Ascidian Fertilization
— Its Morphological Aspects —

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Halocynthia roretzi (12×15cm)

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Review
Ascidian Fertilization
— *Its Morphological Aspects* —

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Abstract

The ascidian spermatozoa have an acrosome(s), albeit a small one. In *Ciona intestinalis* and *Halocynthia roretzi* spermatozoa, the acrosome reaction occurs by fusion between the plasma membrane overlying the acrosome and the outer membrane of the acrosome at its peripheral margin. A flat sac bounded by hybrid membranes of the acrosomal outer membrane and the overlying plasma membrane forms at the sperm tip. Membrane fusion proceeds along the peripheral margin of the acrosome. The hybrid sac transforms into a vesicle that eventually detaches from the tip of the sperm head. During this process, acrosomal substance is released. This type of acrosome reaction actually occurs in the perivitelline space after passage through the chorion during the fertilization of *H. roretzi*. *This is a new type of acrosome reaction which has not been described previously.*

In *H. roretzi*, sperm can pass through the chorion with an intact acrosome, leaving a distinct hole in the chorion. This suggests that *chorion lysin(s) are nondiffusible and intercalated into the plasma membrane enclosing the sperm head.* It has been proposed that the fuzzy extracellular material (surface ornament) at the tip of the sperm head in ascidians is the site where the lysin(s) are found and that it plays an important role sperm-chorion interactions at fertilization.

In the perivitelline space, apical processes protrude mainly from the peripheral region of the apex of the acrosome-reacted sperm head. Gamete fusion occurs between some of these processes and the egg plasma membrane, resulting in the incorporation of the sperm into the egg from the anterior tip of its head, in the same way that it does in other marine invertebrates. In this respect, *the apical process may be functionally homologous to the acrosomal process(s) of some other marine invertebrates.*

Morphological studies on ascidian species with external fertilization have made the sequential events associated with fertilization clearer. There are many other groups of ascidians in which fertilization is internal. For a satisfactory understanding of the general mechanisms of ascidian fertilization, intensive studies on the morphological events associated with the internal fertilization are indispensable.

Key words: acrosome, acrosome reaction, apical process, fertilization, Ascidians

[I] Introduction

The chordate subphylum Tunicata comprises three classes: Appendicularia, Ascidiacea and Thaliacea. Most of the work on the fertilization biology of these animals has been done on ascidians. One pecuriality of ascidian sperm is the greately reduced size of these acrosome. In the appendicularian, *Oikopleura dioica* spermatozoa have the so-called "typical acrosome"[31]. According to Holland *et al.*[60], fertilization in *O. dioica* occurs almost in the same way that it does in other marine invertebrates. As this review will show, this is not the case in ascidians. Two fairly recent reviews [28, 78] have summarized the biochemical and physiological events associated with ascidian fertilization. Another review [46] describes morphological events associated with fertilization in ascidians. This review will attend to integrate the morphological and biochemical aspects of external fertilization, mainly

in *Ciona intestinalis* and in *Halocynthia roretzi.*

It will also examine fertilization in ascidians in a comparative context.

[II] Sperm Morphology

Ascidian sperm have several characteristic features including a fairly long head with a single mitochondrion [28] that extends along the nucleus; a midpiece is lacking [29, 32]. In some ascidian species, tubular or filamentous structures appear in the mitochondrion during spermiogenesis [6, 38, 39, 40, 41, 49]. In *Pyura vittata* spermatids, tube-like structures which are 30 nm in diameter filled with an electron-opaque substance have been observed. A fairly regular banding pattern is observed along the structures. Each band is about 7 nm in width. These structures extend longitudinally in the mitochondria from its anterior tip to its posterior end along the inner membrane adjacent to the nucleus. There is an increase in the number of these structures as the mitochondria continue to elongate along the nucleus during spermiogenesis. Mitochondria of mature spermatozoa have 14-16 in number of thses structures which are arranged parallel to one another along their longitudinal axes [38]. Similar structures have been recognized in *Pyura haustor* [40]. Tubular structures (about 20 nm in diameter) have been recognized in the sperm mitochondria of *C. intestinalis* (Fukumoto, unpublished).

