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SPERM ENTRY IN OYSTER (*Crassostrea gigas*) OOCYTES[1]

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Sperm entry in the polyspermic oocytes of the oyster, *Crassostrea gigas*, was observed with scanning and transmission electron microscopes. The oocytes were inseminated in sea water containing 0.0075 M procaine. The sperm underwent acrosome reaction on the surface of the chorion and bound to it by an electron-dense material derived from the acrosomal vesicle. The acrosomal process elongated from the subacrosomal region penetrated into the chorion. The apical part of the acrosomal process passed through the chorion. It penetrated into the oocyte cortex and then seemed to fuse with the oolemma. Oocyte cytoplasm surrounding the acrosomal process protruded and developed into a small fertilization cone. The sperm head passed through the chorion with the absorption of the fertilization cone. The sperm entered the oocyte cortex within 5 min after insemination.

The interphylum crossing between sea urchin eggs and pelecypod sperm suggests that the cross-fertility varies in sperm species (OSANAI and KYOZUKA, 1984). Oyster spermatozoa easily enter intact eggs of sea urchins (OSANAI and KYOZUKA, 1982). *Mytilus* spermatozoa somewhat differ in structure and behavior at fertilization from other pelecypod spermatozoa. Their acrosomal process is derived from a tubular subacrosomal structures, which consists of a bundle of microfilaments. (NIIJIMA and DAN, 1965a, b). On the other hand, the acrosomal process of oyster spermatozoa elongated from the subacrosomal region during acrosome reaction (OSANAI and KYOZUKA, 1982). A lysin secreted from the acrosome of *Mytilus* sperm is able to destroy the egg chorion (WADA *et al.*, 1956; TAMAKI and OSANAI, 1985), however, the chorion (the vitelline layer) is not dissolved in the vicinity of attached sperm of *Chama*, *Spisula* and *Modiolus* (HYLANDER and SUMMERS, 1977). These differences in sperm species lead us to examine the reaction of oyster spermatozoa on the egg surface for the analysis of the relation between sperm morphology and interphylum cross-fertility.

To increase the chance of observation in sperm entry, we attempt to induce polyspermy in oyster oocytes. Polyspermy in sea urchin eggs is induced by the pretreatment with procaine (VACQUIER, 1975). Thus, it was examined whether the procaine treatment is also effective for the induction of polyspermy in oyster oocytes.

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Materials and Methods

Material. The material used was the oyster, *Crassostrea gigas*, which was collected around the Asamushi Marine Biological Station. The gametes were obtained according by the previous method (Osanai and Kyozuka, 1982).

Procaine treatment. The oocytes were pretreated with sea water containing various concentrations of procaine hydrochloride (Sigma, 0.001-0.02 M) for 10 min and inseminated with 10^{-3} dilution of dry sperm. Ten min after insemination, they were washed in sea water and incubated in 20°C. At 60 min after insemination, monospermic and polyspermic oocytes were counted by bipolar and multipolar nuclear division, respectively.

Isolation of chorion. Oocytes (0.5 ml) were suspended in 10 ml of sea water and homogenized by a teflon homogenizer. The homoginate was centrifuged at 1,000 g for 5 min. The sediment (isolated chorions) was washed by removing the supernatant and adding sea water for 3 times.

Electron microscopy. The oocytes were fixed at various period up to 10 min after insemination in 1% glutaraldehyde in sea water containing 0.5 M saccharose for 30 min. The isolated chorions were fixed at 60 min after insemination in the same fixative. They were washed in sea water and postfixed in 1% osmium tetroxide in sea water for 45 min. After dehydrated in ethanol, they were transferred in liquid CO_2, dried at critical point, coated with gold and observed with a scanning electron microscope (Hitachi-Akashi MINISEM-4). The another series of samples were dehydrated in acetone and embedded in Sppurr's resin. Thin sections were stained with ulanyl acetate and lead citrate, then observed with a transmission electron microscope (JEM T-8).

