The Fertilizing Capacity of Frog Sperm in the Homologous and Heterologous Egg-jellies and Polyvinylpyrrolidone (PVP)[1]

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(With 5 Text-figures and 5 Tables)

The role of the egg-jelly in the amphibian fertilization has recently been studied along two different lines. The one is the immunological approach to the fertilization of *Rana pipiens*, which involves both the effects of the treatments of gametes with the antiserum directed against the jelly, and the demonstration of tissue- as well as species specificity of jelly antigens (Barch & Shaver, 1963; Shaver & Barch, 1960; Shaver *et al.*, 1962; Shivers, 1965; Shivers & Metz, 1962). The other approach to the problem has been made with the gametes of the toad, where the role of egg-jelly was analyed by inseminating completely dejellied uterine eggs in the presence of several substances including homologous and heterologous egg-jellies and a chemically well-defined material (Katagiri, 1966; 1967).

In view of the prominent effectiveness of a variety of substances as a substitute for the role of toad egg-jelly in fertilization, it seems necessary to determine if the similar situation applies also for the fertilization of other anuran species. The results presented below will provide evidence that a quite similar mechanism is present in the early phases of fertilization of both *Bufo* and *Hyla*.

Materials and Methods: The mature eggs and spermatozoa of a tree frog, *Hyla arborea japonica*, were used. Besides *Hyla*, spermatozoa were also obtained from *Bufo bufo formosus* and *Rana chensinensis*[2]. Physiologically balanced salt solution used was De Boer's solution (DB). Its dilutions into 1/2 and 1/20 will be called 1/2 DB and 1/20 DB, respectively, in the following pages. Dejellied *Hyla* eggs were obtained by the method of Bataillon (1919): *viz.*, Unfertilized uterine eggs were immersed in 0.5% KCN dissolved in DB. Since fertilization occurs when a trace of the jelly remains unremoved, care was taken particularly to achieve complete removal of the innermost jelly layer. The time required for the complete removal of jelly envelopes varied among the batches used, ranging from

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2) Formerly described as *Rana temporaria temporaria*, but recently shown to be different from that species (*cf.*, Kawamura, 1962).

50 to 75 min. The dejellied eggs thus obtained were washed thoroughly with 1/20 DB and submitted to the experiments. The results of insemination were expressed as a percentage of fertilization by counting the cleaved eggs at blastula or gastrula stage (18–24 hr. after insemination). The "homogenized jelly" of *Rana chensinensis* was prepared in the following way: Unfertilized uterine eggs were placed in 1/20 DB for 3–4 days at 5°C to allow complete hydration of jelly envelopes. The outer three layers of the envelopes were removed with forceps, collected, and were homogenized until they became homogeneous viscous solution. The homogenized jelly thus obtained was stored at –20°C until use. The methods for preparation of the "dialyzed jelly" and polyvinylpyrrolidone (PVP) solution have been described elsewhere (*cf.*, Katagiri, 1966). Other particular procedures used will be presented in each experimental part.

Results

The first experiment was performed to determine if dejellied eggs are fertilized in the presence of PVP, as found with the dejellied *Bufo* eggs (Katagiri, 1966). The dejellied eggs were placed in 5.0% PVP solution and were inseminated by spermatozoa of *Hyla*, *Bufo* and *Rana*. The results are presented in Table 1. As shown in the table, *Hyla* and *Bufo* sperm fertilize dejellied eggs in approximately the same rate. In the same condition *Rana* sperm do not fertilize *Hyla* eggs. Additional experiments using various concentrations of PVP proved that PVP in 5.0% is necessary to obtain high percentage of fertilization: *viz.*, the fertilizability considerably decreases when the concentration of PVP becomes below 2.5%. The dejellied eggs fertilized by *Bufo* sperm develop in normal fashion during cleavage stages until they attain blastula stage, and form a dorsal lip (Figs. 1 and 2). However, the gastrulation proceeds to a slight degree and the development ceases as an arrested gastrula which is frequently found in the variety of hybrid crosses (*cf.*, Moore, 1955). The results presented in Table 1 thus prove that PVP acts as a substitute for the *Hyla* jelly in its role in fertilization. It is also suggested that *Hyla* sperm behave similarly to *Bufo* sperm with respect to their ability to fertilize eggs.