The filamentous structures in the sperm mitochondria have been reported in *Perophora formosana* [39], in *P. annectens* [41], in *Diplosoma listerianum* [6] and in *D. macdonaldi* [49]. In *P. formosana*, filamentous structures (approximately 10 nm in thickness) are observed exclusively in the mitochondrial matrix. They are arranged parallel to one another along the long axis of the mitochondrion. During spermiogenesis, they appear in the mitochondria of spermatids as the mitochondrion begins to elongate into the lateral body. Similar structures have been recognized in mitochondria in some other compound ascidians: *Clavelina picta, C. huntsmani, Distaplia occidentalis* and *Aplidium californicum* (Fukumoto, unpublished). These structures are thought to be neccessary for mitochondrial elongation [6, 38, 39, 41, 49].

The tail of ascidian spermatozoa is a simple flagellum with a 9+2 microtubular axoneme pattern. In ascidians with internal fertilization, the sperm head is generally longer and more specialized than in ascidian species with external fertilization. For example, the sperm head of *P. formosana* (internal fertilization) is about 90 μm long and has an apical structure, approximately 4 μm in length, at the

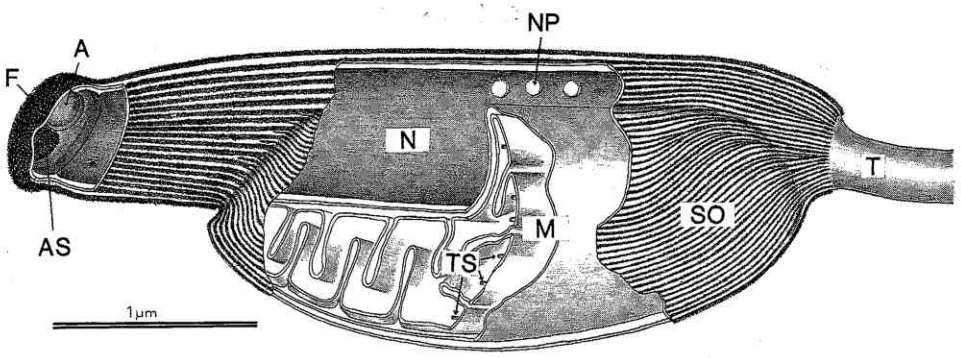

Fig. 1 Schematic illustration of *Ciona intestinalis* Spermatozoon
The spermatozoon of *C. intestinalis* has architectural features that are characteristic of ascidian spermatozoa. It has an elongated head (approximately 4 μm in long) with a wedge-shaped tip and a mitochondrion which is closely applied laterally to the nucleus. An acrosome (A) is present at the anterior region of the head, which appears as a flattened vesicle (about 150nm x 160nm x 60nm). At the anterior-most tip of the head, apical substance (AS) which is accumulation of granular material, approximately 5-7 nm in diameter, is recognized. Fuzzy materials (surface ornamentation) decorate the external sueface of the plasma membrane enclosing the head. Nuclear pores (NP) are present, which appear sometimes in the anterior region of the head. Tubular structures (TS), approximately 20 nm in diameter, are present in the mitochondrion just inside its inner membrane running antero-posteriorly. [Fukumoto [46]].

anterior tip of its head [39]. On the other hand, the sperm head of *C. intestinalis* (external fertilization) is about 4 μm in length [121]. The morphological description of ascidian sperm in the evolutionary aspects was recently published [68]. As a representative of the sperm used in external fertilzation, the differentiated spermatozoon of *C. intestinalis* is schematically illustrated in Figure 1 [46].

[Ⅲ] The Acrosome of Ascidian Spermatozoa

There have been a long debate concerning the presence or absence of an acrosom(s) in ascidian spermatozoa [46, 78].

There are a number of papers which have questioned the presence of an acrosome in differentiated ascidian spermatozoa: in *Corella parallelogramma* [33], in *Ascidia malaca* [115], in *C. intestinalis* [121], in *H. roretzi* [72], in both *Molgula impura* and *Styela plicata* [116], in *Microcosmos sabatieri* [117], in *Ascidia malaca, Ascidiella aspersa* and *Phallusia mammillata* [118], and in *Pyura stolonifera* [3].

