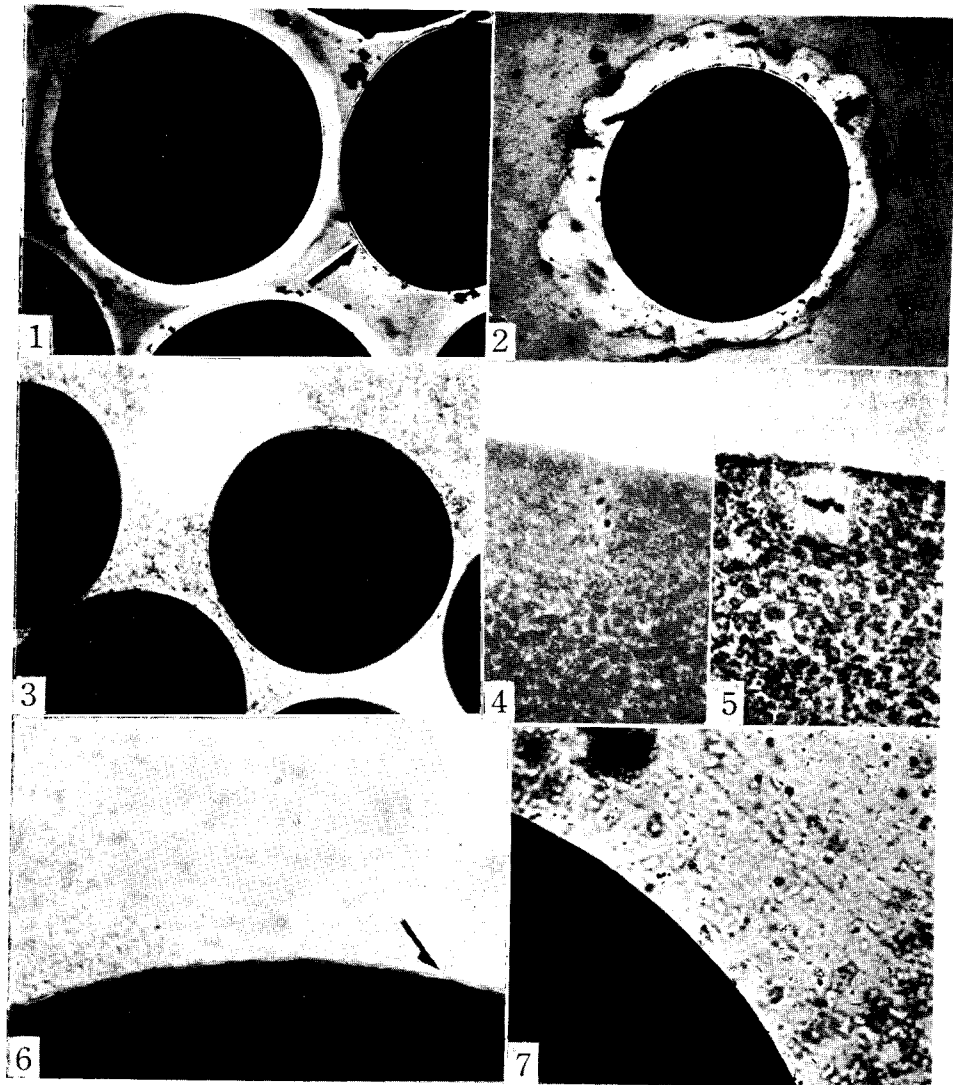
Fig. 1.   Unfertilized eggs subjected to sodium thioglycolate-trypsin treatment.   Jelly envelopes have been completely digested.   Compare the swollen vitelline membrane of unpricked eggs and the unswollen membrane (arrow) of the egg which had been pricked before the treatment.   Photographed in the medium containing India ink. ca. × 20.

Fig. 2.   Unfertilized egg immersed in tap water for 30 min. and then treated as eggs shown in Fig. 1.   The jelly layer J_1 remains undigested, surrounding the swollen vitelline membrane. Photographed in the medium containing India ink. ca. × 20.