Table 1. Fertilizability of dejellied *Hyla* eggs inseminated in 5.0% PVP by homologous and heterologous sperm

Inseminated in	Sperm used	No. of eggs used	Percentage cleaved
PVP	*Hyla*	52	88.5
PVP	*Bufo*	64	92.2
PVP	*Rana*	52	0
Control (1/20 DB)	*Hyla*	48	0

Then the possibility arises that the penetration of *Hyla* sperm into homologous eggs could be assured by the presence of *Bufo* jelly. In order to test this, dejellied eggs were placed in the dialyzed jelly solutions from *Hyla* and *Bufo*, and were inseminated. The results are shown in Table 2. It is evident that the jelly from both species is highly effective for the occurrence of fertilization. Lower efficiency of *Hyla* than *Bufo* jelly presented in the table seems to be due to the rather lower concentration of jelly material in the former. This is illustrated by the observation that the higher percentage of fertilization is obtained when the more concentrated dialyzed jelly from *Hyla* is employed. The results thus indicate that *Hyla* sperm attain fertilizing capacity in response to *Bufo* as well as to the species jelly.

Table 2. Effectiveness of dialzyed jellies of *Hyla* and *Bufo* for fertilization of dejellied *Hyla* eggs

Inseminated in	No. of eggs used	No. of eggs cleaved	Percentage cleaved
Hyla jelly	49	30	61.2
Bufo jelly	45	37	80.4
Control (1/20 DB)	44	0	0

Table 3 represents the results of a similar experiment in which the dejellied *Hyla* eggs were inseminated in the egg-jelly from *Rana*. It is clear that the *Hyla* sperm again utilize homogenized jelly, but not the dialyzed one, of *Rana* as a substitute for the species jelly. The *Rana* sperm, on the contrary, fail to fertilize *Hyla* eggs even in the presence of the species egg-jelly. Supplementarily it was noted that a percentage of the eggs fertilized in the homogenized *Rana* jelly display the retardation in the initiation of cleavage, presumably due to the delay of the sperm entry into eggs. Additional attempts to fertilize eggs by *Hyla* sperm in the dialyzed *Rana* jelly were not successful, after careful check on the pH of the solutions, the movements of sperm, *etc.*

Table 3. Effectiveness of *Rana* jelly for fertilization of dejellied *Hyla* eggs

Inseminated in	Sperm used	No. of eggs used	Percentage cleaved
Rana homogenized jelly	*Hyla*	48	60.4
Rana homogenized jelly	*Rana*	54	0
Rana dialyzed jelly	*Hyla*	49	0
Rana dialyzed jelly	*Rana*	41	0
5.0% PVP	*Hyla*	40	80.0
Control(1/20 DB)	*Hyla*	44	0

The results obtained in the above experiments were further confirmed by the observations on the ordinary cross-fertilization. Table 4 is a summary of the results of the crosses where fully jellied uterine eggs of *Hyla* were inseminated by spermatozoa of *Hyla, Bufo* and *Rana*. As shown in the table, the results are quite comparable with those presented in the above tables in showing that essentially no difference is found in the fertilizing capacity of both *Hyla* and *Bufo* sperm. A remarkable observation in this experiment was that no *Rana* sperm do penetrate into the jelly envelopes, in contrast to *Bufo* and *Hyla* sperm which are found in great number at the vitelline membrane. The failure of fertilization by *Rana* sperm here obtained is therefore attributable to the inability of spermatozoa to penetrate into *Hyla* jelly.

