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Observation by Scanning Electron Microscopy on the Time Sequence of Sperm Penetration into the Vitellus of Hamster Eggs

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Summary

In the golden hamster, the time sequence and mode of sperm head incorporation in to the vitellus *in vivo* were studied by the phase contrast microscope and the scanning electron microscope.

From the observation by the phase contrast microscope, the process of sperm penetration into the vitellus was classified into five phases. Also, the results from the observation by the scanning electron microscope demonstrated the two modes of sperm head incorporation in the golden hamster ova: one is the entry from the anterior end protion of the head including the perforatorium and the other, like that observed in rat, is the enter first from the posterior portion of the head and then from the perforatorium. In the golden hamster, the former way was more frequent than the later.

It was suggested that the spermatozoon including the entire tail in the golden hamster must be completely incorporated into the vitellus with one hour from the onset of the head attachment on the oocyte surface.

The changes in the fine structure during the sperm penetration reported in the rat and mouse ova were not observed in the golden hamster.

Fertilization in mammalian ova has been studied extensively by electron microscope e.g. transmission (TEM) (1～5) and scanning (SEM) (6～13) in experimental animals.

There are few reports on the time sequence of sperm incorporation into egg cytoplasm *in vivo*, using SEM. Yanagimachi & Noda (1972) have studied by SEM the penetration of sperm into the cytoplasm in the denuded hamster ova *in vitro*. They have suggested a possible mode of sperm penetration into vitellus during fertilization.

In a previous paper, we observed that the entire length of rat sperm tail is incorporated in the mode which the flagellum formed an incorporation wave on the oocyte surface (Sugawara *et al.* 1974, 1975).

Recently, we disclosed a sequence in the sperm head incorporation into vitellus in the rat *in vivo* (14).

Although previous reports have elucidated a number of important features of sperm penetration (or incorporation) into mammalian ova, information on the relationship between oocyte surface, and the behavior and roles of the structural elements of spermatozoon during fertilization is still very limited.

The experiment had attempted to elucidate the time sequence and mode of sperm head incorporation into the vitellus in golden hamster *in vivo*, using techniques of SEM.

Materials and Methods

Animals: Nullparous and primiparous female golden hamsters were maintained on a 12 hour light cycle. The vaginal discharge was daily recorded for two weeks and the animals with a regular cycle of 4 days were selected for the experiment.

Proestrus females were caged with fertile males from 17:10 hours and the mating was confirmed by the presence of spermatozoa in the vaginal smear. The mating time was recorded for each individual and the animals which had no mated by 19:15 hours were omitted from the experiments.

Ova collection: On the morning after the mating, the hamsters were killed at intervals of 15 minutes in the clock time from 4:00 to 7:15 hours and at 60 minutes intervals from 8 to 13:30 hours. The ova which were surrounded with cumuli were liberated by tearing the ampulla with a pair of needles.

The recovered ova were immediately fixed for 4 to 6 hours in 2.5% glutaraldehyde buffered with 0.2 M phosphate (pH 7.2) at 4°C.

Cumuli of the fixed ova were mechanically removed under a dissecting microscope and the denuded ova were re-fixed for 1 hour at 4°C in the glutaraldehyde solution mentioned above. The ova were transfered to a 0.2 M phosphate buffer (pH 7.2) and examined immediately by light microscope.

Microscopic examination:

a) **Light microscope**:

The presence of spermatozoon in the denuded ova was examined by phase contrast microscope and a number of ova with sperm were recorded. The ova with sperm were classified into I~V process groups, according to the mode of sperm tail attachment to the ova.

b) **Scanning electron microscope**:

The ova examined by light microscope were stored in 0.2 M phosphate buffer at 4°C until critical-point drying was performed. In this study, a special container devised by us was used for dehydration and critical point drying of ova (Fig. 1). The ova were washed twice by distilled water and dehydrated through a graded series of ethanol to isoamyl acetate.

The specimens were critical-point dried with CO_2 in a Hitachi HC P–I critical-

point drying apparatus, and coated with gold in a Hummer sputtering coater, and examined with a Hitach S-700 scanning electrone microscope operated at 20 KV.

We have attempted to analyse the time lag consumed for the entry of spermatozoon i.e. from the time of contact with the oocyte surface to the time of incorporation of the entire length of the flugellum into the vitellus. We have applied the procedure for the estimation of the time lag as follows:

The ova with spermatozoon were classified into the following three categories A) Sperm head had just attached to the oocyte surface or a part of spermatozoon head was still seen on the surface, B) The head had entered into the vitellus. C) The sperm tail was in the process of entry which formed in incorporation wave on the surface of the vitellus. The percentage of ova in each category was recorded according to their recovery time.

