J. Embryol. exp. Morph. Vol. 31, 3, pp. 573–587, 1974
Printed in Great Britain

A high frequency of fertilization in premature and mature coelomic toad eggs after enzymic removal of vitelline membrane

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SUMMARY

The jelly-less coelomic eggs of the toad, *Bufo bufo*, even if chromosomally mature and reactive to parthenogenetic stimuli, are not fertilized or fertilized in a low frequency under the conditions which assure the sperm entry into dejellied uterine eggs. A high frequency of fertilization was obtained, however, when coelomic eggs from which the vitelline membrane had been digested away by pronase or hatching enzyme were inseminated in the presence of polyvinylpyrrolidone (PVP) or the diffusible egg-jelly component. Experimental evidence is presented to show that the conditions important for inducing this success in fertilization are the complete removal of the vitelline membrane and insemination in the presence of one of the following sperm 'capacitating' substances: the diffusible jelly component, the non-dialysable jelly component, PVP, dextran, or Ficoll. Fertilization obtained by this method was monospermic, as defined both by cytological examination and by the mode of early cleavage. However, the eggs invariably ceased development at the gastrula stage, because of the lack of the mechanical support normally provided by the egg envelopes. These results indicate that toad eggs acquire the full ability, without any influences from the oviduct, both to react to a penetrating sperm and to prevent polyspermy.

When eggs undergoing maturation (premature eggs) were demembranated and inseminated in the presence of PVP, they did not show a genuine cleavage. Cytological examination proved the occurrence of highly polyspermic fertilization in these premature eggs, together with a tendency for the rate of pronucleus formation by the incorporated sperm to increase as the eggs were closer to the stage of full nuclear maturity.

On the basis of these results, a possible role of the oviduct in affecting the vitelline membrane-egg surface interrelationship is discussed, as well as the changing reactivity of maturing oocytes to spermatozoa, and vice versa.

INTRODUCTION

Usually, amphibian eggs removed from the coelom are not fertilized by sperm, becoming fully fertilizable only when they acquire the jelly envelopes during their passage along the oviduct to the uterus or ovisac. There are several reasons for thinking that coelomic eggs will mature without a sojourn in the female genital tract although they cannot incorporate sperm. This is suggested by several lines of study: namely cytological observations (for a review of the older

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literature, see Aplington, 1957), competence toward parthenogenetic stimuli (Bataillon, 1919; Barthélémy, 1922; Tchou & Wang, 1956), and the full capability to undergo normal development after receiving transplanted somatic cell nuclei (Subtelny & Bradt, 1961). All these studies have thus led the workers to emphasize the necessity of the jelly envelopes secreted by the oviduct for a successful sperm-egg fusion in fertilization (for a review, see Shaver, 1966).

It is now well established by recent studies that uterine eggs, from which the jelly envelopes have been artificially removed, may be fertilized if they are inseminated in the presence of various egg-jelly preparations or a synthetic high polymer (Katagiri, 1966*a*, *b*, 1973*a*; Barbieri & Raisman, 1969; Elinson, 1971*a*; Wolf & Hedrick, 1971; Barbieri & Oterino, 1972). However, attempts to fertilize coelomic eggs under the same or other conditions have so far led to negative or only partially successful results (Subtelny & Bradt, 1961; Katagiri, 1965; Shivers & James, 1970; Elinson, 1971*a*). It is therefore possible to assume that in addition to jelly the oviduct supplies some other factor required for fertilization (cf. Katagiri, 1965; Elinson, 1971*a*).

Very recently, Elinson (1973*a*, *b*) succeeded in fertilizing coelomic *Rana pipiens* eggs by both homologous and *R. clamitans* sperm in the presence of the diffusible *pipiens* egg-jelly component ('egg-water'), providing strong evidence that the coelomic eggs are mature with respect to their ability to accept sperm. Of particular interest in his experiment (Elinson, 1973*a*) is that the vitelline membrane needs to be modified to obtain high frequencies of fertilization by the homologous sperm.

A previous experiment by the author (Katagiri, 1965) showed that 30–50 % of coelomic toad eggs could be normally fertilized after they had been stored *in vitro* for 1 or 2 days at low temperature. However, the reason for this partial success remained obscure. In the effort to answer this question, it has been found that the enzymic removal of the vitelline membrane before insemination results in a consistently high degree of success in fertilizing both mature and premature coelomic eggs. The details of the technique and the results will be described and discussed below.