Results

1. *Induction of polyspermy by inseminating in procaine sea water*

The oyster oocytes were inseminated in various concentrations of procaine sea water. When the oocytes were inseminated in 0.01 M procaine sea water, 93% of them were fertilized (Fig. 1-a). The fertilization events and the time course of early development did not differ from the control, which were inseminated in ordinary sea water. The rate of polyspermy was low in control. It became high with the increase of the procaine concentration. The fertilization rate decreased in 0.02 M procaine sea water. In 0.0075 M procaine sea water, polyspermy was induced in 85% of oocytes. In those oocytes, the plural number of male pronucleus developed after the germinal vesicle breakdown. The oocytes underwent the multipolar cleavage or the multinuclear division without cytokinesis (Fig. 1-b, c, d). The meiosis did not proceeded in procaine sea water without insemination.

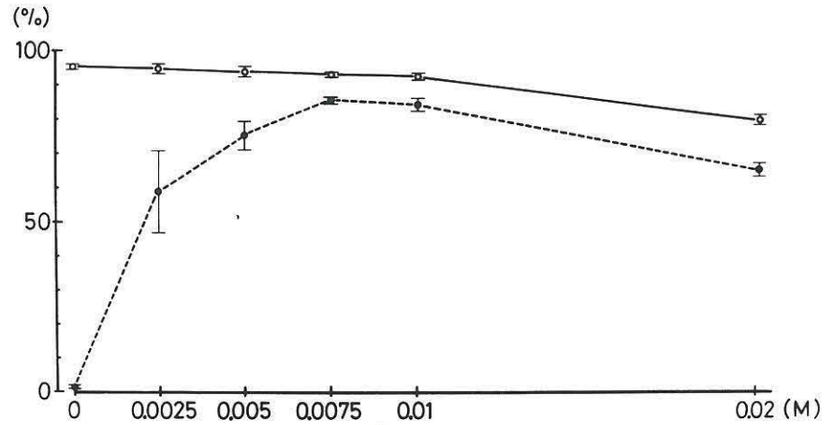


Fig. 1-a. Effect of procaine treatment on the induction of polyspermy in oyster oocytes. Abscissa: procaine concentration in sea water (M); Ordinate: % fertilized oocytes (open circles) or polyspermic oocytes (closed circles). Vertical bars represent standard error.

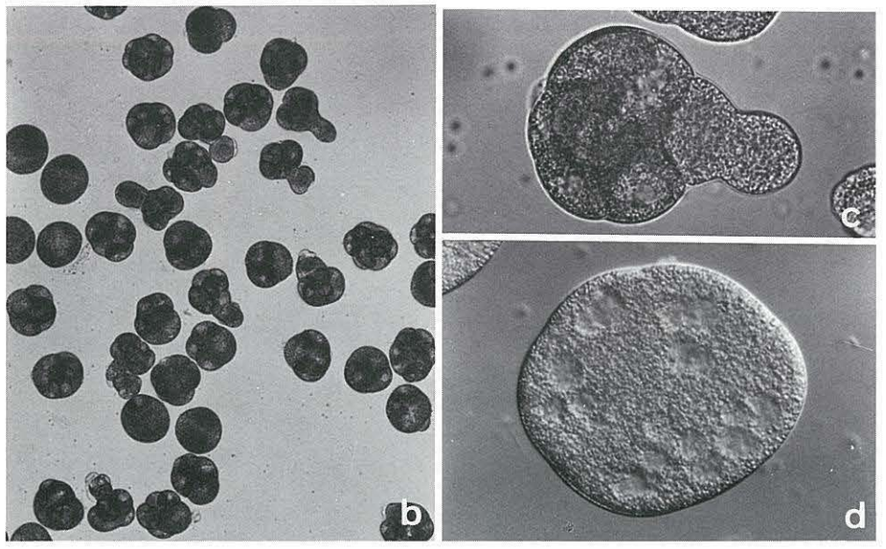


Fig. 1-b, c, d. Oyster oocytes inseminated in 0.0075 M procaine sea water. 80 min after insemination. They performed multipolar cleavage (c) or multinuclear division without cytokinesis (d). b)×110 c, d)×450.