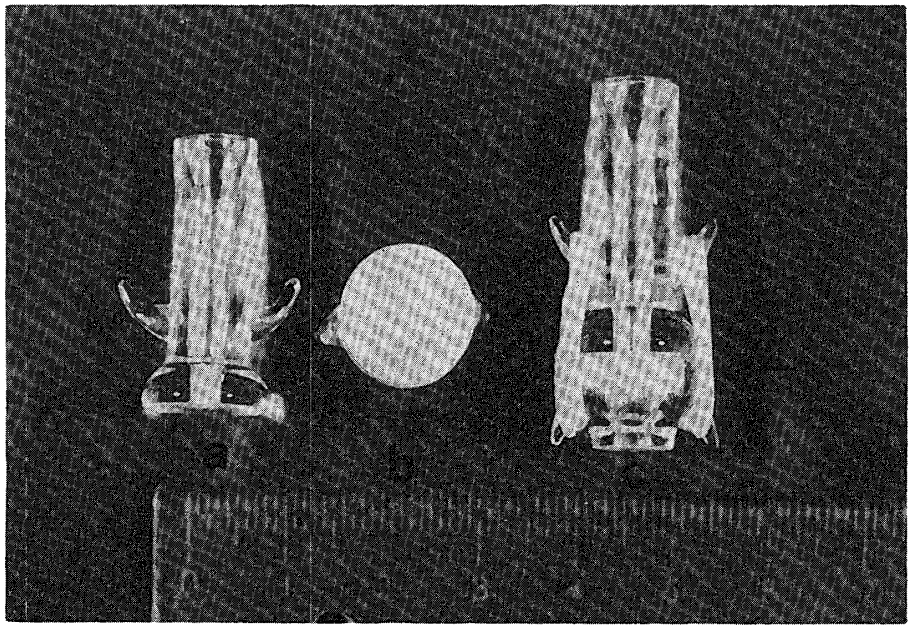

FIG. 1. The container for critical-point drying for the mammalian ova. The apparatus made with a glass was composed of two parts (Fig. 1, a and b). Glassfilter was fixed in center of part b and it's surface was rubbed.
For drying, filter paper cut to 3 or 4 mm in diameter was putted between parts a and b, and part a and b were connected to each rubbed side with a gumband.

Results

Sexual behaviour of hamsters: The animals used for the present experiments came into oestrus from 17:35 hours and 85% of the animals had been mated for 1 hour between 17:30 and 18:30 hours. 95% of all animals mated by 19:15 hours and 3 animals showing ovulating discharge on the next morning had no sperm in the smear. All of the animals had fertile mating for 10~15 minutes after the start of the mating.

Proportion of ova penetrated by sperm: A total of 369 ova were

examined by light microscope, and the proportion of the ova penetrated by sperm was 73.6%. Only one of the ova with sperm had one supplementary sperm and 99.4% of the ova had been penetrated by the spermatozoon. Changes in the proportion of the sperm incorporated ova as a function of the time interval on the morning after the fertile mating are summarized in Table 1. In the hamsters killed from 4:00 to 4:30 hours, sperm penetration was observed in 8% of the ova recovered and thereafter the number of the ova with a sperm increased with time until 6:15 hours.

Sperm penetration in hamster ova had occurred between 5:00 and 6:00 hours on the morning of ovulating discharge. The results obtained from the observation in processes I~V are shown in Table 1.

TABLE 1. Observation by light microscope on sperm attaching and entry on the surface or cytoplasma of the hamster eggs in the progressed time on the morning after fertile mating.

Recovery time of ova A.M.	No. ova penetrated by sperm		Five categories for the sperm penetration No. ova penetrated by spermatozoon/total No. ova with sperm				
	Total No. ova examined	%	I	II	III	IV	V
							
4:00	2/25	8		2/2			
4:30	0/12	0					
4:45	3/10	30		2/3	1/3		
5:00	4/24	17		4/4			
5:15	17/36	48	4/17	3/17	5/17	4/17	1/17
5:30	47/64	74	3/47	23/47	18/47		3/17
5:45	44/48	92	3/44	25/44	16/44	0	0
6:00	27/28	97	1/27	4/27		18/27	3/27
6:15	18/18	100		8/18	7/18		3/18
6:30	15/15	100		4/15	1/15	8/15	1/15
7:00	12/12	100		12/12			
7:15	23/23	100		8/23		5/12	
8:00	15/15	100		15/15			
9:00	13/13	100				10/13	3/13
10:00	12/12	100					12/12
11:30	13/14	93					12/13

The ova classified to process 1 were observed from 5: 15 to 6:00 hours and the ova recovered at 5:15 hours had spermatozoon in the highest proportion (24.0%).

