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Scanning Electron Microscopical Observation on the Penetration Mechanism of Fowl Spermatozoa into the Ovum in the Process of Fertilization

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(Figs. 1–4)

In the process of fertilization, spermatozoa must cross the vitelline membrane surrounding the ovum, whether mamalian or avian. The penetration mechanism of spermatozoa in mammals has been observed morphologically by phase-contrast or electron microscopy. In the mammalian fertilization, it is generally believed that spermatozoa dissolve the corona radiata enclosing the ovum by the aid of a trypsin-like enzyme contained in the acrosome, so that they might enter the ovum with ease. Unlike the mammalian ovum, the fowl one is extremely large in size (about 3 cm in diameter) due to the accumulation of yolk granules in the cytoplasm. Moreover, it is enclosed in a mere vitelline membrane without possessing the covering of a mammalian ovum. This suggests that the entrance mechanism of fowl spermatozoa in the process of fertilization may differ from that of mammalian spermatozoa.

Recently, HOWARTH and PALMER[1], HOWARTH and DIGBY[2], PALMER and HOWARTH[3], and LANGHOLD and HOWARTH[4] reported that cock spermatozoa also contained a trypsin-like enzyme like mammalian ones. The existence of the enzyme in spermatozoa makes it possible to presume that hydrolysis of the vitelline membrane may be induced by the time of spermatozoal penetration. The true penetration mechanism of spermatozoa in fowl fertilization, however, remains obscure morphologically.

The present study, therefore, was attempted to observe morphologically the entrance mechanism of cock spermatozoa into the ovum through the vitelline membrane by means of the scanning electron microscope. Observation was made on ova fertilized experimentally *in vitro*.

MATERIALS AND METHODS

All ova and spermatozoa used in this experimental fertilization were collected from White Leghorn hens. The ova were divided into two groups. Those of the first group had been taken by laparotomy from the infundibulum of the oviduct of unmated laying hens just after ovulation (referred to as infundibular ova). The time of ovulation was decided by a clutch of laying. Those of the other group had been collected from laid eggs after oviposition (referred to as oviposited ova). The infundibular ova were taken directly into the experiment. The oviposited ova were used after removing the covering around them. For this purpose, the egg shell was first cracked in two along the equatorial line. Then albumen was poured out by transferring the whole content of each ovum repeatedly from one half of the cracked shell to the other. Next, the denuded ovum was washed gently several times in a modified Ringer solution of OLSEN and NEHER[5]. The use of the oviposited ovum was to examine whether spermatozoa could cross the completely formed vitelline membrane or not.

Semen samples were collected from healthy cocks by abdominal massage. They were pooled and diluted about 40 times with the modified Ringer solution before use. Fertilization *in vitro* was carried out by placing a collected ovum in about 20 m*l* of the diluted semen contained in a small beaker 50 m*l* in capacity and warmed at 40°C beforehand. The beaker was held in an incubator at 40°C for about 10 minutes. Then the diluted semen was discarded from the beaker and replaced by a 2.5 % gultaraldehyde solution buffered with phosphate (pH 7.4). The beaker was allowed to stand for a few minutes. This procedure was to kill spermatozoa and to fix the vitelline membrane rapidly. Then the vitelline membrane was minced into small pieces and washed gently with Ringer solution to remove yolk plasm adhering to the inside of the membrane. The membrane pieces were refixed in the same fixative as mentioned above for 10 hours. They were dehydrated through alcohol and isoamyl acetate. Then they were dried in the conventional manner by the critical point drying method with CO_2. After that, they were coated with gold and examined by a scanning electron microscope (JSM type U) at an accelerating voltage of 10 KV.

RESULTS AND DISCUSSION

The present scanning electron microscopy of experimentally fertilized ova revealed clearly the behavior of spermatozoa in the process of fertilization.

Observation on the infundibular ovum

Fig. 1 shows the outer surface of the vitelline membrane of the infundibular ovum. In it, a number of tangled thread-like structures are present on the vitelline membrane. Obviously, they are spermatozoa which are about to enter the ovum through the vitelline membrane. Such distribution of spermatozoa was found all over the surface of