2. *The scanning electron microscopy of sperm penetration*

The sperm penetration in 0.0075 M procaine sea water was observed with a scanning electron microscope. On the surface of chorion, the tips of the microvilli

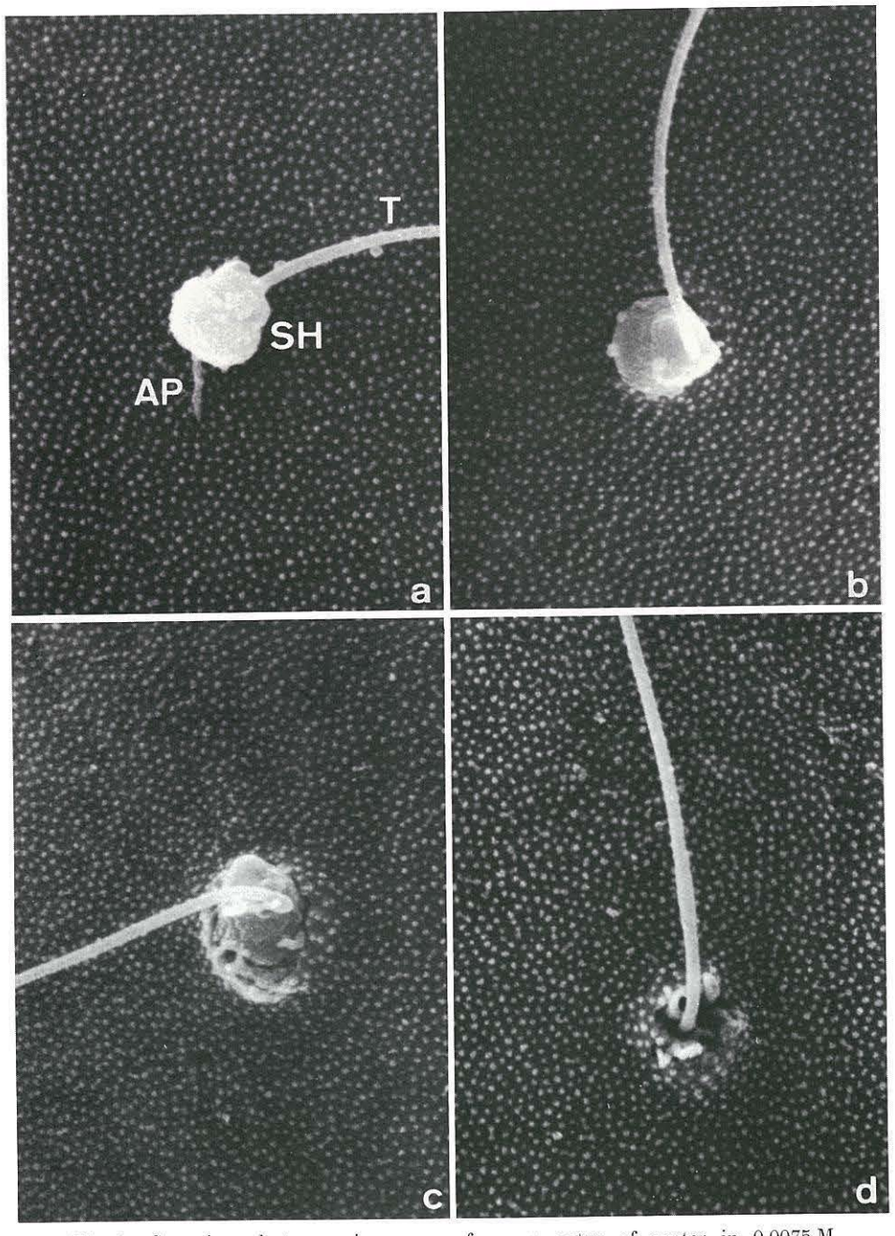


Fig. 2. Scanning electron microscopy of sperm entry of oyster in 0.0075 M procaine sea water (×8,800). a) 1 min after insemination. b) 3 min after insemination. c) 5 min after insemination. d) 7 min after insemination. T : sperm tail, AP : acrosomal process, SH : sperm head.

arranged regularly. The sperm attached their heads on the chorion within a minute after insemination (Fig. 2-a). They underwent acrosome reaction on the surface of chorion, however, the acrosomal process was rarely observed for the most part of them had penetrated into the chorion (Fig. 2-b). At 3 min after insemination, the sperm head began to swell and sink into the chorion. The chorion around the penetrating sperm was spread by swelling sperm head (Fig. 2-c). The arrangement of the microvilli did not change during the sperm penetration. Sperm head sank into the chorion around 5 min after insemination (Fig. 2-d).