The proportion of ova in process 11 from 4:00 to 8:00 hours was highest for the 15 minutes between 5:30 and 5:45 hours. Process III was observed in the ova examined form 5:15 to 6:30 hours. The ova recovered from 6:00 to 9:00 hours belonged to process IV. The ova in process V were observed from 5:15 to 11:30 hours.

Observation by SEM on the sperm penetration:

a) **Occurance of penetration of spermatozoon**: The time of the entry of

the spermatozoon and its time sequence were examined by SEM observation. The results classified to the three processes are summarized in Fig. 2. The proportion of the ova in each process to the ova penetrated by spermatozoon was plotted as a function of the recovery time of ova.

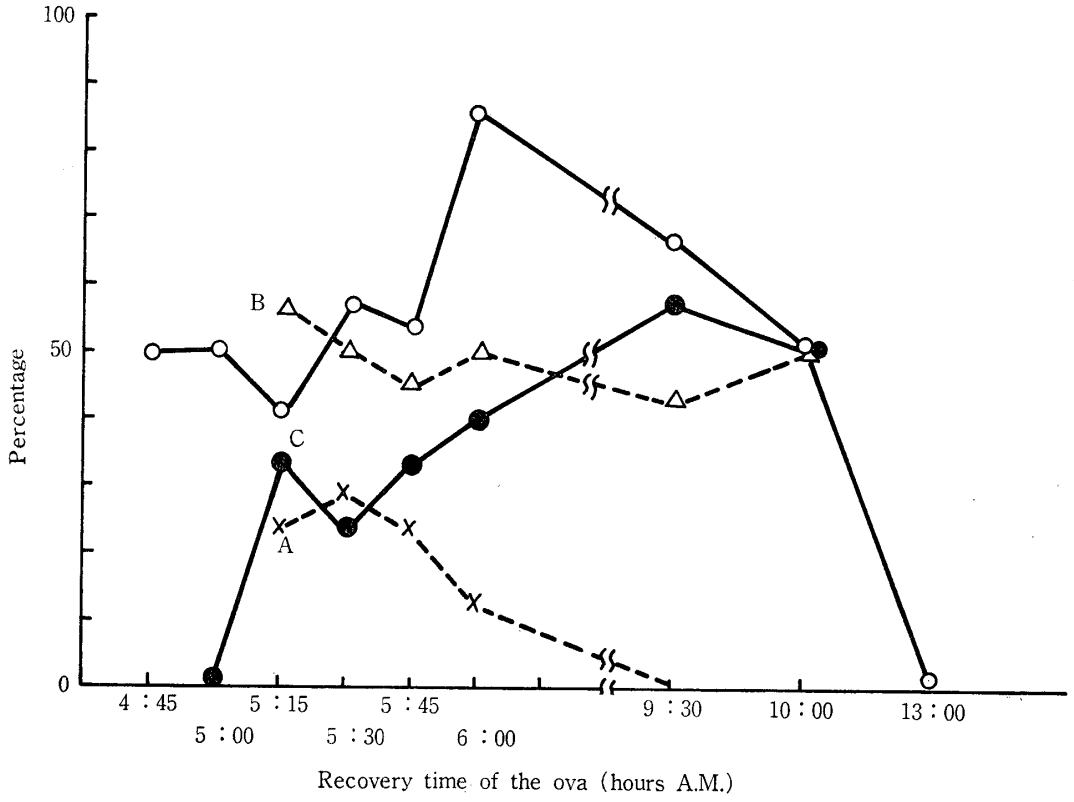

FIG. 2. The proportion of the ova penetrated by spermatozoon in three categories based on the observation by scanning electron microscope.
o—o : Total ova penetrated by spermatozoon.
(A) ×···×: Ova just attached by the head of spermatozoon.
(B) △···△: Ova had the head penetrated completely and the neck of the spermatozoon
(C) ●—● : Ova had penetrating wave of the spermatozoon tail.

23～18% of the ova which had been penetrated by spermatozoon from 5:15 to 5:45 hours belonged to process A and the ova clasified in process B and C at the above mentioned time were 45, 56 and 23, 34%, respectively.

At 6:00 hours, only 11% of the ova with spermatozoon were included in process A, while process B and C were 50 and 40%, respectively.

Although the ova classified in process A were not observed at 9:30 and 10:00 hours, the ova in process B and C were 43～50 and 50～58%, respectively.

