Penetration of Rat Spermatozoa into the Vitellus in vivo : Observations on the Time Sequence

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Penetration of Rat Spermatozoa into the Vitellus *in vivo*: Observations on the Time Sequence

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Summary

Time sequence and mode of sperm penetration into the vitellus *in vivo* were studied in rats by phase contrast and scanning electron microscopes.

In observation by light microscope, the process of sperm penetration was classified into six stages. Spermatozoon in various stages of penetration was found in a large number of ova recovered between 6:00 and 6:45 in the morning after mating. In observation by scanning electron microscope, the process of sperm head incorporation was classified into three stages. Spermatozoon in various stages of head incorporation was found in the majority of ova recovered between 6:15 and 6:45.

Sperm tail, incorporating like a wave was observed on the surface of vitellus. Percentage of ova penetrated by sperm with the incorporation wave was highest at 7:15.

These results suggest that rat spermatozoon including the whole tail must be completely incorporated into the vitellus within an hour after the onset of head attachment on the vitelline surface.

Penetration of spermatozoa into mammalian eggs has been studied extensively using phase contrast microscope and transmission electron microscope (TEM) in rats (1–8), mice (9–13), hamsters (14–17), rabbits (18–21) and guinea pigs (22, 23).

More recently, the study of fertilization using scanning electron microscope (SEM) has been achieved in rats (24–26), hamsters (28, 29) and rabbits (3). Yanagimachi & Noda (16, 17, 27) and Noda & Hidaka (28) reported the mode of sperm penetration into the vitellus of zona-free hamster eggs *in vitro* by SEM as compared with observations by TEM. The relationship between ovum microvilli and sperm head in hamsters (27) and rats (26) was observed by SEM, which was not elucidated in detail by TEM.

In a previous report (25), we disclosed a possible mode of incorporation of sperm head into vitellus in rats *in vivo* by SEM. However, the time sequence of sperm incorporation into vitellus has not been elucidated. Further, there are few

reports (29) concerning the incorporation process and time sequence of sperm tail on the vitelline surface.

The present study was undertaken to observe the mode of sperm head and tail incorporation by SEM, and to clarify the time sequence of sperm penetration into the vitellus in the rat *in vivo* upon which little has been known (20).

Materials and Methods

Mature female rats of Wistar strain weighing 170–280 g, which had been bred in our laboratory, were used. They were housed under natural conditions and provided with a basic diet and tap water *ad libitum*. Before use, vaginal smear was checked daily to confirm the presence of at least two consecutive regular 4 day cycles.

Females in proestrus were caged with a fertile male for an hour between 19:00 and 20:00. They were killed between 3:00 and 9:00 the next morning after mating. The Fallopian tubes were dissected and placed in physiological saline. The tubes were broken with two subcutaneous needles and the eggs with cumulus cells were extruded.

The eggs surrounded with cumulus oophorus were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4 for 10–12 hours at 4–5°C. After fixation, the eggs were washed three times with 0.2 M phosphate buffer, pH 7.4. Using two fine needles, the cumulus oophorous and zona pellucida were removed mechanically under a dissecting microscope. These eggs were observed by phase contrast microscope and were classified into six stages according to the state of contact between sperm and vitellus, as shown in Table 1. After the observations by phase contrast microscope, the zona-free eggs were kept in a refrigerator for 1-2 days.

In the next step of preparation for scanning electron microscope, the eggs were transfered into watch glasses containing distilled water and washed for a few minutes. The eggs were transfered into a glass container and dehydrated with a graded ethanol series by the method of Sugawara et al (29). After complete dehydration with 100% ethanol, the eggs were transfered into a critical point dryer, HCP–1 (Hitachi Co.). They were laid on filter paper (No. 2 filter, Toyo Roshi Co.), which was glued to an aluminum stub. The specimen was coated with gold by an ion coater, IB–3 (Eiko Engineering Co.). Observations and photographs were made using a Field Emission type SEM, S–700 (Hitachi Co.) and Tri-X Pan film (Eastman Kodak Co.). In the observations by SEM, the penetration process of sperm head into the vitellus was classfied into three pictures (attachment of sperm head on the vitelline surface, incomplete envelopment of sperm head by the protrusion of ovum cytoplasm and complete envelopment of sperm head by ovum cytoplasm) and the relationship between time and frequency distribution of the three types were investigated. The frequencies of appearance of wave-like patterns of tail fragments on the vitelline surface were also recorded at each time. This