3. The transmission electron microscopy of sperm penetration

In the intact sperm the acrosomal vesicle consisted of an electron-dense material occupied the apical part. The subacrosomal space surrounded by the acrosomal vesicle and the nucleus was spherical and contained partially polarized fibrous matter (Fig. 3-a, inset).

The sperm underwent acrosome reaction on the chorion. The inner acrosomal membrane bounded to the chorion by the electron-dense material derived from the opened acrosomal vesicle. The subacrosomal region developed into the acrosomal process with the elongation of the fibrous structure. The protruded acrosomal process passed through the chorion (1.2 μm in thickness). We could not find the figure showing the fusion of the acrosomal process and the microvillus, but obtained two figures in which the apical part of the acrosomal process was penetrated into the cortex (Fig. 3-a, b). The boundary between the acrosomal process and the egg cytoplasm was delimited by the plasma membrane. After the fusion of gamete membranes, the cytoplasm from the oocyte protruded around the acrosomal process and formed the penetration cone (Fig. 3-c). The protruded cytoplasm surrounded the sperm organella (Fig. 3-d). The sperm head passed through the chorion with the absorption of the penetration cone (Fig. 3-e). The formation of peri-vitelline space or the secretion from granules was not observed.

4. Insemination to the isolated chorion

The chorion was isolated almost in size of oocyte (Fig. 4-a). The sperm were added to the suspension of isolated chorions. They bound to the chorion (Fig. 4-b), however, did not dissolved the chorion. The transmission electron microscopy showed that the chorions contained the fragments of microvilli, the bottom of which were cut and became vesicular (Fig. 4-c). The sperm underwent acrosome reaction on the isolated chorions after insemination and protruded the acrosomal process. The acrosomal process penetrated through the chorion, but the heads of sperm remained on the surface of the chorion even 1 hr after insemination (Fig. 4-d). The chorion around the penetrated acrosomal process did not change.