Cloney and Abbott [12] first clearly showed membrane bound vesicle(s) which they referred to as the putative acrosome in the anterior tip of the sperm head in *Ascidia callosa*. They argued that the claim that the spermatozoa of ascidians lack an acrosome

Table Ascidian species with acrosome(s) [Fukumoto [46] modified]

Order	Suborder	Family	Genus and species	References
Enterogona	Aplousobranchia	Clavelinidae	*Clavelina huntsmani*	*
			Clavelina picta	*
			Clavelina lepadiformis	(34)
			Distaplia occidentalis	*
		Polyclinidae	*Aplidium californicum*	*
		Didemnidae	*Diplosoma macdonaldi*	(49)
	Phlebobranchiata	Cionidae	*Ciona intestinalis*	(42)
			Ciona savigney	*
		Perophoridae	*Perophora formosana*[1]	*
			Perophora annectens[1]	*
			Ecteinascidia turbinata	*
		Corellidae	*Corella pacifica*	*
		Ascidiidae	*Phallusia nigra*	(50)
			Phallusia mammillata	(62)
			Ascidia zara	*
			Ascidia gemmmata	*
			Ascidia mentula	*
			Ascidia callosa	(12)
Pleurogona	Stolidobranchiata	Styelidae	*Styela plicata*	(40)
			Styela clava	(44)
			Cnemidocarpa finmarkiensis	(44)
			Botryllus schlosseri	(44)
			Metadrocarpa taylori	*
		Pyuridae	*Pyura haustor*	(40)
			Pyura vittata	*
			Boltenia villosa	(44)
			Herdmania momus	(44)
			Halocynthia roretzi	(52)
		Molgulidae	*Molgula manhattensis*	(43)

[1] After the paper was published, I found an acrosome at the anterior region of the nucleus.
* Unpublished data