Fig. 3.   Unfertilized eggs treated with hatching enzyme, showing the egg envelopes almost

of fertilized eggs if immersed previously in tap water for a long time. The mechanical removal of the outer half of J_3 with microscissors also shortens the time required for the digestion of the egg envelopes. From the experiments described above, the following procedure is concluded to be the best for obtaining denuded unfertilized eggs: Immersion in 1/20 De Boer's solution for 30 minutes to allow imbibition of the jelly envelopes → Mechanical removal of the outer half of J_3 in De Boer's solution → Exposure to hatching enzyme solution prepared in 1/20 De Boer's solution. By this procedure, using an enzyme solution secreted by about 400 embryos in 5 cc of 1/20 De Boer's solution, the denuded unfertilized eggs are obtained 1 to 1.5 hours after exposure to the enzyme solution.

The denuded eggs thus obtained, either fertilized or unfertilized, flatten over the bottom of the petri-dish. Development of the denuded fertilized eggs proceeds quite normally, giving rise to tadpoles. In addition, the section of unfertilized eggs revealed that the egg nucleus is in the state of metaphase of the second maturation division and that the cortical granules remain unbroken (Fig. 5). From these results, it is concluded that the use of a natural hatching enzyme enables successful removal of the egg envelopes from both unfertilized and fertilized eggs without any harmful effects on the egg proper.

Fertilizability of the denuded egg. The denuded unfertilized eggs obtained by means of the hatching enzyme described above were carefully washed with 1/20 De Boer's solution. The eggs were then exposed to a dense sperm suspension made up with 1/20 De Boer's solution. In these eggs, however, no indication of fertilization could be observed, *i.e.* no breakdown of cortical granules, extrusion of the second polar body or cleavage. However, denuded unfertilized eggs obtained by the same method can be normally activated by pricking with a glass needle.

In the next place, fertilizability was studied of the eggs which were covered with the jelly envelopes to a various degree after exposure to the hatching enzyme solution for a varying period. Unfortunately, the digestion of the jelly envelopes by the enzyme does not proceed at the same rate over the whole surface of the same egg. Namely, the envelopes are digested in some areas, but the digestion does not proceed so markedly in other areas (Fig. 3). When inseminated, however, the eggs undergo cleavage if they are still invested with a small amount of the jelly material at the time of insemination. In the area where part of the jelly envelopes remain undigested around the egg surface, spermatozoa are found to

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digested. White area is the jelly remaining undigested. ca. × 20.

Figs. 4 & 5. Nucleus of the egg treated with thioglycolate-trypsin and with hatching enzyme, respectively. Fig 4, Feulgen-light green preparation; Fig. 5, Delafield's hematoxylin preparation. ca. × 1,000, respectively.

Figs. 6 & 7. Surface of the egg treated with hatching enzyme and inseminated. Fig. 6, An area where a part of the jelly envelopes remain undigested. Note a spermatozoon (arrow) in perpendicular contact with the egg surface; Fig. 7, Another area where the envelopes have been completely removed. ca. × 150, respectively.

swim straight forwards in the direction of the egg and make a direct perpendicular contact with the egg surface (Figs. 6 & 7). Some of them penetrate through the vitelline membrane with the top of their head: they twist their head and tail violently just like spermatozoa in normal fertilization. Careful observations proved that the perpendicular contact of the sperm head with the egg surface is always found if only a trace of the jelly envelopes, the innermost layer J_1, is present around the vitelline membrane. But when the egg is completely deprived of its envelopes or is covered only by the vitelline membrane, the movement of spermatozoa is entirely at random even on the surface of the egg proper or the vitelline membrane; no spermatozoa find perpendicular contact with the egg surface and adhere.

Discussion

The susceptibility of egg envelopes to enzymatic proteolysis as described in the present paper gives similar results to those obtained in other amphibian species (Bliss '40, Spiegel '51, Townes '53). Unlike the case of some other amphibian egg envelopes, however, single treatments with proteolytic enzymes are of no use for the complete digestion of the present material, *viz.* it is necessary to apply the successive treatment of proteolytic enzymes with reducing agents such as thioglycolate or KCN. In this respect, it is supposed that the disulfide linkages occupy a position of significance for the integrity of the structure of the egg envelopes. Preliminary cytochemical tests for S-S or SH groups were positive in the vitelline membrane and rather faintly so in the jelly envelopes.