On the other hand, the ova belonging to process C were not observed in those recovered before 5:15 hours and also the ova examinated at 11:30 hours were not classified in any process, because the spermatozoon had incorporated completely into the vitellus.

From the proportion in the distribution of the ova to the three processes as described above, we observe that the attachment and the penetration of the spermatozoon head begun between 5:15 and 5:45 hours on the morning after mating which was verified at 17:30~18:30 hours in proestrus and the incorporation of the tail occurred between 5:15 and 5:45 hours. The entire length of the tail had incorporated into the vitellus from 6:00 to 10:00 hours.

b) **Mode in the incorporation of the spermatozoon head and tail**:

The unfertilized and fertilized hamster oocytes were covered with long microvilli. No difference in fine structure of the surface was observed in either fertilized or unfertilized ova which were examined about 13 hours after the ovulation.

On the initial stage of the spermatozoon-egg fusion, the head of the spermatozoon had attached itself parallel to the oocyte surface and microvilli began to cover the portions of the head attached and soon covered them completely (Plate 1). Incorporation of the head of spermatozoon had occurred in two patterns as follows: i) entry from the anterior end including perforatorium (Plate 1, A~D) and and ii) entry from the poterior portion of the head in which the perforatorium is still outside the vitellus after the complete entry of the nuclear portion of the sperm head (Plate 2, B, C).

The former process occurred more frequenty than the latter one.

The trace formed by the movement of spermatozoon during the head incorporation was observed on the surface of the ova (Plate 2, B).

The microvilli around the incorporated sperm head did not differ from those of other areas (Plate 3, A, B).

In the fertilized ova, protrusion denuded from microvilli was generally observed on the side apart from first polar body.

After the incorporation of the sperm head, the spermatozoon was still active and the incorporation of the entire length of the tail progressed from the anterior portion toward the mid- and end-piece of the tail which was trapped by microvilli, forming waves on the surface of the oocytes.

The incorporation wave of the sperm tail began to form in the ova recovered from 5:15 hours and reached a peak at 6:00 hours (Plate 3, C, D).

The pieces of the tail which were still visible on the oocyte surface had fused with the microvilli (Plate 3, D).

Site of sperm penetration: Spermatozoon head penetrated around the first polar body extruded were observed in 46 of the 50 ova which were penetrated.

Discussion

Harvey *et al* (15) have studied the onset of estrus and ovulation time in the golden hamsters kept under controlled lightning (12 hours light and 12 hours dark). They found that the majority of the animals ovulated 8 to 9 hours after the onset of estrus and that the time of ovulation was not influenced by the mating.

Because the hamsters used for the present experiment came into estrus at the time reported by Harvey *et al.*, they should ovulate between 1:30 and 2:30 hours on the morning of vaginal discharge.

Yanagimachi (16) studied by phase contrast microscope on the time and process of sperm penetration into hamster ova *in vivo* and *in vitro*, and he indicated the penetration of the spermatozoon into the ovum started about 3 hr after the commencement of ovulation or about 1.5 hr after the first ovum passed into the tube.

From the results by the light microscopic observation, it was found that the majority of the ova recovered between 5:15 and 6:00 hours had spermatozoon in various stages of penetration.

Therefore, in the golden hamsters, the spermatozoon should pass through the cumulus cells and zona pellucida and then penetrate the vitellus within 2～3 hours after ovulation.

The observation by the phase contrast and scanning electron microscope in the present experiment supports the following conclusions: Complete penetration of the spermatozoon including the entire length of the tail into the vitellus in golden hamsters requires one hour from the onset of the head attachment on the surface of the oocytes.

In the investigation on the sperm penetration into the hamster oocyte *in vitro* (3, 17, 18), it was found that the plasma membrane of the sperm head attached to the surface of the oocyte began to fuse their plasma membranes to each other within 5 minutes after the trapping of the head by the microvilli and that about 30 minutes later, the sperm head could no longer be seen on the oocyte surface.

Although the sperm penetration in the hamsters *in vitro* was observed to occur first by the mid-lateral region of the head, it was observed in our experiment that the head of the spermatozoon penetrated in two ways: one is the entry from the anterior end including the perforatorium and the other as observed in rat, the entering first from the posterior portion of the head and the perforatorium last. In the hamster, the former way was observed more frequently than that in the later one.

It is well known that in the rat and mouse, the surface of their oocyte around the point of sperm head absorption is relatively devoid of microvilli and that the microvilli-free region becomes increasingly more extensive after the complete incorporation of the head (12～15).

However, in the hamster, no change in the fine structure of the oocyte surface at the site and around where the sperm head incorporated was observed. Especially the microvilli did not change in their structure and appearance before or after the sperm penetration.