incorporation process of sperm head and tail into ovum cytoplasm was shown in Figs. 1–12.

Results

Proportion of ova penetrated by sperm: A total of 627 ova were examined by phase contrast microscope, and 60.6% were penetrated by sperm. In the Table 1, the frequencies of ova classified under phase contrast microscope were shown at each time between 3:00 and 9:00. No ova with spermatozoon were observed for the two hours between 3:00 and 5:00. Eight of 36 ova (22%) were penetrated by the spermatozoon by 5:15. The percentage of ova with spermatozoon increased gradually toward around 7:00, when all ova had spermatozoon.

Sperm penetration in rat ova occurred between 5:45 and 6:45 in the morning of estrus. The results of the observation on stages I-VI are shown in Table 1.

The ova classified as stage I were observed from 5:15 to 6:45. The highest

TABLE 1. *Observation by Phase Contrast Microscope on Sperm Attachment and Entry on the Surface or Cytoplasm of the Rat Eggs*

Recovery time of ova (hours)	No. of ova examined	No. of ova penetrated (%)	* Stages of sperm penetration (%)					
			I	II	III	IV	V	VI
3:00	9	0(0)						
3:30	9	0(0)						
4:00	7	0(0)						
4:30	8	0(0)						
5:00	29	0(0)						
5:15	36	8(22)	1(13)	7(87)				
5:30	53	4(8)	2(50)	2(50)				
5:45	38	13(36)	4(30)	5(39)	3(23)	1(8)		
6:00	65	29(53)	5(17)	8(28)	2(7)	1(3)	5(17)	8(28)
6:15	58	28(48)	7(25)	9(32)		1(4)		11(39)
6:30	49	37(76)	4(11)	10(27)	3(8)	3(8)		17(46)
6:45	53	47(89)	2(4)	9(19)	7(15)	2(4)	10(22)	17(36)
7:00	47	47(100)		2(4)	4(9)	7(15)	8(17)	26(55)
7:15	25	23(92)		1(4)	2(9)	6(26)	9(39)	5(22)
7:30	21	21(100)					11(52)	10(48)
7:45	25	24(100)			2(18)	4(16)	1(4)	18(72)
8:00	47	47(100)				4(9)	1(2)	42(89)
8:15	11	11(100)				1(9)		10(91)
8:30	25	24(100)				2(8)	2(8)	21(84)
8:45	11	11(100)						11(100)
9:00	11	11(100)						11(100)

* Stage I: Sperm head attaches to the ovum surface and sperm tail appears straight.
Stage II: Sperm tail appears in arc.
Stage III: Sperm tail attaches to several places of ovum surface.
Stage IV: Sperm head disappears in the ovum cytoplasm and the end of sperm tail is observed outside of ovum surface.
Stage V: Sperm tail attaches completely to the ovum surface.
Stage VI: Sperm tail is observed as a dotted line on the surface of ovum or an unclear line in the ovum.

percentage (50%) appeared at 5:30. The proportion of ova at stage II was the highest (87%) at 5:15 and thereafter decreased gradually. Stage III was observed extensively between 5:45 and 7:45. Stage IV was observed from 5:45 to 8:30 with the highest percentage (26%) at 7:15. Stage V was observed from 6:00 to 8:30 with the highest percentage (52%) at 7:30. The proportion of ova at stage VI increased gradually to 100% at 8:45.