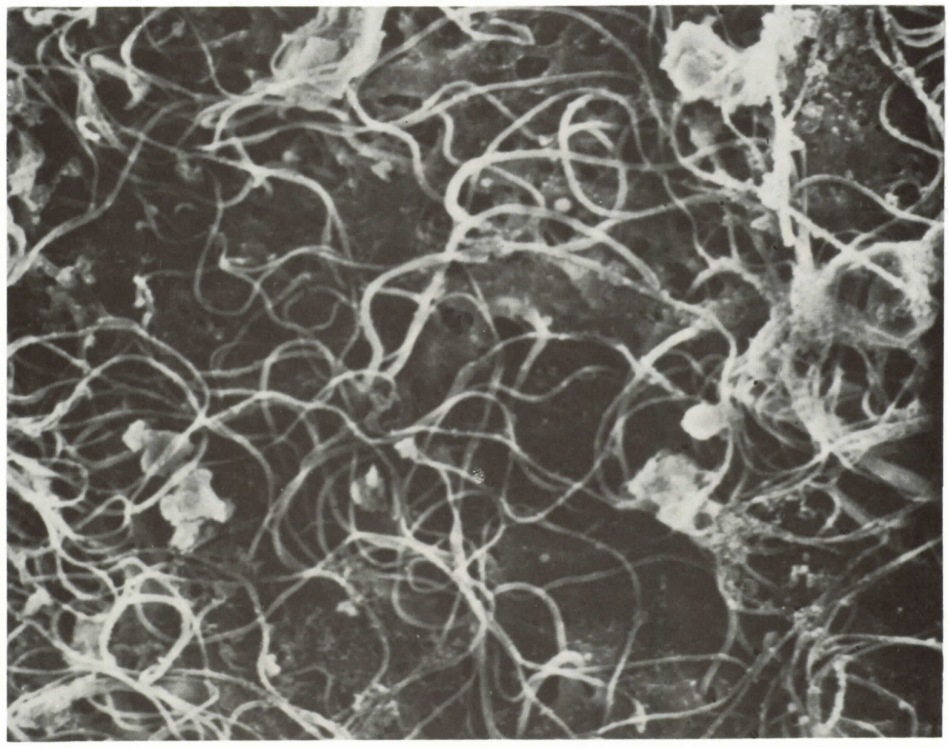

Fig. 1. The outer surface of the vitelline membrane of an infundibular ovum fertilized experimentally. A number of spermatozoa are about to enter the ovum through the inner layer of the vitelline membrane. × 2,400.

the vitelline membrane around the ovum. Although the spermatozoa show curious outlines, they had their head directed to the vitelline membrane.

When observed at a high-power magnification, almost all the spermatozoa uniformly had their head inserted into the interstice of the lattice-like structure of the vitelline membrane (Fig. 2). Their crooked tails remained outside. Occasionally, a few spermatozoa lost themselves and could not enter the spaces of the vitelline membrane. They were generally abnormal in structure.

As is well known, a cock spermatozoon is very long and slender in appearance. According to LAKE[6], it is about 100 μ in total length, with the head 25 μ, the middle piece 4 μ, and the tail 80 μ in length. Its width is 0.5 μ in the broadest part of the head. The general structure of cock spermatozoa is such that the spermatozoa lying on the vitelline membrane seem to penetrate into the vitelline membrane up to the level of the middle piece or the more caudal region of the body. Unfortunately, the present study failed to determine whether spermatozoa had certainly crossed the layer of the vitelline membrane or how many spermatozoa had reached the cytoplasm of the ovum.

On the other hand, the vitelline membrane presented here is constructed roughly

with three-dimensionally arranged coarse fibers, as shown in Fig. 2. Accordingly, it looks like a sieve with holes of variable sizes. It is definitely the inner layer of the vitelline membrane, or the perivitelline membrane, because it is identical with the one