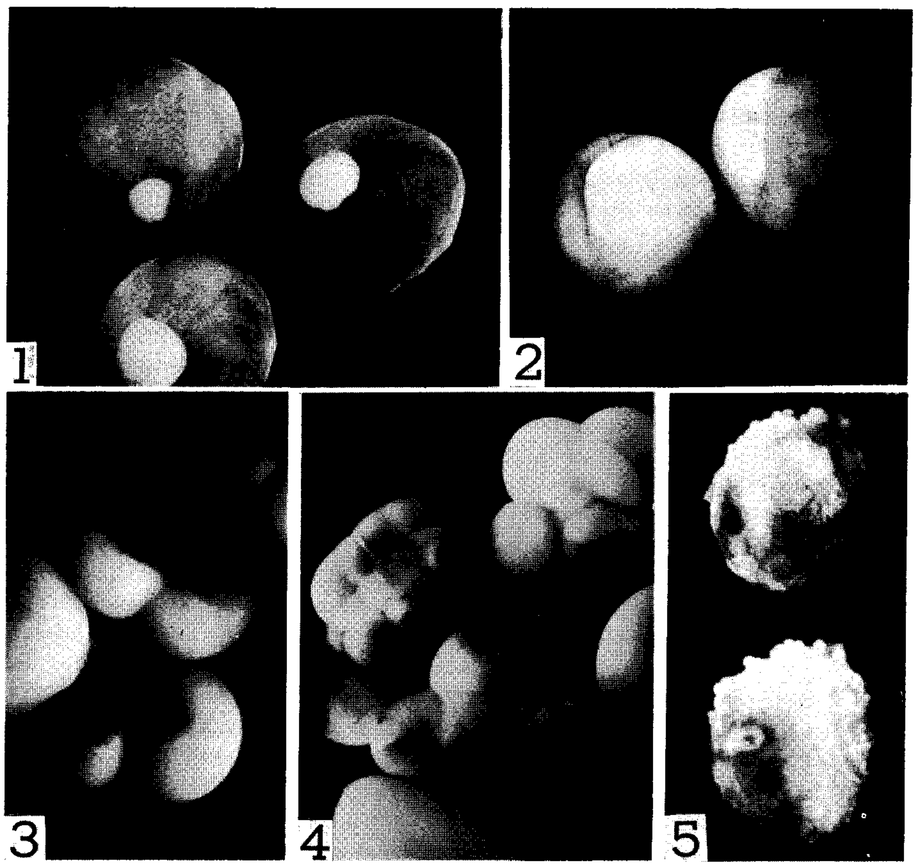
Table 4. Fertilizing capacity of heterologous sperm into fully jellied *Hyla* eggs

Sperm used	No. of eggs used	Percentage cleaved	Remarks
Bufo	41	97.6	Many sperm in jelly Developmental arrest at gastrula stage
Rana	47	0	No sperm in jelly
Hyla	38	92.1	Many sperm in jelly

In the cross *Rana chensinensis* ♀ × *Bufo* ♂, the evidence has been presented that the blockage of sperm entry into eggs occurs at the vitelline membrane (Katagiri, 1967). Thus it seemed of interest to study whether or not the *Rana* sperm could fertilize *Hyla* eggs in the presence of *Rana* jelly, if the eggs had been freed from the vitelline membrane. For this purpose, preliminary tests were made to remove the vitelline membrane of *Hyla* eggs without loss of the egg viability. The eggs dejellied by KCN were treated with 0.3% trypsin dissolved in 1/20 DB (pH 9.0) in a petri-dish with agar base. In 15–30 min. after the treatment, the vitelline membrane became digested and the eggs were freed from the membrane. The eggs thus obtained, briefly called "denuded eggs" in the following pages, flattened over the bottom of the petri-dish but were healthy as defined by the pigmentation changes and the second polar body emission upon pricking. Then the fertilizability of the denuded eggs was studied in the following way. The petri-dishes with agar base were filled with the experimental media such as the homogenized jelly of *Rana*, 5.0 % PVP or 1/20 DB. Concentrated sperm suspensions of *Hyla* and *Rana* were added dropwise to the media, and the denuded eggs were placed gently onto it. The results of this experiment are summarized in Table 5. The table indicates that, coincident with the case of dejellied eggs, spermatozoa of both species are unable to fertilize denuded eggs when inseminated in 1/20 DB. The presence of PVP or jelly material evidently assures the successful entry of *Hyla* sperm into the eggs. It is also clear that *Rana* sperm fail to fertilize *Hyla* eggs freed from the vitelline membrane, in spite of the presence of the species egg-jelly. Among the eggs inseminated

Table 5. Fertilizability of eggs denuded by successive treatments with KCN and trypsin (Explanation in text)

Time treated with trypsin (min.)	Inseminated in	Sperm used	No. of eggs used	Percentage cleaved
28	*Rana* jelly	*Hyla*	34	23.5
28	*Rana* jelly	*Rana*	51	0
28	5.0% PVP	*Hyla*	38	55.3
28	5.0% PVP	*Rana*	44	0
28	1/20 DB	*Hyla*	32	0
0	5.0% PVP	*Hyla*	24	79.2
0	1/20 DB	*Hyla*	34	0



Figs. 1 and 2. Dejellied eggs inseminated in PVP by sperm of *Hyla* (Fig. 1) and *Bufo* (Fig. 2), respectively. Photographed at gastrula stage. Note arrested yolk plug closure in Fig. 2.