It is interesting that in the hamster the fertilization cone was not observed any place on the oocyte surface, unlike that previously indicated in the study on rat and mouse ova.

In the rat ova, we demonstrated the formation of a second polar body which protruded on an area apart from the first polar body and apparently was distinguished from the fertilization cone (Sugawara and Takeuchi 1974).

From the observation on the protruding area formed on the side apart from the first polar body in the fertilized ova, we could conclud that the protrusion may be the formation of a second polar body, because the protruding area was formed in no relation to the penetrating site of the spermatozoon and also, as in most mammals, a second polar body should be extruded by the beginning of sperm penetration. There is no predilection for the site of penetration of the sperm head in relation to the first polar body.

It was confirmed that when the entire length of the sperm tail has been incorporated into the vitellus a penetration wave is formed, just as in the rat oocytes during fertilization.

In the present study, sperm head penetration around the first polar body was observed in many cases. It is interesting that hamster should be easily penetrated in the relaiton to first polar body.

Acknowledgement

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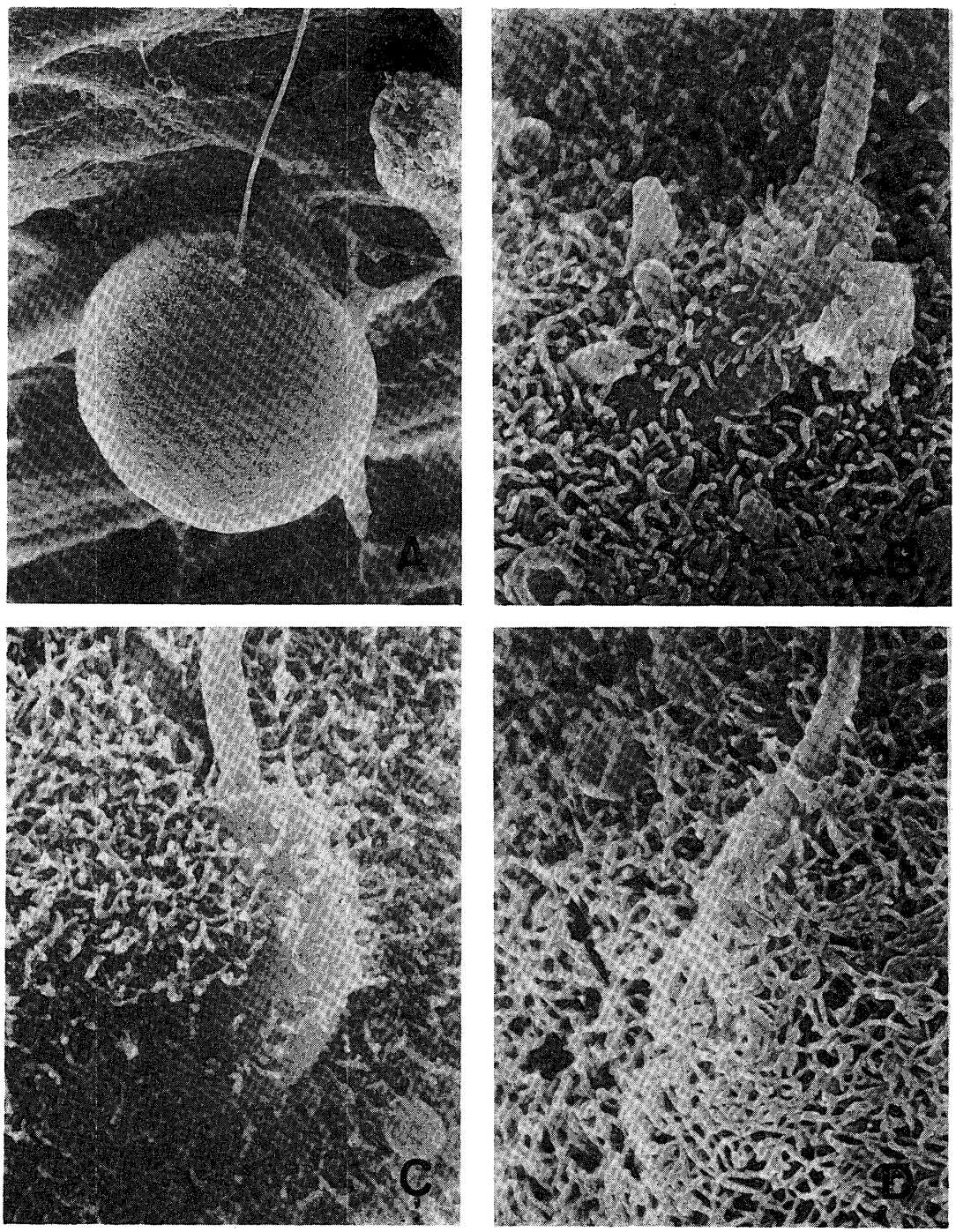

PLATE 1.