MATERIAL AND METHODS

Mature *Bufo bufo* were purchased from a dealer in Tokyo during the hibernation period, and stored at 4 °C until use. All the experiments reported here were done with toads which had been in hibernation for more than 3 months.

Coelomic eggs were obtained by the method described previously (Katagiri, 1965), and placed in De Boer's solution (DB). Approximately 20 eggs from each batch were taken out of DB, and pricked with a glass needle in 1/20 dilution of DB (1/20 DB). When more than 90 % of the eggs reacted normally, as defined by both the rotation of orientation and polar body emission, the eggs were regarded as mature and stored at 5 °C for subsequent use.

In order to obtain premature eggs, i.e. those from the stage of germinal vesicle breakdown to the stage before the second meiotic metaphase, maturation was induced *in vitro* in the following way: Portions of the ovarian lobes were excised from a pithed female, and placed in DB. Individual fully grown oocytes were freed from the surrounding ovarian tissues with watchmaker's forceps, leaving the follicle cells and the vitelline membrane intact (cf. Masui, 1967). About 50 such oocytes were incubated at 18 °C in 10 ml DB containing 1 macerated pituitary and antibiotics. Under these conditions, oocytes of several maturation stages were obtained 10–20 h after the beginning of treatment. The approximate stages of the oocyte maturation were checked periodically according to the criteria described by Smith, Ecker & Subtelny (1968) for *Rana pipiens*. Aliquots from each experimental lot were fixed for a further cytological confirmation of the maturation stages.

The digestion of the vitelline membrane was accomplished with 0·05 % pronase-E (Kaken Chem. Co.) in DB (pH 7·2). The demembranated eggs were then washed by transferring them with a wide-mouthed pipette to another dish containing DB. The enzymic digestion and the washing were carried out in a Petri dish with a 2 % agar base. The hatching enzyme used for digesting the vitelline membrane was prepared from pre-hatching embryos according to the method described for the *R. chensinensis* enzyme (Katagiri, 1973*b*). The enzyme used in the present study was fairly crude, and the actual concentration of the enzyme involved could not be determined.

'Dialysed jelly' (DJ) and the diffusible jelly component (DF) used as insemination medium were prepared from uterine eggs in the manner described elsewhere (Katagiri, 1966*a*, 1973*a*). Synthetic polymers, 5 % polyvinylpyrrolidone (PVP; Tokyo Kasei Chem. Co., mol. wt., 350000), 10 % dextran (British Drug Houses Ltd, mol. wt., 150000–200000), and 10 % Ficoll (Pharmacia) were dissolved in 1/20 DB.

Sperm suspensions were prepared by macerating one testis in 5 ml 1/20 DB. For insemination of denuded eggs, drops of the sperm suspension (approximately 0·1 ml) were first added to 3 ml insemination medium, and the denuded eggs were then placed gently into it. One and a half hours after insemination, the insemination medium was replaced by 1/2 dilution of DB (1/2 DB) or full strength Steinberg's solution to assure that cleavage took place in the fertilized eggs. Care was also taken to observe the time of occurrence of the first cleavage as well as the mode of the early cleavages. The percentage of fertilization was recorded 20–24 h after insemination by counting the number of eggs which attained the blastula–early gastrula stages. Unless otherwise stated, all the experiments on fresh material were carried out at room temperature (18–22 °C).

For the cytological observations, eggs were fixed in Helly's solution, and 8 μm thick serial sections were prepared by the methylbenzoate-paraffin method. The sections were stained by Feulgen's nuclear reaction, and counterstained with light green.

Table 1. *Effect of pronase treatment on the fertilizability of coelomic toad eggs. Eggs were treated with 0·05 % pronase-DB, and inseminated in PVP or 1/20 DB*