Observation by SEM on sperm penetration into the vitellus

a) *Incorporation mode of sperm head and tail into the vitellus*

The unfertilized and fertilized rat oocytes were covered with short microvilli. There were no differences in fine structure of the surface between the unfertilized and fertilized ova as previously reported (25).

On the initial stage of sperm-egg fusion, the rat spermatozoon attached the postnuclear cap region of sperm head to ovum surface (Fig. 1). That is, the numerous microvilli were in contact with the postnuclear cap region of sperm head (Fig. 2). After that, the protrusion of ovum cytoplasm with a few microvilli enveloped the sperm head from the postnuclear cap region (Fig. 3, 4). Then, the sperm head, except the perforatorium, was covered with the protrusion of ovum cytoplasm with few microvilli (Fig. 5, 6). Eventually, the sperm head was completely enveloped by the ovum cytoplasm (Fig. 7, 8).

After the incorporation of the head, the spermatozoon was still movable to some extent. Therefore, the incorporation of the sperm tail progressed from the anterior portion to the mid- and end-pieces, which were trapped by microvilli at the contact site of the tail (Fig. 9–12). Because of flagellum movement, the sperm tail formed waves on the surface of the oocytes (Fig. 10, 12). Finally, the sperm tail was completely incorporated into the ovum cytoplasm.

The penetration site of the sperm head was not always around the first polar body extrusion. Only 9 of 63 ova were penetrated around the first polar body extrusion or at the site where the second polar body region was expected to extrude.

b) The time sequence of sperm penetration

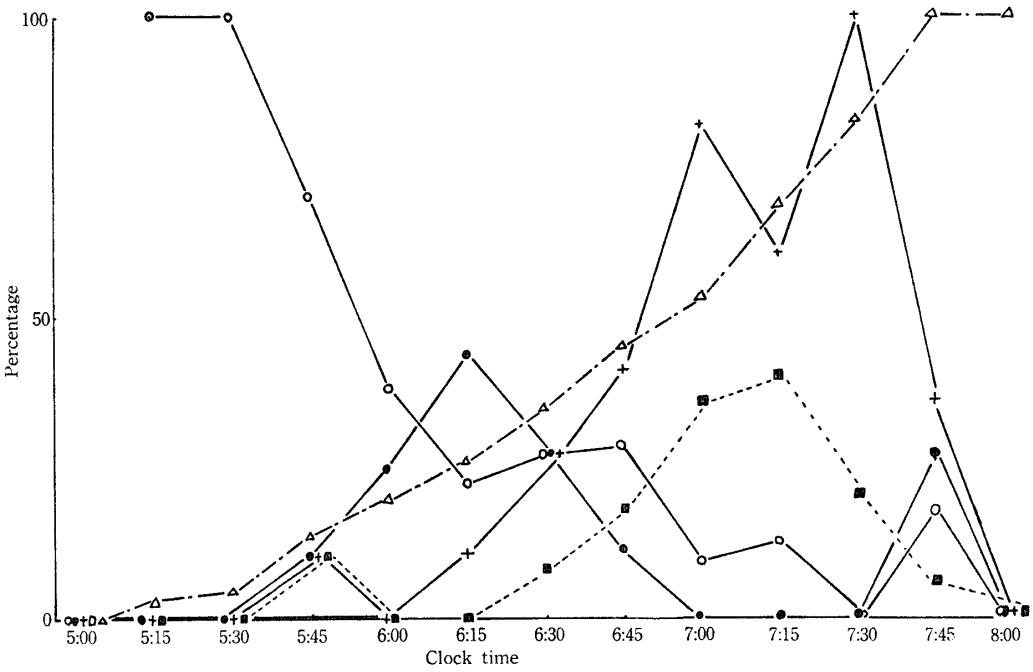
The time of entry of the sperm head and tail and its time sequence were examined by SEM observation. The results are shown in Table 2 and Text-Fig. 1. The ova observed by SEM were classified into three processes according to the entry of sperm head and the sperm tail which formed waves on the surface of vitellus. The proportion of the ova in each process to the ova penetrated by spermatozoon was observed.