Figs. 3–5. Denuded eggs inseminated in PVP, showing the occurrence of cleavage. Photographed 2.5 hr. (Fig. 3), 3 hr. (Fig. 4), and 20 hr. (Fig. 5) after insemination, respectively.

by *Hyla* sperm, the lower fertilizability of denuded than of dejellied eggs is likely to be due to the apparent mechanical damage of the former during the experimental manipulation. The fertilized denuded eggs do not continue cleavage beyond 4 cell stage when cultured in the media suitable for fertilization; *i.e.*, PVP or jelly solution. This was ameliorated by replacing the eggs to 1/2 DB 1–1.5 hr. after insemination: *viz.*, in 1/2 DB the cleavage and development of denuded eggs proceed at the latest until mid-gastrula stage (Figs. 3–5).

Discussion

Comparison of the present results with those obtained in the gametes of toad (*cf.*, Katagiri, 1966; 1967) indicates that in both cases fertilization of dejellied eggs is assured by quite the same substances; namely, the egg-jellies of *Bufo*, *Hyla* and *Rana* or PVP. Hence it may be reasonable to suppose the presence of a common mechanism by which both *Bufo* and *Hyla* sperm are stimulated similarly to be capable of fertilizing eggs. In this respect the spermatozoa of *R. chensinensis* appear to behave in somewhat different manner. Failure of fertilization by *Rana* sperm observed in the present study, however, cannot be simply interpreted as indicating that the fertilizing capacity cannot be induced by the egg-jellies used or PVP. As pointed out elsewhere (Katagiri, 1967), the vitelline membrane will also act as a barrier to the successful union of heterologous gametes. Further it is not conceivable that *Rana* sperm is not stimulated by the homologous egg-jelly. Inability of *Rana* sperm to fertilize *Hyla* eggs freed from the vitelline membrane, is therefore attributable to the failure of interaction of heterologous gametes themselves. In order to determine the nature of the fertilizing capacity of *Rana* sperm, then, similar experiments are needed with the dejellied *Rana* eggs.

One of the criteria for the occurrence of the fertilizing capacity of *Hyla* sperm is their adherence to the vitelline membrane, just as found in the toad sperm (Katagiri, 1966). As for the role of egg-jelly in fertilization, a tentative explanation has been presented by Shaver (1966), who suggests that the interaction of the complementary molecular configurations of jelly and sperm surfaces may be an important factor in the initial step of fertilization. This view is based on the extensive immunological approaches which involve the treatments of gametes with the anti-jelly serum as well as the agar diffusion analyses of several jelly antigens (Barch & Shaver, 1963; Shaver & Barch, 1960; Shaver *et al.*, 1962; Shivers, 1965; Shivers & Metz, 1962). There is of course good reason to anticipate that the interaction of some kinds takes place in the reciprocal gametes during the process of their successful union. However, the exact reactive sites of the jelly participating in the interaction with sperm, if present, have been shown to be of a nature that cannot be identified by the precipitation analysis (Katagiri, 1967). Further support of this is the failure of the immunological cross-reaction between the egg-jellies of *Bufo* and *Hyla*, in spite of their high efficiency in stimulating reciprocal heterologous sperm.

In view of the high efficiency of a variety of egg-jellies as well as PVP in fertilization, it seems more reasonable to assume that the interaction of sperm and jelly may be a type of stimulant-reactant mechanism in a wide sense. This assumption may involve recognition that the specific reactivity of the sperm surface in response to the surrounding substances is a predominant factor of the interaction in question. Further analyses of this problem, in connection with those of the fine structure of spermatozoon, will be presented elsewhere.

Summary

In order to determine the conditions necessary for the sperm entry into eggs, experiments were performed in the tree frog, *Hyla arborea japonica*, with the uterine eggs freed from the jelly envelopes. It was found that *Hyla* sperm utilize either PVP, dialyzed jellies of *Bufo* and *Hyla*, or homogenized jelly of *Rana chensinensis*, as a substitute for the species intact jelly envelopes. Also in the eggs freed from the vitelline membrane, the presence of PVP or jelly material was found to be necessary for the successful sperm entry into eggs. Spermatozoa of *Bufo* also fertilize the dejellied *Hyla* eggs in response to PVP, *Hyla* as well as the species jelly material. In the same conditions, the spermatozoa of *R. chensinensis* fail to fertilize *Hyla* eggs. The results were discussed in comparison with those obtained in the toad eggs, with particular attention to the nature of the fertilizing capacity of sperm.

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