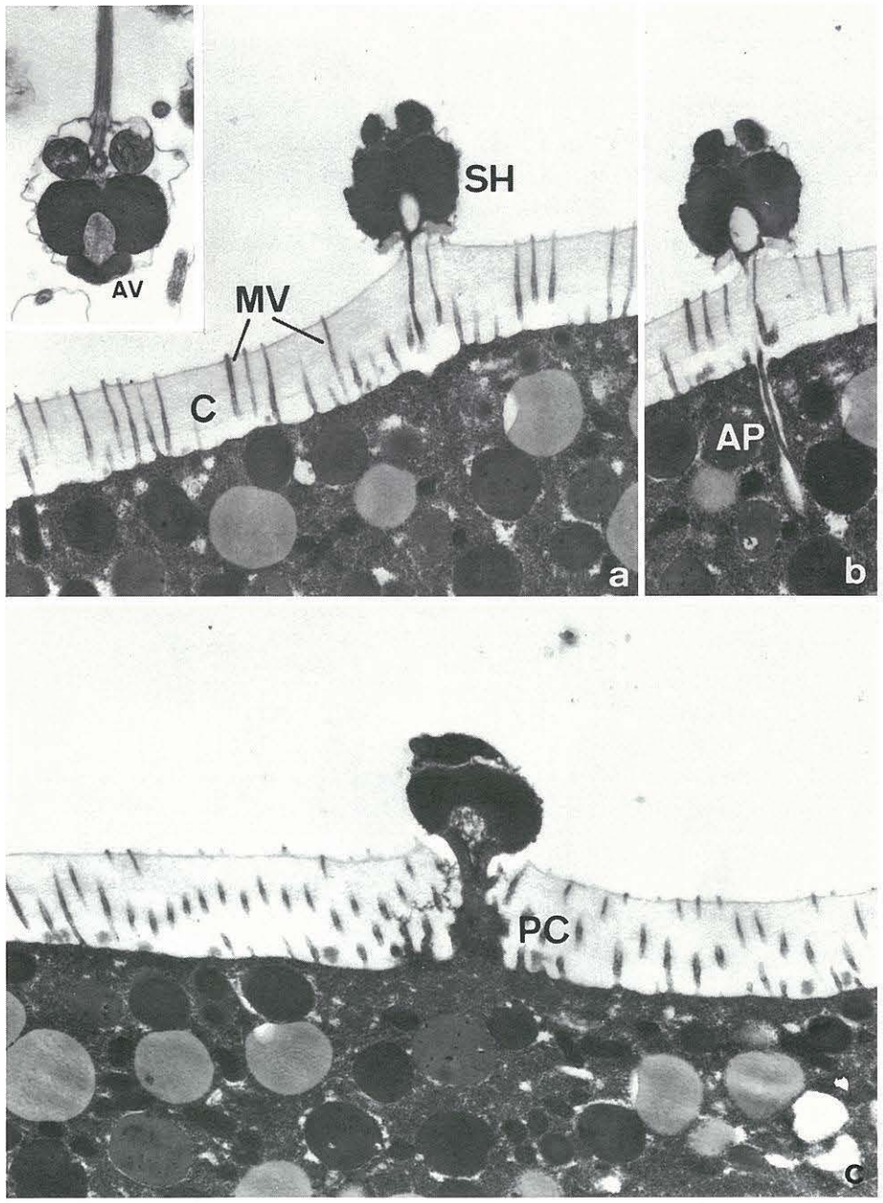
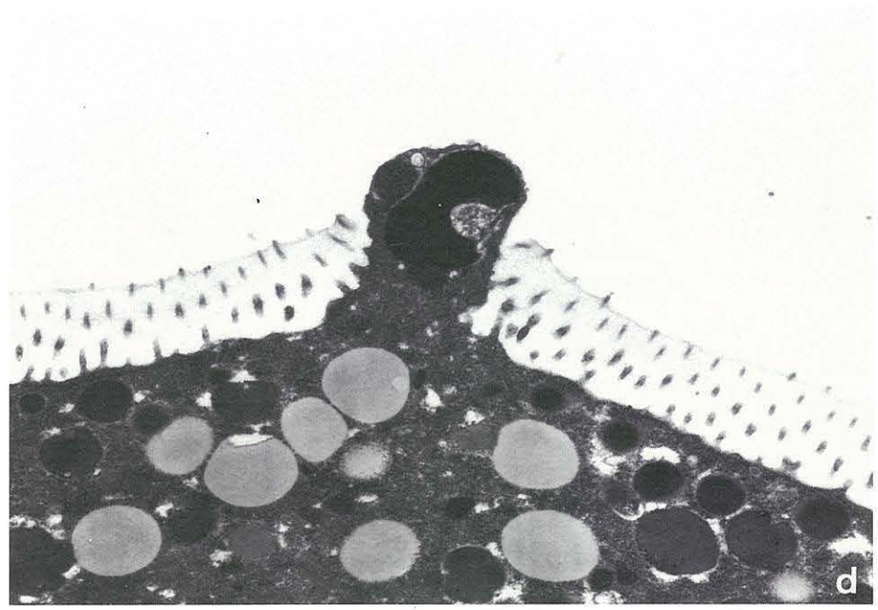
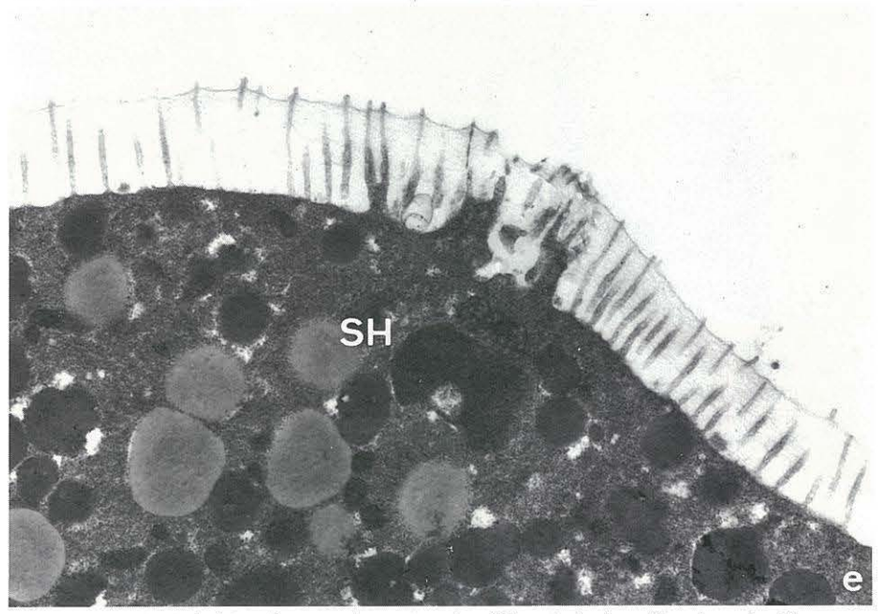