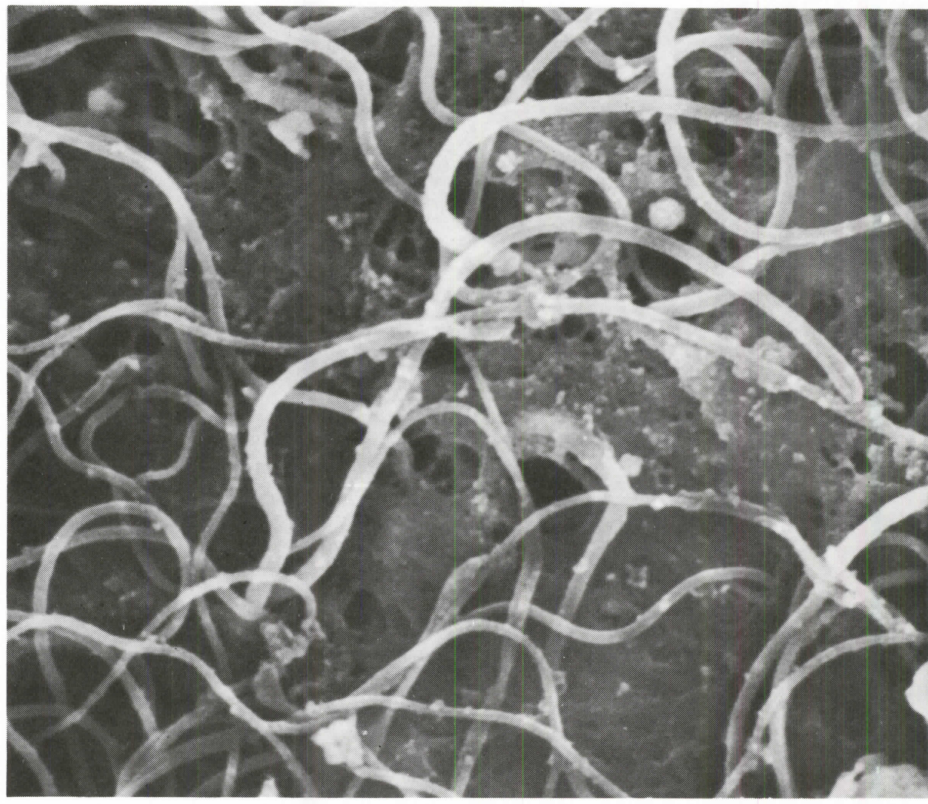

Fig. 2. Magnification of Fig. 1. Most spermatozoa have invaded spaces of the network of the vitelline membrane. Few spermatozoa have entered this membrane. × 4,800.

observed by the present author[9].

The fine structure of the vitelline membrane of the hen's ovum has already been studied in detail by electron microscopy by BELLAIRS *et al.*[7] and BAIN and HALL[8], and by scanning electron microscopy by FUJII *et al.*[9] According to BELLAIRS *et al.*[7], the compleied vitelline membrane is about 10 μ in total thickness and composed of two distinct layers, an inner and an outer layer. Both layers are connected with a thin, granular membrane called the "continuous membrane" by BELLAIRS *et al.*[7] The inner layer is formed by a three-dimensional network of coarse fibers 0.2 to 0.6 μ in diameter. The same authors stated that the ovulated ovum was surrounded only by the inner layer of the vitelline membrane until it reached the upper end of the magnum of the oviduct.

From the results mentioned above, it is concluded that spermatozoa may readily enter the ovum by way of spaces in the meshwork of the inner vitelline membrane. Accordingly, fertilization takes place in hens by such a penetration mechanism of spermatozoa into the ovum as this. In this mechanism, spermatozoa do not penetrate straight into the vitelline membrane, but work their way strenuously by looking for spaces of sufficient size through which they pass to enter the vitelline membrane, because this membrane is made of a three-dimensional meshwork with spaces of variable sizes.

Observation on the oviposited ovum

Fig. 3 shows the outer surface of the vitelline membrane of the oviposited ovum.

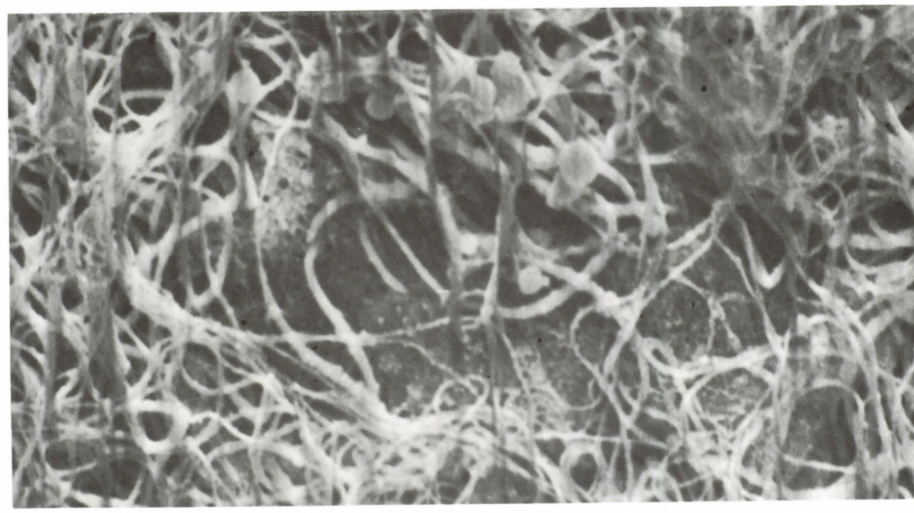

Fig. 3. The outer surface of the vitelline membrane of an experimentally fertilized ovum of a laid egg. A number of spermatozoa are distributed on the vitelline membrane. X 2,400.

In it, a number of spermatozoa are found on the vitelline membrane. At a glance, the distribution of spermatozoa around the oviposited ovum resembles that around the infundibular ovum. There is, however, a remarkable difference in the behavior of spermatozoa. When observed at a high-power magnification, each spermatozoon exhibited its whole body consisting of the head, middle piece, and tail on the vitelline membrane (Fig. 4). A noticeable finding was that few spermatozoa had penetrated into the vitelline membrane, as is shown in Fig. 4. Most spermatozoa only touched this membrane, or inserted part of the apex of the acrosome into this membrane. Some spermatozoa were free without touching the membrane.