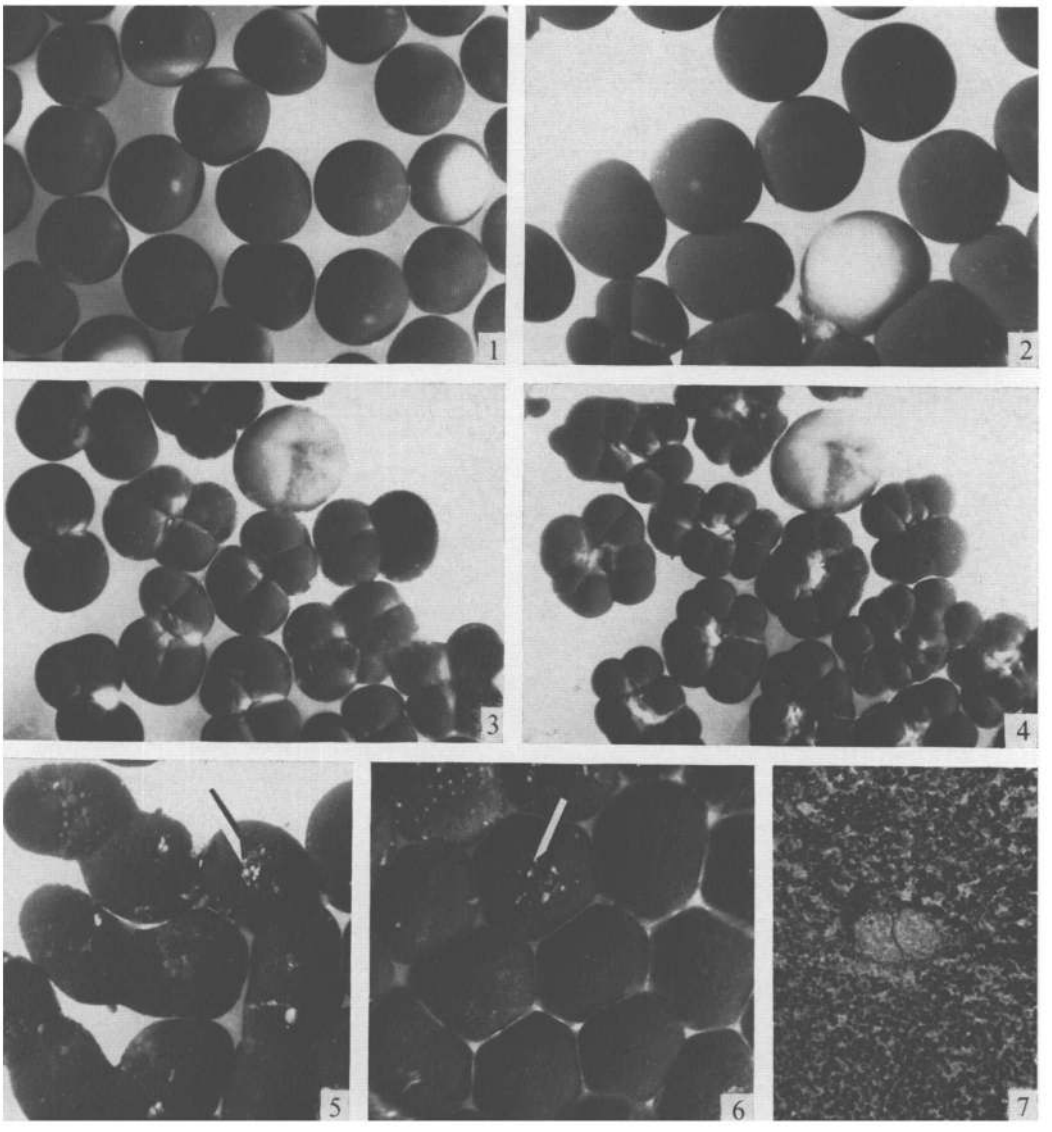
Treatment	(min)	Inseminated in	No. of eggs used	Percentage cleaved
Pronase	15	PVP	75	0
	15	1/20 DB	60	0
	30	PVP	72	6·9
	30	1/20 DB	61	0
	45	PVP	93	75·3
	45	1/20 DB	68	0
	60	PVP	71	94·4
	60	1/20 DB	52	1·9
DB (control)	60	PVP	71	0
	60	1/20 DB	67	0

RESULTS

Fertilization of 'mature' coelomic eggs

When placed in 0·05 % pronase-DB at 20 °C, coelomic eggs showed no appreciable changes for the first 30–40 min. At about 40 min, a prominent wrinkling of the egg surface became apparent, and this lasted for about 15 min. Then the egg surface became smooth, accompanied by the flattening of the egg proper over the agar base. A microscopical observation revealed that the last step described above was due to the peeling off of the wrinkled vitelline membrane from the egg surface, thus completely freeing the eggs from the membrane. A few minutes after the wrinkling of the membrane, its removal was possible by a gentle shaking of the dish, or even by transferring the eggs to another dish containing DB. The time required for the digestion of the vitelline membrane varied among individual eggs, the batches of eggs used, and the enzyme preparations. In most cases, demembranated eggs were obtained within 50–75 min. The viability of the eggs was not affected by the enzymic treatment, since pricking the flattened eggs with a glass needle induced pigmentation changes and polar body emission in a quite normal fashion.