Fig. 3. Transmission electron microscopy of sperm entry of oyster in 0.0075 M procaine sea water (×11,500). (inset : intact spermatozoon with acrosomal vesicle. AV : acrosomal vesicle). a, b) 1 min after insemination. Same spermatozoon of different section. Apical part of acrosomal process (AP) penetrated into the oocyte cortex in (b). c) 3 min after insemination. Cytoplasmic protrusion began to form the penetration cone (PC). C : chorion MV : microvilli. d) 5 min afterr insemination. e) 7 min after insemination. SH : sperm head.





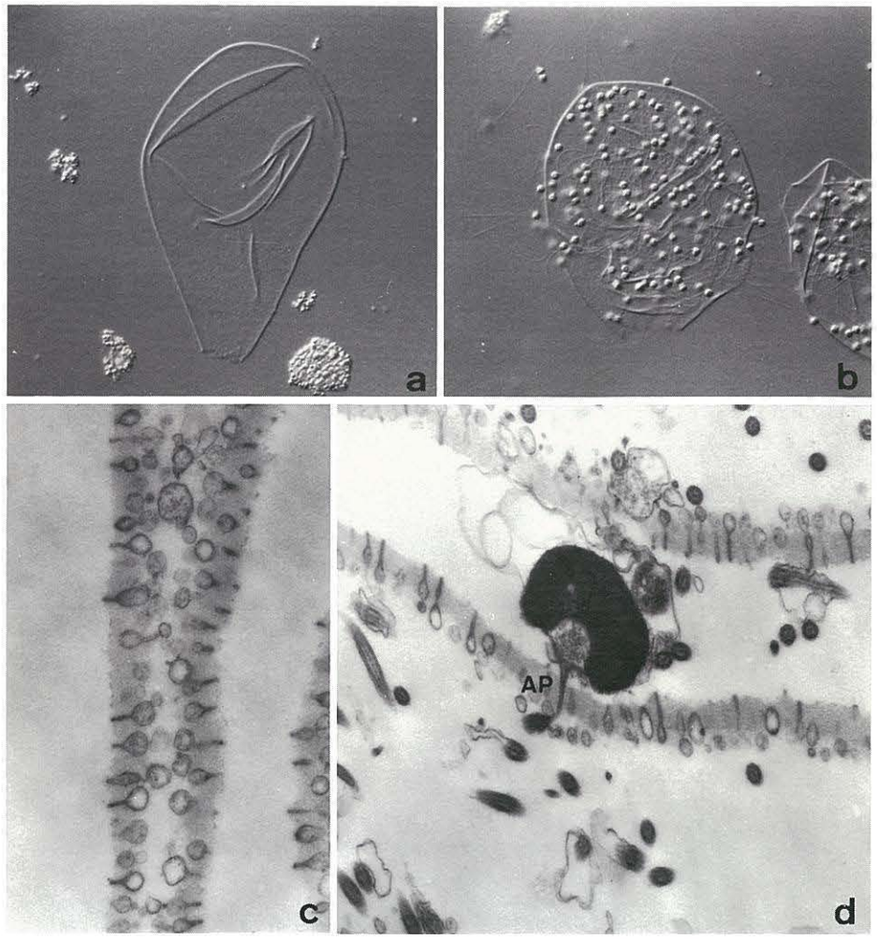


Fig. 4. Insemination of oyster sperm to the isolated chorion. a) Isolated chorion. ×450. b) 60 min after insemination to the isolated chorion. ×450 c) Transmission electron microscopy of isolated chorion. The tip of microvilli remained in the chorion. ×11,500. d) 60 min after insemination to the isolated chorion. ×11, 500, Sperm acrosomal process (AP) penetrated through the chorion.