In all ova penetrated by spermatozoon at 5:15 and 5:30, the sperm head was attached on the surface of vitellus. Of 10 ova penetrated by spermatozoon, 70% was attached to the sperm head at 5:45. After that, the percentage decreased gradually. Sperm head enveloped by the protrusion of ovum cytoplasm was

TABLE 2. *Observation by Scanning Electron Microscope*

The time of killing (hours)	No. of ova examined	No. of ova after critical point drying	No. of ova observed	No. of ova with sperm
5:00	24	20	18	0(0)
5:15	36	32	30	4(13)
5:30	53	37	36	3(8)
5:45	38	28	27	10(37)
6:00	55	32	31	8(26)
6:15	56	40	37	9(24)
6:30	49	34	29	11(38)
6:45	53	42	41	17(41)
7:00	47	32	31	11(35)
7:15	25	24	24	15(63)
7:30	21	21	18	10(56)
7:45	25	18	16	11(69)
8:00	24	21	21	0(0)
Total	508	381(75)	359(71)	

* The sperm tail was observed, but the stage of penetration



Text-FIG. 1. The observation by SEM on the time sequence of the penetration of rat sperm into the vitellus *in vivo*.

△ —·— △ The ova penetrated by spermatozoon.
○ —— ○ The ova attached by the sperm head on the surface of vitellus.
● —— ● The ova which partially enveloped sperm head by the protrusion of ovum cytoplasm.
\+ —— + The ova which completely enveloped sperm head by the protrusion of ovum cytoplasm.
■ ----- ■ The ova which incorporated the sperm tail like a wave.

on Sperm Penetration into Vitellus in vivo in Rat

No. of ova unestimated*	The mode of sperm head incorporation			Wave-like tail
	attachment	incomplete envelopment	complete envelopment	
	4(100)			
	3(100)			
1	7(70)	1(10)	1(10)	1(10)
3	3(38)	2(25)		
2	2(22)	4(44)	1(11)	
2	3(27)	3(27)	3(27)	1(9)
3	5(29)	2(11)	7(41)	3(18)
1	1(9)		9(82)	4(36)
4	2(13)		9(60)	6(40)
			10(100)	2(20)
2	2(18)	3(27)	4(36)	1(9)

was not estimated correctly due to various problems.

observed for the first time at 5:45 and its proporition to the ova penetrated by spermatozoon was 10%. The highest percentage (44%) was observed at 6:15. Complete sperm head envelopment by the protrusion of ovum cytoplasm was first observed at 5:45 and its proportion to the ova penetrated by spermatozoon was 10%. The percentage of this process was higher (60–100%) between 7:00 and 7:45. Ova with wave-like sperm tails were initially observed at 6:15. The highest percentage (40%) of this stage was found at 7:15.

By the distribution of the three stages as described above, the penetration and the attachment of the sperm head was found to begin at 5:15 in the morning after mating which was verified at 19:00 and 20:00 in proestrus. That time accords to about 2–3 hours after ovulation. The incorporation of the tail occurred between 5:45 and 6:30. The whole length of the tail incorporated into the vitellus from 6:30 to 7:45.

Discussion

In this study, penetration of rat sperm into the vitellus began at 5:15. This time was about 10 hours after copulation and 2–3 hours after ovulation, since ovulation started between 2:00 and 3:00. In *ad libitum* mated rats, Shalgi & Kraicer (4) showed that the penetration of spermatozoa into oocytes started about 3–4 hours after the onset of ovulation and this time coincided with two hours after median time of ovulation. By about two hours after the beginning of sperm penetration, all eggs were penetrated. Austin (2) reported that penetration of all eggs in any one rat took a mean time of 3.5 hours. These delicate shades of timing of sperm penetration were due to the variance of ovulation and were affected by the

use of different species or different breeding conditions.