The effect of pronase treatment on egg fertilizability was tested in the following way. Coelomic eggs were placed in 0·05 % pronase-DB. Some of the eggs were taken out of the enzyme at 15 min intervals, washed with DB and inseminated in 5 % PVP-1/20 DB or 1/20 DB. The results presented in Table 1 clearly show that longer enzyme treatment greatly improves egg fertilizability. It is also evident that, as repeatedly shown for dejellied uterine eggs (Katagiri, 1966*a*, *b*), demembranated eggs require the presence of PVP in order to be successfully fertilized by sperm. The observed increase in the egg fertilizability apparently paralleled the visual damage of the vitelline membrane by pronase: namely at the end of a 45-min treatment, a wrinkling of the membrane



FIGURES 1–7

Fig. 1. Coelomic eggs which failed to be fertilized, inseminated in PVP. Photographed 6 h after insemination. × 4.

Fig. 2. Coelomic eggs demembranated by pronase, showing flattening on the base of dish. Photographed 6 h after insemination in 1/20 DB. One fertilized egg in four-cell stage is seen at the lower left. × 4.

Fig. 3. Coelomic eggs demembranated by pronase and inseminated in PVP, showing four-cell stages. 6 h after insemination. × 4.

Fig. 4. Eight-cell stages of the same eggs as shown in Fig. 3, 6·5 h after insemination. × 4.

Figs. 5 and 6. Blastulae from the demembranated and fertilized eggs shown in Figs. 3 and 4, 20 h after insemination. Note fusion of neighbouring blastulae in Fig. 5. Arrows indicate blastomeres which were separated out of the blastocoel roof. × 4.

Fig. 7. Section of demembranated and fertilized coelomic egg, showing male and female pronuclei in close contact. Fixed 2 h after insemination. × 400.

Table 2. *The fertilizability of coelomic toad eggs demembranated by hatching enzyme or pronase. Demembranated eggs were inseminated in diffusible jelly component (DF) or 1/20 DB*

Treatment	(min)	Inseminated in	No. of eggs used	Percentage cleaved
Hatching enzyme	30	DF	53	71·7
	30	1/20 DB	42	4·8
Pronase	50	DF	46	87·0
	50	1/20 DB	43	2·1
DB (control)	50	DF	55	0
	50	1/20 DB	41	0

was already apparent, and in a large proportion of the eggs, the membrane actually became torn off during the manipulation of the eggs for insemination. At 60 min, the digestion of the membrane was so complete as to cause all the eggs to be flattened down on the agar base (Figs. 1 and 2).

The eggs fertilized by the above method underwent cleavages in normal time as compared with fertilized uterine eggs. In addition, there was no significant deviation among individual eggs in the same dish with respect to the time of the onset of the first cleavage. Because of the flattening of the eggs, the third and fourth cleavages deviated from the normal fashion in that the blastomeres were divided in a horizontal direction (Figs. 3 and 4). The development of these eggs proceeded to blastula or early gastrula stages, with an occasional fusion of neighbouring blastulae (Figs. 5 and 6), but finally deteriorated as arrested gastrulae. Examination of sections proved the occurrence of monospermic fertilization, the union of male and female pronuclei (Fig. 7), as well as the formation of blastocoel and early blastopore, although the blastomeres localized at the blastocoel roof tended to move out of line (Figs. 5 and 6, arrows). It seems that the observed failure of fertilized coelomic eggs to develop beyond the gastrula stage can be accounted for by the flattening of the egg proper in the absence of the vitelline membrane.

Besides pronase, the homologous hatching enzyme was also effective in removing the vitelline membrane without affecting the viability of coelomic eggs. Since isotonic saline is known to be rather inhibitory to the hatching enzyme activity (cf. Katagiri, 1973*b*), the lyophilized crude enzyme preparation was dissolved in 1/2 DB (pH 7·4). In one experiment, the coelomic eggs placed in the enzyme were freed from the vitelline membrane in 30 min. Along with this series, eggs from the same batch were demembranated by pronase and inseminated with same sperm suspension to compare the egg fertilizability. As will be seen in Table 2, both methods for removing the vitelline membrane improved the egg fertilizability to a similar degree, when the eggs were inseminated in the presence of diffusible jelly component (DF). From these results, it may well be

Table 3. *Effect of insemination medium on the fertilizability of demembranated coelomic eggs of the toad. Eggs were demembranated by 0·05 % pronase-DB, and inseminated in 3 ml of various media containing the same amount of sperm suspension*

Inseminated in	No. of eggs used	Percentage cleaved
DF	41	80·5
Dialysed jelly	43	72·1
5 % PVP	38	84·2
10 % dextran	47	78·7
10 % Ficoll	42	66·7
1/20 DB (control)	53	10·4

concluded that the complete removal of the vitelline membrane renders coelomic eggs fully fertilizable provided that the spermatozoa are properly 'capacitated'.