Initial stage of the sperm-egg surface interaction.

A: The head of spermatozoon penetrated through the zona pellucida had just attached on the oocyte surface. Note that the head is attached from the perforatrium and that microvilli are beginning to cover the head. × 1,125

B: The area of spermatozoon attachment magnified to 7.500

C: Note that the microvilli cover the posterior portion of the head 7,500

D: Nonte that the microvilli completely cover the speratozoon head. 7,500

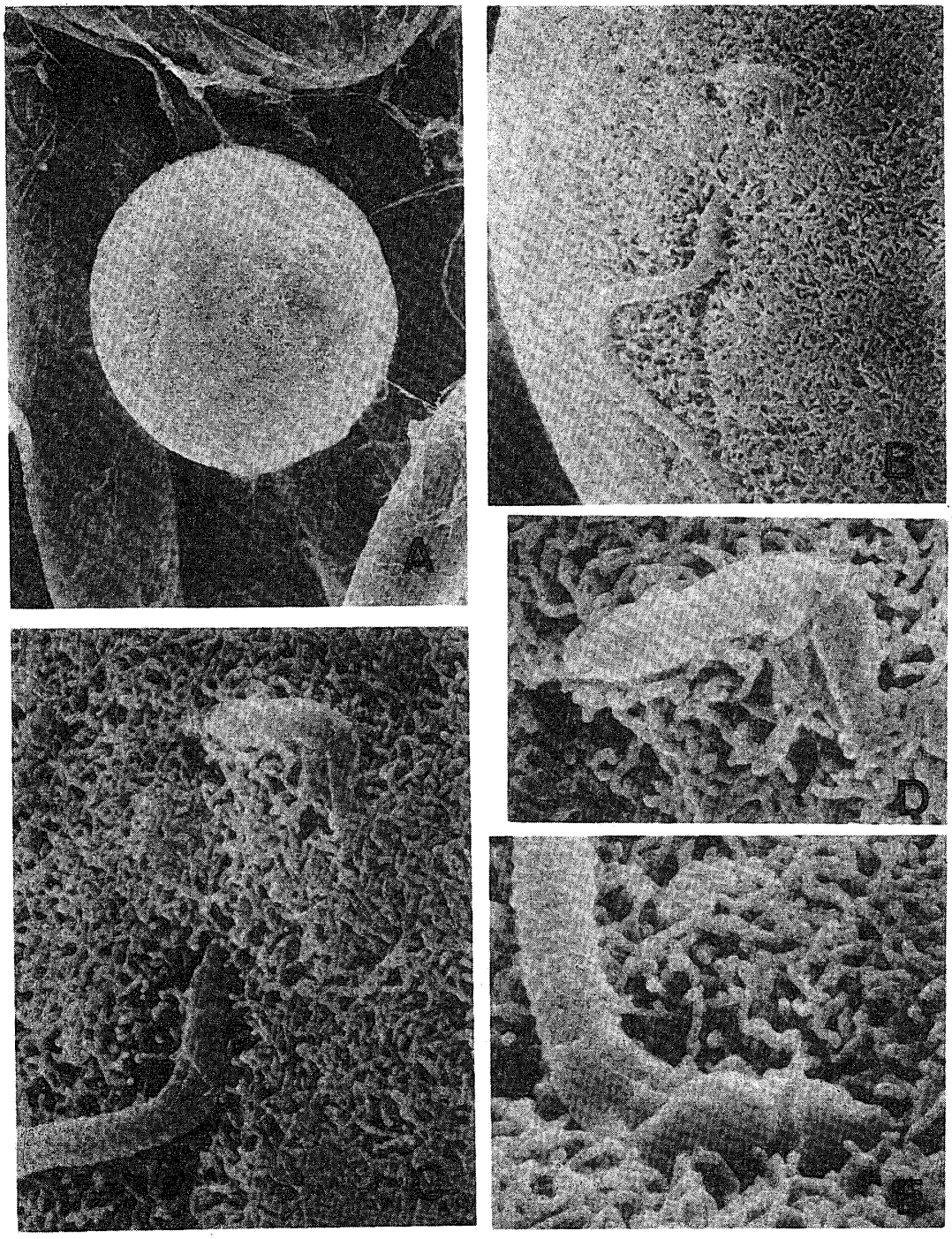

PLATE 2.