The results of successive treatment with thioglycolate and trypsin provide some information concerning the change of the innermost jelly layer, J_1, and the vitelline membrane. It has been revealed in the previous paper that when the egg is immersed in tap water for 30 minutes prior to insemination, J_1 changes to prevent sperm penetration, and consequently the egg fertilizability is completely lost (Katagiri '62). The reduced susceptibility of the jelly layer, J_1, after immersion in tap water, therefore, serves as a strong demonstration of the change of that layer. Moreover, the results indicate that the vitelline membrane after fertilization or artificial activation becomes resistant to the treatment which induces the complete digestion of the membrane before fertilization. This fact reminds us of the well-known evidence found in the vitelline membrane or chorion of the sea urchin and teleost fish eggs that the change in the susceptibility to enzymatic treatments occurs at the time of fertilization (Monroy & Runnström '48, Yamamoto '57). In amphibian eggs, no attention has hitherto been paid to the change of the vitelline membrane at fertilization.

As cited above, Bliss ('40), Spiegel ('51) and Townes ('53) have succeeded in enzymatic proteolysis of amphibian egg envelopes without harmful effects on the developing embryos. This holds true also in the present material at least for

fertilized eggs. For unfertilized eggs, however, the enzymatic treatments which result in the complete digestion of the egg envelopes exert harmful effects, as revealed by the breakdown of cortical granules or by the induced abnormal state of egg nucleus. Barch & Shaver ('59) have noted that they could not fertilize or even parthenogenetically activate *R. pipiens* eggs dejellied by the papain-thiogly-colate method. This result can be easily accepted in view of the present observa-tion that unfertilized eggs treated in this way were activated during the treatment. It must be noted that unfertilized and fertilized eggs cannot be con-sidered in the same context when subjected to experimental treatments such as enzymatic proteolysis. Thus far studied, the use of a natural hatching enzyme is the only favorable method for the present purpose. The method employed for preparation of the hatching enzyme for the present material is essentially the same as that devised by Cooper ('36) for the developing eggs of *R. pipiens*. That the removal of the egg envelopes by the hatching enzyme is caused by some proteinase can be demonstrated by the alcohol-titration method, as noted by Ishida ('47). The enzyme may also attack gelatin, with its optimal activity at pH 7.0–8.0 (unpublished data).

Although unfertilized eggs denuded by the hatching enzyme can be activated by pricking, they are not fertilized by spermatozoa. The present result is there-fore in harmony with the observations that the jelly-less eggs of amphibians are generally unfertilizable (Kambara '53, Tchou & Wang '56, Shaver & Barch '60, Subtelny & Bradt '61). Kambara has reported that toad eggs, surrounded only by the innermost jelly layer, were not fertilizable; the fertilizability increased as the next three jelly layers were added. Tchou & Wang state, on the other hand, that jelly-less toad eggs become fertilizable when introduced into any region of an oviduct. Although more extensive experiments are needed to clarify the role of the jelly envelopes in the present material, the data in hand seem to coincide with the result of Tchou & Wang in showing that the eggs are fertilizable when only a trace of the jelly is present. That the presence of an innermost jelly layer is sufficient to enable successful fertilization is well recognized also in *Hyla* eggs (unpublished data). In addition, the present observation on the behavior of spermatozoa at the surface of the jelly-less eggs may indicate that the failure of fertilization in the denuded eggs is attributable to the failure of spermatozoa to adhere to the surface of the egg. A more detailed account of this problem will be given elsewhere.

Summary

Several attempts were made to remove the envelopes of the unfertilized frog egg. Treatments with NaOH, KOH, KCN and successive treatment with papain and sodium thioglycolate or with sodium thioglycolate and trypsin result in the removal of the egg envelopes. With these treatments, however, unfertilized eggs undergo cytolysis or activation. Healthy denuded unfertilized eggs are

obtained when the natural hatching enzyme of the same species is applied.   The method most favorable for the removal of egg envelopes by the hatching enzyme was described.

The denuded eggs obtained with the aid of the hatching enzyme are normally activated by pricking, but they are not fertilized by spermatozoa.   On the basis of these results, discussion was offered with particular emphasis on the nature of the egg envelopes and the relation of the envelopes to sperm penetration.

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