The results presented in Tables 1 and 2 suggested the necessity of 'capacitating' sperm for fertilizing demembranated coelomic eggs, as was found true for dejellied uterine eggs (cf. Katagiri, 1973*a*). To determine the spermatozoan capacity for fertilizing coelomic eggs, the pronase-treated eggs were inseminated in the presence of egg-jelly preparations or synthetic polymers which have been shown to be effective for capacitating sperm in the dejellied egg bioassay system. As will be evident from Table 3, all the substances which assure the fertilization of dejellied uterine eggs similarly do so in fertilizing demembranated coelomic eggs. It is apparent that there is no difference with respect to the requirement for sperm capacitation between uterine and coelomic eggs if the latter are deprived of the vitelline membrane. Another observation to be mentioned in connexion with Table 3, as well as Tables 1 and 2, is that frequently a low percentage of demembranated eggs were fertilized in 1/20 DB, which was never observed in eggs invested with the vitelline membrane.

Fertilization of premature eggs

Employing the technique described above, an attempt was made to fertilize the eggs of the maturation stages before the second polar spindle formation. To do this, fully grown ovarian oocytes were induced to mature *in vitro*. After a 10 h incubation with pituitary-DB, all the oocytes had started maturation, as defined by the appearance at the animal pole of a less pigmented area with or without black dots (cf. Smith, Ecker & Subtelny, 1968). On treatment with pronase, the vitelline membrane surrounding the premature eggs was digested as described in the above section. The follicular layer outside the membrane was not digested, but became so fragile in the enzyme that it was eventually torn off by a gentle shaking of the dish, thus causing the eggs to flatten on the agar base within 60–70 min after the beginning of treatment. The oocyte

maturation proceeded during and after the enzymic treatment. The demembranated eggs were washed with DB, and inseminated in the presence of 5 % PVP at the 12th, 15th and 20th hour after the initiation of pituitary treatment. More than 75 % of the eggs inseminated after 20 h of treatment underwent cleavage in the manner described in the previous section, indicating that they had attained full maturity. However, those inseminated at the 12th and 15th hour did not show genuine cleavage. A conspicuous feature in these lots was the occurrence on the egg surface of numerous pits with a concentration of pigment granules, and occasional irregular grooves which later disappeared. Particularly, the eggs inseminated at the 15th hour occasionally showed unsynchronized, partial cleavages usually confined to the pigmented animal hemisphere, followed by deterioration of the egg within 24 h.

To determine what occurs in the premature eggs, the eggs were fixed 60 min after insemination (i.e. 13, 16 and 21 h after the initiation of pituitary treatment), and processed for cytological examination. The cytological aspects of the eggs inseminated at the 20th hour and fixed 1 h later were comparable to those found in normally fertilized uterine eggs: the female pronucleus was just under the egg surface and, more interiorly, a sperm pronucleus with the well-pigmented path pursued by the migrating sperm was seen (Figs. 8 and 9). This observation forms the basis for interpreting the cell division described above as genuine total cleavage.

The eggs inseminated at the 12th and 15th hour after pituitary treatment exhibited without exception a high incidence of polyspermy. In Table 4 are summarized the results of the cytological observations as expressed in terms of the state of egg and sperm nuclei, as well as the counted number of sperm in individual eggs. As shown in the table, the number of sperm counted per egg was fairly variable among the eggs even at the same maturation stage. Besides many sperm within the egg, there was a frequent occurrence of cytoplasmic

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FIGURES 8–15

Photomicrographs of eggs induced to mature *in vitro*, demembranated by pronase, and inseminated thereafter. All specimens fixed 1 h after insemination.