Austin & Braden (2) observed details of spermatozoon penetration by phase contrast microscopy in rodents (rats, mice and hamsters). They illustrated four stages in the penetration of rodent eggs by spermatozoon. These details concluded 1) sperm head made contact with the vitellus immediately on penetrating the zona pellucida, 2) sperm head was lying upon the vitelline surface. 3) whole of sperm mid-piece entered the perivitelline space and sperm head invaded the vitellus and 4) transformation of sperm head proceeded to the stage immediately before the appearance of nucleoli. The same process was confirmed by phase contrast microscope in this study. Furthermore, a number of ova were recovered between 6:00 and 6:45 in various stages of penetration. Therefore, complete penetration of the whole spermatozoon into the vitellus in rats requires an hour from the onset of head attachment to the vitelline surface. Similarly, Sugawara et al (29) reported that it took an hour for spermatozoon to penetrate the vitellus in hamsters *in vivo*.

By the investigation of *in vitro* fertilization of hamster zona-free ova, Yanagimachi & Noda (16, 17) clarified the incorporation time of sperm head. They reported by TEM that the sperm plasma membrane contacted physically or fused with the ovum plasma membrane within five minutes after insemination, and concluded that it took fifteen minutes after insemination for entire sperm head incorporation into the ovum cytoplasm. Therefore, the incorporation time of sperm head in rats may be similar to that in hamsters.

Preliminary reports (25, 26) outlined SEM observation on the mode of incorporation of sperm head into the vitellus in rats *in vivo*. That is, the sperm head attaches to the vitelline surface soon after penetration to the zona pellucida. Subsequently, ovum microvilli transform and trap the sperm head. After that, the protrusion of ovum cytoplasm begins to cover the postnuclear region of the sperm head until it envelopes the head completely. Ovum microvilli disappear gradually, in this process. This penetration process was confirmed by the present study which showed the time relations of the sperm incorporation process by SEM. Further, the majority of ova recovered between 6:15 and 6:45 had the spermatozoon in various stages of head incorporation, suggesting that it took a half hour for the sperm head to invade the vitellus. In addition, there was a high percentage of ova in which sperm tail was incorporated like a wave on the vitelline surface between 7:00 and 7:15. Therefore, it took an hour for the sperm tail to incorporate into the vitellus after sperm head attachment.

The time sequence of penetration of rat sperm into vitellus was eleucidated by SEM, but the process of fusion between sperm and vitelline membrane in early efrtilization remained for future study.

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Palte I

Explanation of the plate (Scale line=1 μ)

Fig. 1. The attachment of sperm head to ovum surface.