Discussion

The present examination reveals that the oyster sperm are not able to pass unaided their head through the chorion. The sperm head bounded to the surface of the chorion by an electron-dense material derived from the acrosomal vesicle. It must be the bindin of oyster which Brandriff *et al.* (1978) isolated from the oyster sperm. Sperm head on the chorion remains unless the fusion of acrosomal process

and egg plasma membrane did not take place. Heavy insemination does not result in the dissolution of the isolated chorion, and no morphological sign suggesting the destruction of the chorion was observed in the vicinity of the inserted acrosomal process. These results suggest that the penetration of the oyster sperm head depend on the movement of egg cytoplasm relating to the development and absorption of the fertilization cone. The binding of the sperm head to the chorion seems to be required for the orienting,the protruding acrosomal process toward the egg cortex.

It has not firmly established as to the actual site of gamete fusion in mollusks whether it occurs at microvillar or intervitelline regions of the egg surface (Longo, 1983). Hylander and Summers (1977) showed the evidence that gamete membrane fusion occurs between the acrosomal tube and a microvillus in *Chama macerophylla*. In *Crassostrea*, we could not find the figure suggesting the fusion between the acrosomal process and microvillus. The apical part of the acrosomal process passed through the chorion penetrated into the egg cortex and then the membrane delimiting the acrosomal process seems to fuse with the oolemma. In crossing of oyster sperm and sea urchin eggs, the multilayered, intricate membranous structure is observed on the boundary between the inserted acrosomal process and the egg cytoplasm. The vesiculation of the membranous layer seems to follow the fusion between the tip of the acrosomal process and egg cytoplasm (Osanai and Kyozuka, 1982). The oyster sperm seems to fuse at the intervillar region of the oocyte cortex by inserting the acrosomal process into it.

References

Brandriff, B., G.W. Moy, and V.D. Vacquier, 1978 Isolation of sperm bindin from oyster (*Crassostrea gigas*). Gamete Res., **1**: 89-99.

Hylander, B.L. and R.G. Summers, 1977 An ultrastructural analysis of the gametes and early fertilization in two bivalve molluscs, *Chama macerophylla* and *Spisula solidissima* with special reference to gamete binding. Cell Tiss. Res., **182**: 469-489.

Niijima, L. and J.C. Dan, 1965a The acrosome reaction in *Mytilus edulis*. I. Fine structure of the intact acrosome. J. Cell Biol., **25**: 243-248.

Niijima, L. and J.C. Dan, 1965b The acrosome reaction in *Mytilus edulis*. II. Stages in the reacton, observed in supernumerary and calcium-treated spermatozoa. J. Cell Biol., **25**: 249-259.

Longo, F.J., 1983 Meiotic maturation and fertilization. In, *The Mollusca*, edited by K.M. Wilbur, Academic Press, New York, 49-89.

Osanai, K. and K. Kyozuka, 1982 Cross-fertilization between sea urchin eggs an oyster spermatozoa. Gamete Res., **5**: 49-60.

Osanai, K. and K. Kyozuka, 1984 Cross-fertilization between sea urchin eggs and mussel spermatozoa: Sperm entry without egg activation. Zool. Sci., **1**: 245-254.

Tamaki, H. and K. Osanai, 1985 Re-initiation of meiosis in *Mytilus* oocytes with acrosome reaction product of sperm. Bull. Mar. Biol. Stn. Asamushi, Tōhoku Univ., **18**: 11-23.

Vacquier, V.D., 1975 The isolation of intact cortical granules from sea urchin eggs: Calcium ions trigger granule discharge. Develop. Biol., **43**: 62-74.

Wada, S.K., J.R. Collier, and J.C. Dan, 1956 Studies on the acrosome. V. An egg-

membrane lysin from the acrosomes of *Mytilus edulis* spermatozoa. Exptl. Cell Res., **10**: 168-180.