Figs. 8 and 9: Egg inseminated 20 h after beginning of pituitary treatment, showing a male pronucleus (Fig. 8, arrow) with pigment trail, and a forming female pronucleus (Fig. 9). Arrow in Fig. 9 indicates second polar body. × 100 and × 1000, respectively. Figs. 10–13: Sections from one egg inseminated 12 h after beginning of pituitary treatment. Figs. 10 and 12 show clear cytoplasmic areas surrounded by sperm asters, and a dense pigment accumulation (arrow) formed by a penetrating sperm. Collapsed fragments of follicular layer with adhering sperm heads are seen outside the egg. × 100. Fig. 11, showing egg nucleus at first meiotic metaphase. × 1000. Fig. 13, showing a sperm head just after incorporation, accompanied by contraction of the cytoplasm. × 1000.

Figs. 14 and 15. Egg inseminated 15 h after beginning of pituitary treatment. Fig. 14, showing swelling male pronuclei in enlarged clear cytoplasmic areas. × 250. Fig. 15, first polar body in the process of being extruded. × 1000.

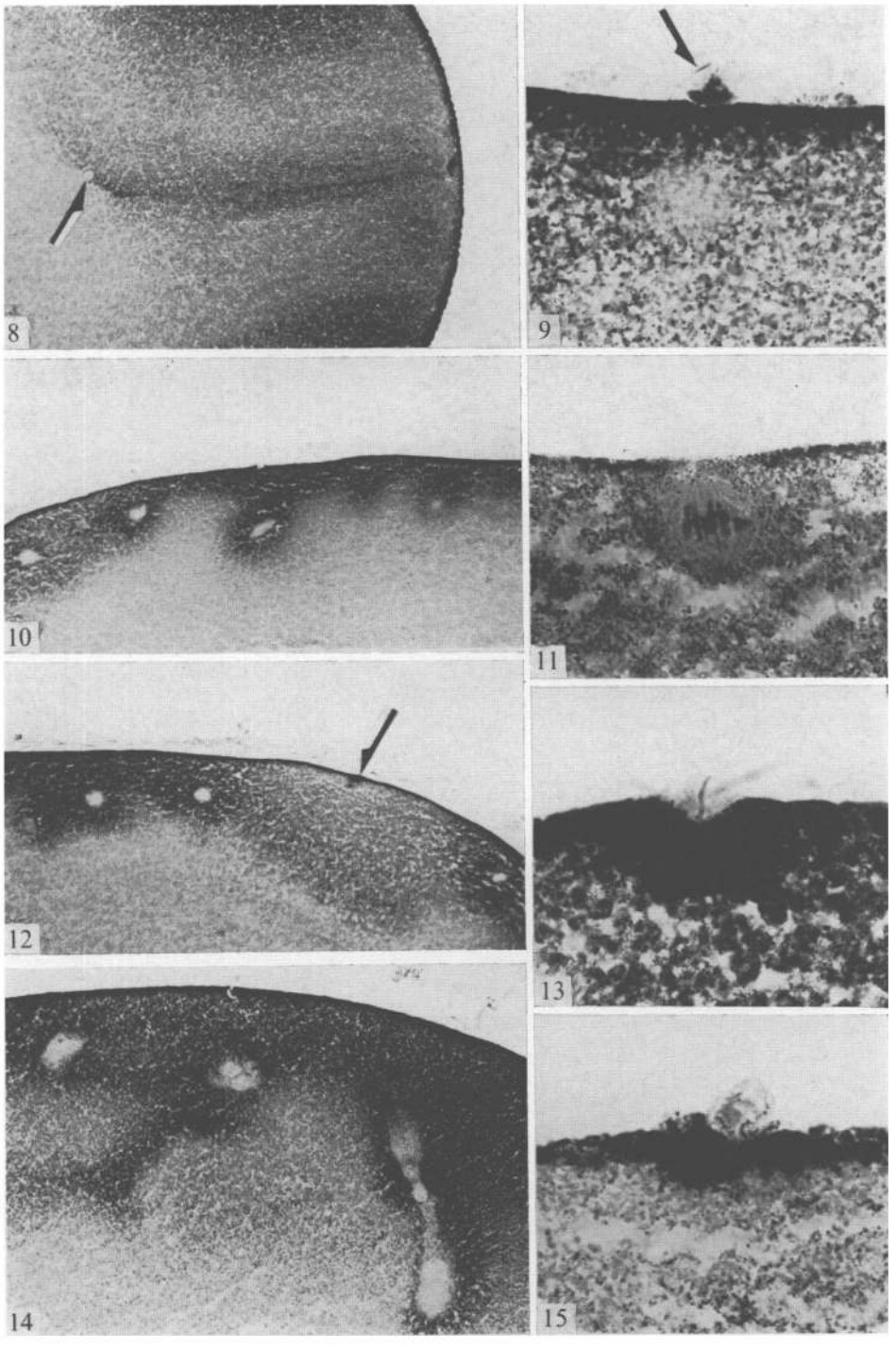
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Table 4. *Results of observations on sectioned eggs which were demembranated and inseminated at various maturation stages. Eggs were fixed 1 h after insemination. Numbers of sperm counted per egg are recorded, together with the state of the egg nucleus at the time of fixation*