This phenomenon indicates that spermatozoa were prevented from entering the ovum by the vitelline membrane. It seems to be attributed to the structure of this membrane. In Fig. 4, the vitelline membrane is presented as a sticky mucous membrane without a meshwork structure of fibers. Such spaces as observed in the vitelline mem-

brane of the infundibular ovum are not present in the oviposited ovum. The structure of the vitelline membrane mentioned above is characteristic of the completed oviposited ovum.

According to BELLAIRS *et al.*[7], the outer layer of the vitelline membrane is 3.0 to 8.5 μ in thickness and consists of a dense network of fibrils about 150 Å in diameter. It is added to the inner layer of the vitelline membrane formed already while the ovum descends in the upper magnum of the oviduct. Therefore, the vitelline membrane presented here is considered to be the completed one, because the ovum has been collected from a laid egg.

From the results mentioned above, it is concluded that spermatozoa may be unable to enter the ovum after the vitelline membrane has once been completed. In other words,

Fig. 4. Magnification of Fig. 3. Spermatozoa have only touched the outer layer of the vitelline membrane or inserted part of the apex of the head into this membrane. No spermatozoa entered the vitelline membrane. × 4,800.

spermatozoa are allowed to enter the ovum only before completion of the vitelline membrane. Accordingly, spermatozoal penetration into the ovum takes place within a limited period of time while the ovum descends in the infundibulum of the oviduct. This conclusion has an important meaning to the site of fertilization of the fowl's ovum.

As is well known, the site of fertilization in fowls has been a subject of controversy for a long time. Since the report of OLSEN and NEHER[5], it is generally accepted that fertilization in fowls takes place at the infundibulum of the oviduct. No direct evidence for this problem, however, has been given morphologically as yet. In the present investigation, spermatozoal penetration was permitted only into the infundibular ovum, which was enclosed only in the inner layer of the vitelline membrane. This result may provide a strong clue for the clarification of the site of fertilization.

As already stated, HOWARTH and his associates[1~4] observed that fowl spermatozoa contained a trypsin-like enzyme in their acrosome. Although this enzyme may dissolve the vitelline membrane as spermatozoa penetrate into this membrane, no visible injury has been observed in the membrane examined. If the enzyme plays any role in the process of fertilization, it may act to liquefy the substance having filled spaces of the meshwork of the vitelline membrane.

Judging from the structure of the vitelline membrane, BELLAIRS *et al.*[7] assumed that the spermatozoal penetration into the ovum might occur physically, since the head of the spermatozoon is about 0.5 μ in width and gaps of the meshwork of the inner layer of the vitelline membrane are about 2.0 μ in diameter. These gaps are large enough to allow spermatozoa to pass. The results of the present observation actually lend support to the assumption of BELLAIRS *et al.*[7]

SUMMARY

In the process of fertilization in hens, the entering mechanism of spermatozoa into the ovum through the vitelline membrane was observed morphologically by scanning electron microscopy. Two types of ova were fertilized experimentally *in vitro*. One of them was taken directly from the infundibulum of the oviduct, where the ovum was enclosed only by an inner layer of the vitelline membrane. The other was obtained from laid eggs, in which the ovum was enclosed by a completed vitelline membrane. When the ovum of the first type was used, spermatozoa readily entered the ovum through gaps in the meshwork of the inner layer of the vitelline membrane. When the ovum of the second type was used, no spermatozoa could penetrate into the vitelline membrane at all. This difference was considered to be attributed to the differentiation or development of the vitelline membrane. The results of the present study may lend a morphological support to the conception that fertilization takes place in fowls at the infundibulum of the oviduct.

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鳥類精子の卵子進入機構の走査電子顕微鏡的観察

藤井俊策

鳥類の受精に際して，精子が卵黄膜を通って卵子へ進入する機構を，走査電子顕微鏡を用いて形態的に観察した。方法は，2型の鶏卵子を in vitro において受精させ，この受精卵について観察した。一型の卵子は，排卵後直ちに卵管漏斗部から取出されたものである。卵子はいわゆる内卵黄膜のみによって包まれた状態にある。他の一型は，放卵後の卵から得られた卵子である。卵子は内卵黄膜に加えて外卵黄膜をも具えた完成した卵黄膜に包まれている。

結果は，漏斗部卵子では，精子は網状構造の内卵黄膜の網眼内に深く進入していた。一方，放卵後卵子では，精子は卵黄膜に進入することなく，外卵黄膜表面に軽く接触するに過ぎなかった。このことは一度外卵黄膜が形成されると，精子の卵子進入は阻止されることを示唆する。

以上の所見から，鳥類の受精に際しては，精子は内卵黄膜の網眼を通って卵子内へ進入するものであり，しかも卵子が卵管漏斗部通過時のみ精子の進入が可能であるものと考えられた。このことは同時に，鳥類の受精は卵管漏斗部で行われるという従来からの説を，形態的観点から支持したことになる。