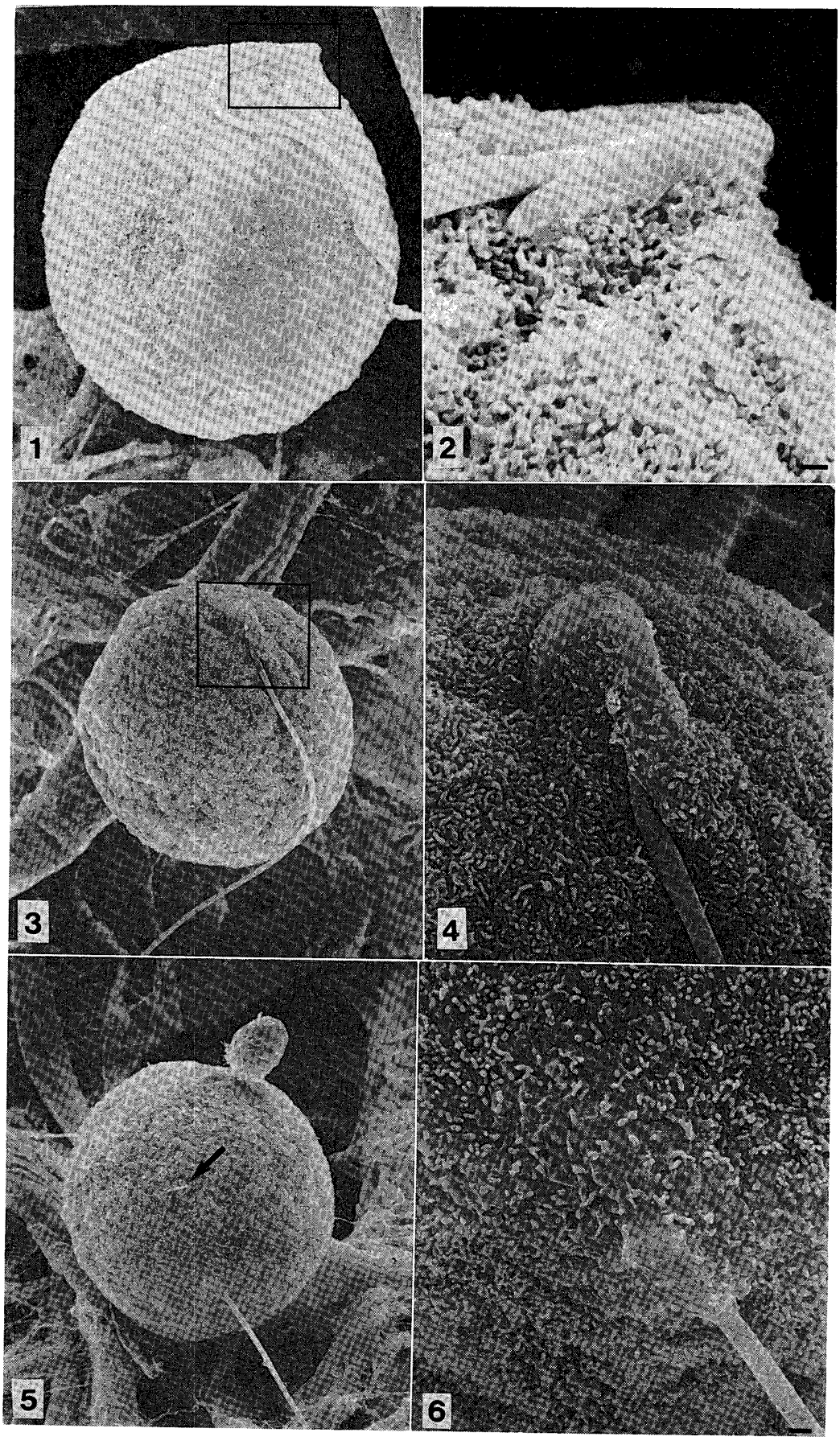
Fig. 2. Enlarged view of the area inside the rectangle shown in Fig. 1. Note the contact between sperm head and ovum microvilli.

Fig. 3. Envelopment of sperm head with the protrusion of ovum cytoplasm with a few microvilli.

Fig. 4. Enlarged view of the area inside the rectangle shown in Fig. 3. Note the protrusion of the ovum cytoplasm covers the postnuclear region of sperm head.

Fig. 5. The covering of sperm head with the protrusion of the ovum cytoplasm without microvilli except the perforatorium.