Time after pituitary treatment	State of egg nucleus	Number of sperm per egg: Forming pronucleus	Without pronucleus	Total number
13 h	First meiotic metaphase	3	59	62
		2	24	26
		0	14	14
		1	9	10
		0	10	10
		0	8	8
	First meiotic ana- and telophase	1	23	24
		2	15	17
		0	13	13
		0	5	5
	First polar body emission	10	14	24
		9	4	13
	Unidentified	3	11	14
		6	3	9
		0	9	9
16 h	First meiotic ana- and telophase	2	11	13
		0	6	6
	First polar body emission	16	15	31
		10	17	27
		10	9	19
		9	4	13
		2	4	6
	Second meiotic anaphase	0	1	1
	Unidentified	9	36	45
		22	20	42
		9	11	20
21 h	Second meiotic metaphase	0	0	0*
		0	0	0*
	Second meiotic anaphase	0	1	1
	Pronucleus	0	1	1†

* Probably unfertilized. † Ten other eggs were found to be in the same state.

contractions at the egg surface which included a penetrating spermatozoon (sperm entrance cone), even at 1 h after insemination (Fig. 13). As in the monospermic mature eggs, sperm penetration into the premature eggs was always confined to the pigmented animal hemisphere (Figs. 10, 12 and 14). Once inside the egg, sperm were associated with large asters, either with or without

formation of a clear, reticular male pronucleus (Figs. 10, 12 and 14). A trend may be seen in Table 4, that the earlier the egg maturation stage, the smaller the number of sperm nuclei which transformed into distinct pronuclei. Particularly, in eggs at the first meiotic metaphase (Figs. 10–12), most sperm nuclei either failed to transform into large pronuclei or seemed to have degenerated to form a homogeneous cytoplasmic area associated with the astral rays. Although the data presented in Table 4 must be interpreted with the limitation that several of the spermatozoa observed in each egg possibly had a 60 min difference in the length of time spent in the egg cytoplasm, the observed trend in the behaviour of incorporated sperm indicates the probability of irregular cytokinesis, leading to the failure of genuine cleavage, as observed in the living material.

DISCUSSION

In discussing the significance of the results above described, a comparison should first be made of the present methods with those which induced the reported success in fertilizing coelomic *Rana pipiens* eggs (Elinson, 1973*a*). The methods used for *R. pipiens* included a visible weakening of the vitelline membrane either by brief trypsinization or cyanide treatment, as well as a manual ripping of the membrane. The present methods employing pronase and the hatching enzyme are comparable to the last method used by Elinson, in the sense that complete removal of the membrane was required in order to increase the egg fertilizability. The possibility that the enzymic treatments employed here affected the egg plasma membrane or cortex to cause the observed improvement, is hardly likely in view of the cytological confirmations reported above, together with the occurrence of the first two cleavages in normal time and normal fashion. In another line of studies, it was shown that trypsinization affects the vitelline membrane of *R. chensinensis* uterine eggs, enabling them to be cross-fertilized by *Bufo* sperm (Katagiri, 1967). However, preliminary efforts with the present material to improve the fertilizability of coelomic eggs with trypsin have thus far ended in total failure, although the outer part of the vitelline membrane was digested by a dual treatment with reducing agents and trypsin. Evidence is also available that the hatching enzyme activity is unaffected by a trypsin inhibitor (unpublished observation). From an overall point of view, the reported differences in the methods for improving the fertilizability of *R. pipiens* and *Bufo* coelomic eggs obviously reflect differences in the chemical nature of the vitelline membrane of the species used.

It is evident that the spermatozoa, even if 'capacitated' by the jelly molecules or the synthetic polymers, are unable to pass through the vitelline membrane surrounding the coelomic eggs, and thus fail to fuse with the egg plasma membrane. The proteolytic enzymes used in the present and Elinson's (1973*a*) studies have supposedly aided the spermatozoa in this respect. The present results therefore support the view proposed by Elinson (1973*a*), that the vitelline

membrane surrounding the coelomic egg is acting as a barrier to the penetration of sperm into the egg. There is evidence suggesting that the fertilizing sperm utilizes lytic enzymes for opening a path through the vitelline membrane (Raisman & Barbieri, 1969; Elinson, 1971*b*; Greenslade, McCormack, Hirsch & Davanzo, 1973). However, the exact bases for such spermatozoan activity in relation to the demonstrated anuran acrosomal ultrastructures (Burgos & Fawcett, 1956; Poirier & Spink, 1971; Katagiri, unpublished) are at present only the subject of speculation.