Spermatozoon entered from the posterior portion of the head.

A: Whole span of the oocyte had spermatozoon attached on the surface and the trace formed by the still movement of spermatozoon. × 3,750

B: High magnification of the area penetrated by the sperm head.

C: Incorporation of spermatozoon from the posterior portion of the head. Note the perforatorium is still seen outside the vitellus after the complete entry of nuclear portion of the head which is covered completely with microvilli. × 7,500

D: Note that the perforatorium has not yet penetrated × 15,000

E: Note the neck and the coudal part of the head. × 15,000

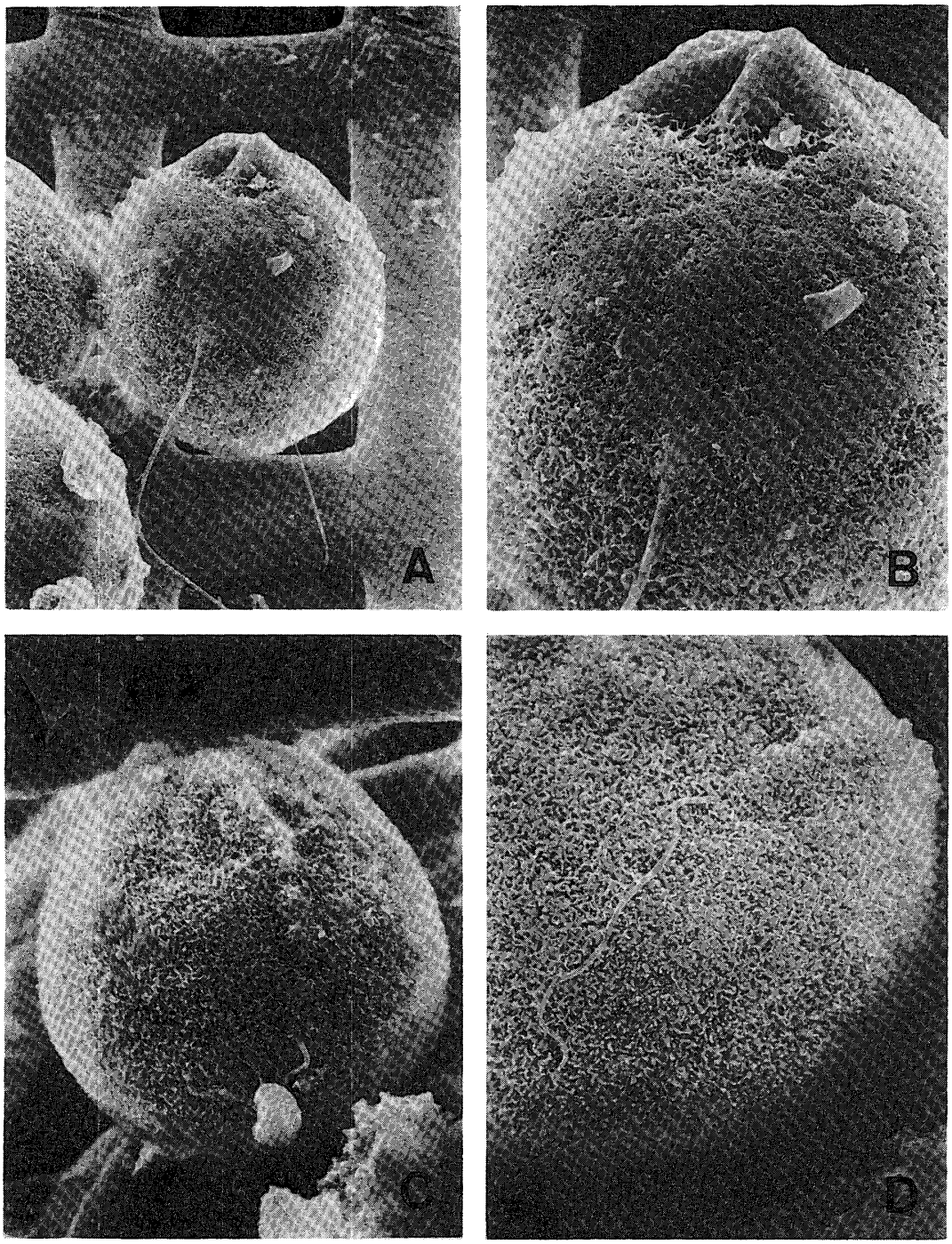

PLATE 3.

Incorporation of spermatozoon tail into the vitellus.