Fig. 6. A high magnification of Fig. 5.
Note the region of sperm head incorporation with a few microvilli. An arrow indicates the perforatorium.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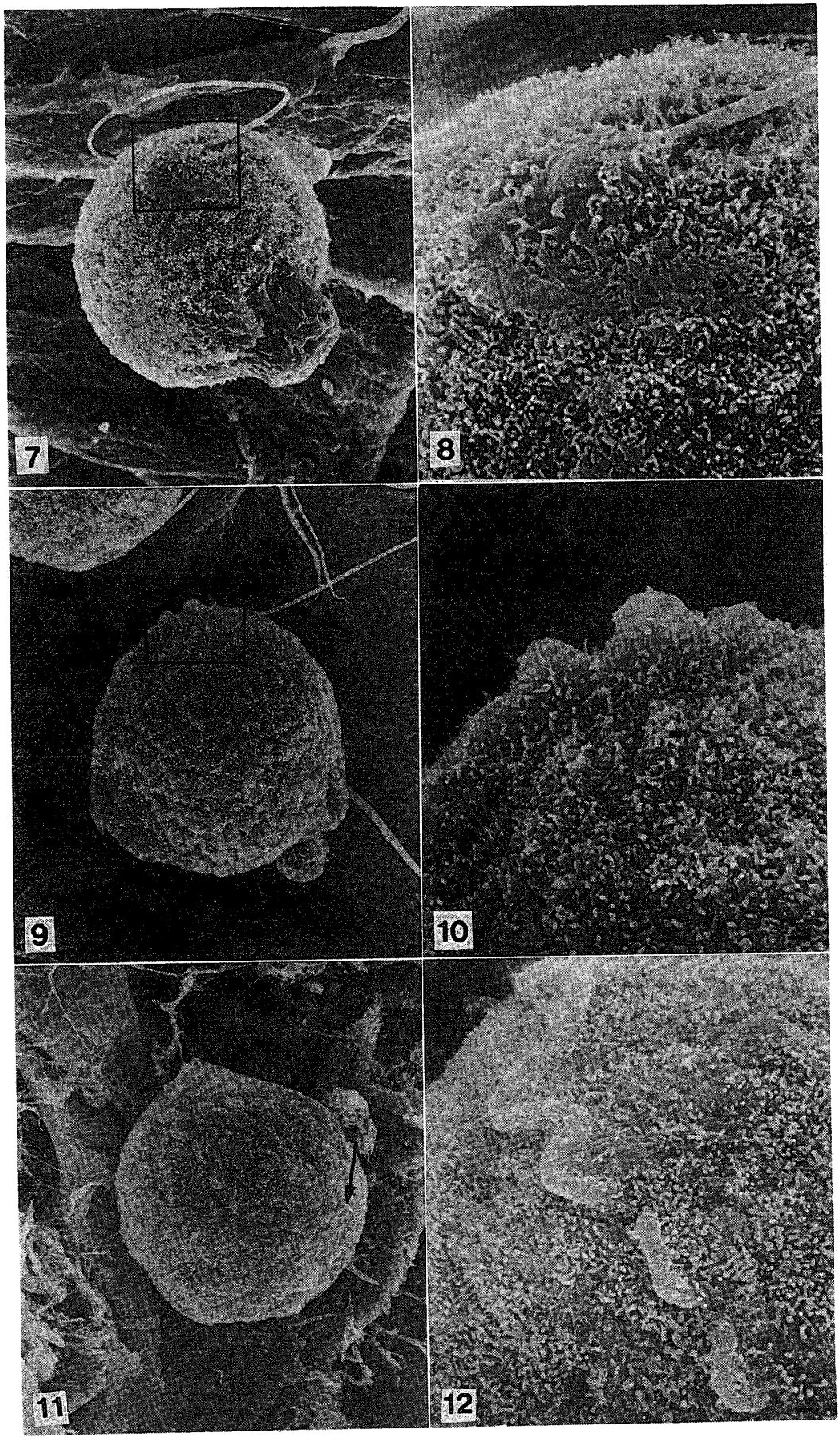
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PALTE II

FIG. 7. The complete envelopment of whole sperm head by ovum cytoplasm.
FIG. 8. Enlarged view of the area inside the rectangle shown in Fig. 7.
Note the region of sperm head incorporation.
FIG. 9. Incorporation of the midpiece of sperm tail.
An arrow indicates the mid-piece of sperm tail.
FIG. 10. Enlarged view of the area inside the rectangle shown in Fig. 9. Note the incorporation wave of the mid-piece of sperm tail. An arrow indicates the end-piece of sperm tail.
FIG. 11. Whole span of the spermatozoon penetrated ova with incorporation wave.
FIG. 12. A high magnification of Fig. 11.
Note the tail covered with microvilli.