Another implication of the above considerations concerns the role of the oviduct in amphibian reproduction. Its role in supplying the jelly envelopes has been repeatedly stressed by several workers (cf. Shaver, 1966). The success of fertilization by the present and the above cited methods suggests a possible participation of the oviduct in modifying the vitelline membrane so that previously capacitated sperm are able to attach to and fuse with the egg plasma membrane. This scheme might involve an enzymic supply from the oviducal epithelium which causes the postulated modifications. Alternatively, a modification might occur as an ultrastructural reorganization of the interrelationship between the egg plasma membrane and the inner part of the vitelline membrane. Although important changes with respect to these structures during the oocyte maturation stages have been reported (cf. Wischnitzer, 1966), no attention has been paid to these structures during the passage of the egg through the oviduct. Whatever the mechanisms involved may be, extremely slight changes will suffice to induce the effects concerned, since an occasional increase in fertilizability can be induced by placing coelomic eggs in physiological saline for a long period at 5 °C (Katagiri, 1965). The possibility of other contributions of the oviducal epithelium in relation to fertilization or subsequent embryonic development cannot be denied, in view of the demonstrated secretion of a particular antigen other than jelly (Nace, Suyama & Smith, 1960), as well as the changes in metabolic behaviour of the eggs during their passage down the genital tract (Legname *et al.* 1972). Evidently, the most likely study for disclosing the answers to these questions will be that of the activities of the epithelial tissues composing the first several centimetres of the oviduct (cf. Katagiri, 1965; Legname *et al.* 1972).

The lower survival of the fertilized eggs seems to be due to the total lack of the vitelline membrane, rather than to the intrinsic inability of coelomic eggs to undergo morphogenesis. In support of this can be cited a previous experiment with *Hyla* (Katagiri, 1966*b*), in which demembranated *uterine* eggs showed a cleavage pattern and a gastrula arrest that are quite similar to the present observations. As extensively analysed by Tchou & Wang (1964), the developmental ability of the eggs should be regarded as a function of the time after the onset of maturation and the environmental temperature, rather than the location of the eggs in the female. The problem is therefore beyond the scope of the present study.

The observed high incidence of polyspermy is similar to that reported earlier by Tchou & Chen (1942), who observed several degrees of atypical fertilization in premature eggs removed from the ovisac. A strong indication from the present experiments is that the block against polyspermy in the toad egg primarily resides in the egg plasma membrane and is established along with oocyte maturation. It should also be noted that the mechanism concerned, once established, is not affected by the proteolytic enzymes used here. Besides the mechanism localized in the egg surface, suggestive evidence has been presented in the frog and toad that the vitelline membrane will also function to avert fertilization by supernumerary sperm, by changing its chemical properties after fertilization or artificial activation (Katagiri, 1963; Raisman & Barbieri, 1969).

The behaviour of spermatozoa after their entry into premature eggs can best be interpreted in the context of the cytoplasmic control over nuclear activity, as clearly evidenced by the artificial introduction of somatic cell nuclei (Dettlaff, Nikitina & Strova, 1964; Gurdon, 1968) and accessory sperm (Graham, 1966), as well as the transfer of specific cytoplasmic factors (Masui & Markert, 1971). An apparent difference in the situations of the 'donors', between the present and the above cited works, is that in the former the sperm to be incorporated in the egg first have to make contact with the egg cortical cytoplasm, instead of being introduced directly into the endoplasm, as in the latter. The present attempts to fertilize premature eggs did not deal with the earliest stages of oocyte maturation, in which spermatozoan responses quite different from those of the present observations have been suggested (cf. Tchou & Chen, 1942). Thus, a more extensive application of the present fertilization technique to several phases of maturing oocytes will provide a convenient system with which the cytoplasmic states of the maturing oocytes can be determined in terms of the spermatozoan responses, under conditions entirely free from the oviducal influences.

The author wishes to express his appreciation to Dr J. C. Dan for reading the manuscript.

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(*Received 13 August 1973, revised 16 October 1973*)