A: Whole span of the egg penetrated completely by the sperm head. × 1,125

B: Note the area into which the head of spermatozoon incorporated × 3,750

C: Note the incorporation wave of the sperm tail beginning to form on the oocyte surface. ×1,500

D: Note the middle and end piece of the tail still outside the oocyte surface.

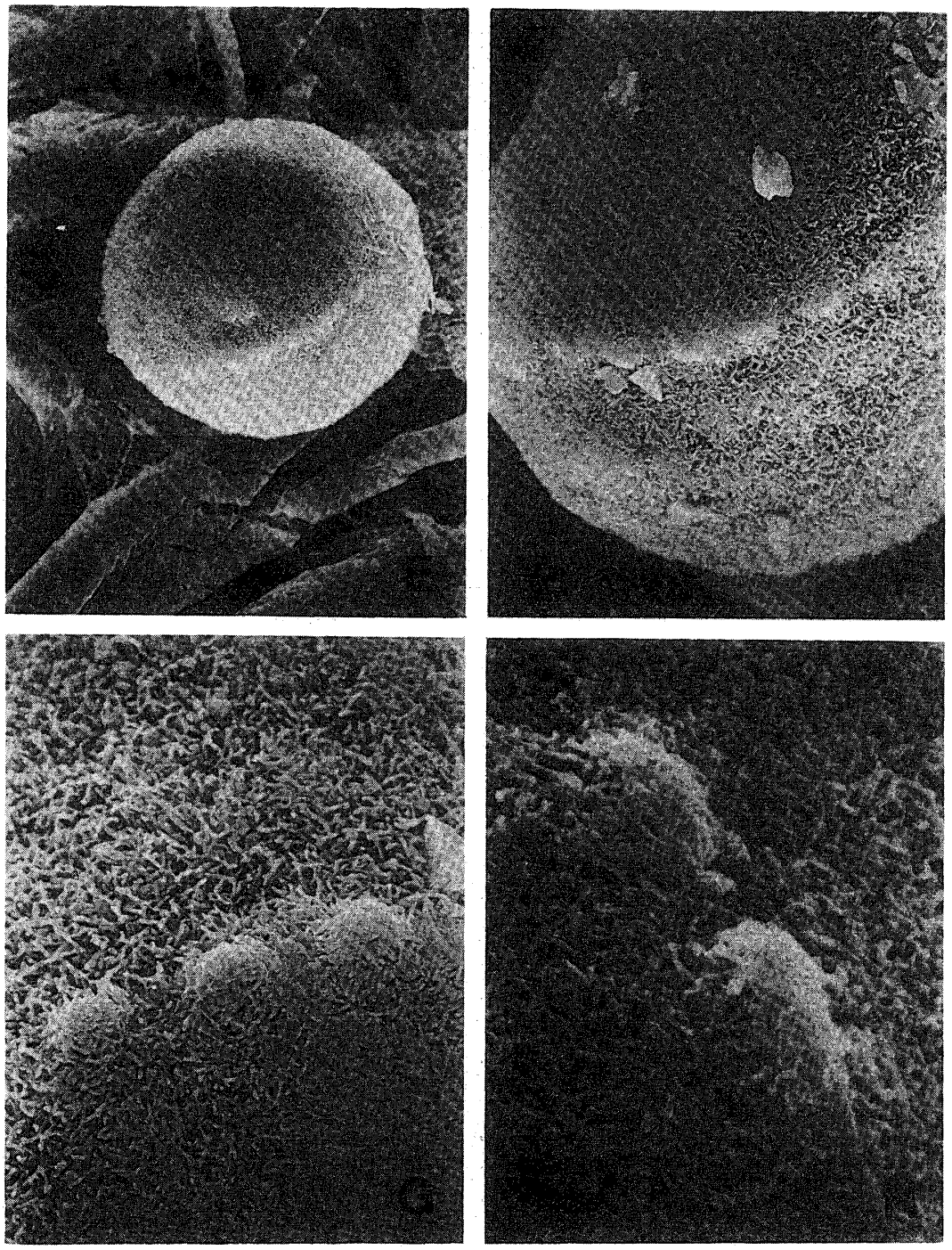

PLATE 4.

Incorporation wave of entire length of the tail formed under the oocyte surface.

E: Whole span of the spermatozoon penetrated egg with incorporation wave. Note the incorporation wave formed an equetrial ring on the oocyte surface. ×1,125

F: The area of incorporation wave in A was magnified to × 2,250 Note the regularity of the tail wave.

G: The part of the wave was magnified to ×3,750. Note that the tail between the wave has been completely incorporated.

H: High magnification of the wave. Note the wave it covered already with microvilli.