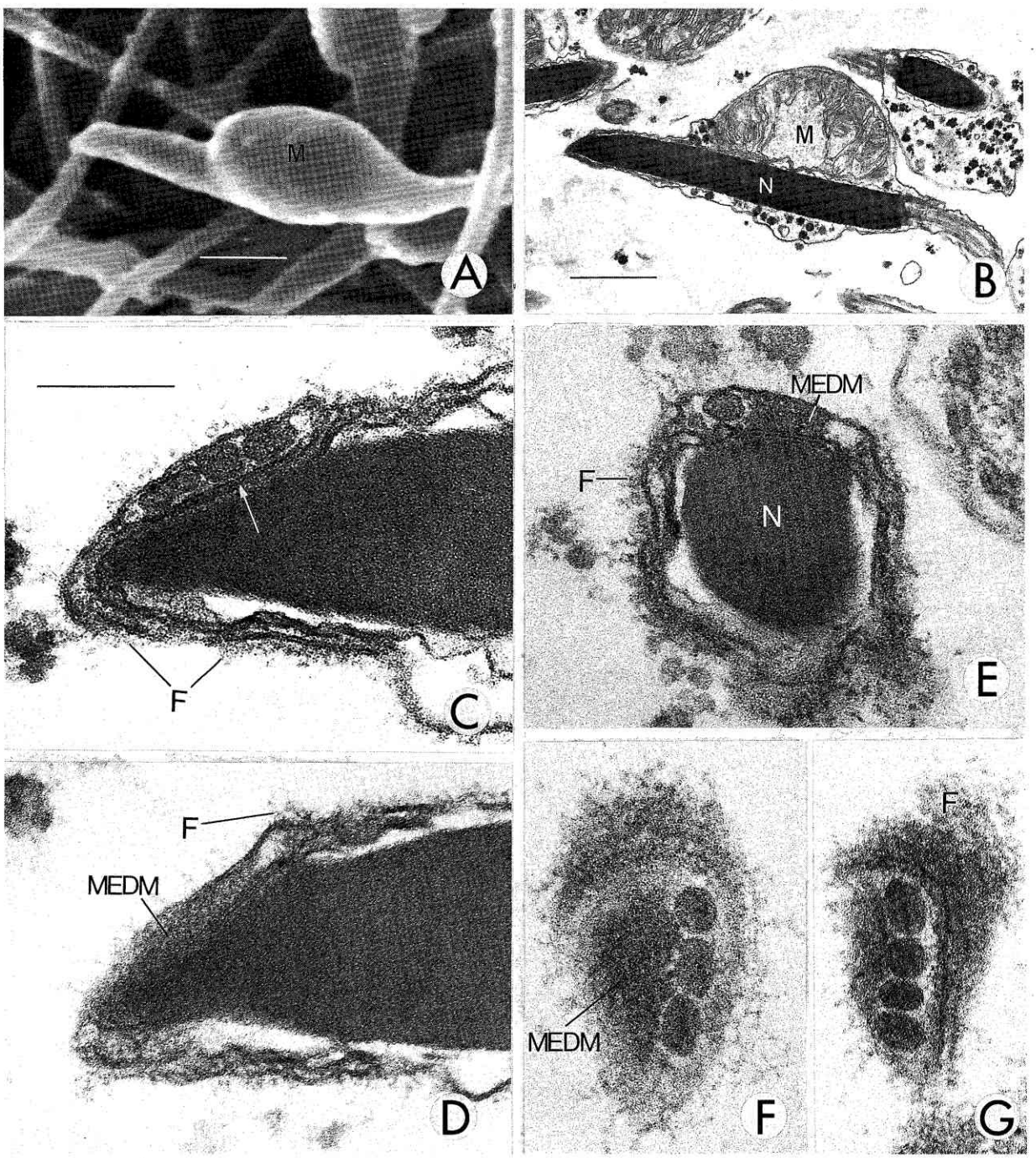

Fig. 2. Acrosome vesicles in *P. nigra* spermatozoa.
A, Scanning electron microscope (SEM) image of *P. nigra* spermatozoon. Bar, 1 μm. **B**, Sagittal section through the head. There is no midpiece. Bar, 1 μm. **C** and **D**, Serial sagittal sections through the apex of the head. Three acrosomal vesicles and moderately electron-dense material (MEDM) are present at the apex between the plasma membrane and the nuclear membrane,respectively. The inner and the outer nuclear membranes come into close contact with each other to make a pedestal for acrosomes (arrow). Bar, 200 nm (This is also the magnification for D-G). **E**, Transverse section through the apex of the head at the level of the acrosome. The MEDM occupies the central region of the apex between the plasma membrane and the nuclear membranes. **F** and **G**, Frontal sections parallel to the apical surface of the head. Three and four acrosomal vesicles are present in a line alongside the MEDM, respectively. F, fuzzy material; M, mitochondrion; N, nucleus.
[Fukumoto [50]].

should be reconsidered.

After the discovary of a putative acrosome in *A. callosa*, a membrane bound vesicle(s) was found in the anterior region of the spermatozoa of a number of species of ascidians including both the Enterogonan and Pleurogonan Orders that made up this Class (Table).

Before the discovery of the acrosome [12], there had been several papers which reported the presence of an acrosome in ascidian spermatozoa: in *Ascidia nigra* [100], in *C. intestinalis* [14, 20, 54, 89, 92], in *Diplosoma listerianum* and *Lissoclinum pseudoleptoclinum* [109], in *Polysyncraton lacazei* and *Tridemnum cereum* [110]. However, these papers were not convincing because they did not demonstrate the existence of a membrane bound acrosomal vesicle.

The view that ascidian spermatozoa have no acrosome can be attributed to the difficulty of getting good fixation and to the greatly reduced size of the ascidian acrosome [12].

Lambert and Koch [78] have insisted that the vesicles in ascidian spermatozoa should be called "apicl vesicles" until the criteria for positive identification as acrosomes could be more closely met (see their review for details).

However, if a vesicle(s) is present in the proper location for an acrosome, this would be one kind of criteria, because acrosomes are anteriorly located vesicles in the sperm of other animals. Furthermore, the vesicle in ascidian spermatozoa is not an oddity which is only found in one or two species, but a feature of all species examined in the Orders of Pleurogonan and Enterogonan ascidians (Table). ***For these reasons, the vesicle(s) located at the apex of sperm head in ascidian spermatozoa should be referred to as an "acrosome"*** [43, 44, 46].

There is more than one acrosomal vesicle in *P. mammillata* [63], in *P. nigra* [50], in *A. callosa* [12], in *A. zara*, *A. gemmata*, *A. mentula* and *A. samea* (Fukumoto, unpublished). During spermiogenesis, proacrosomal vesicles do not coalesce to make a single mature acrosome in these species (in preparation). Figure 2 shows the acrosomal vesicles in *P. nigra* spermatozoa [50].

[IV] Acrosome Differentiation

During acrosome differentiation in other animal

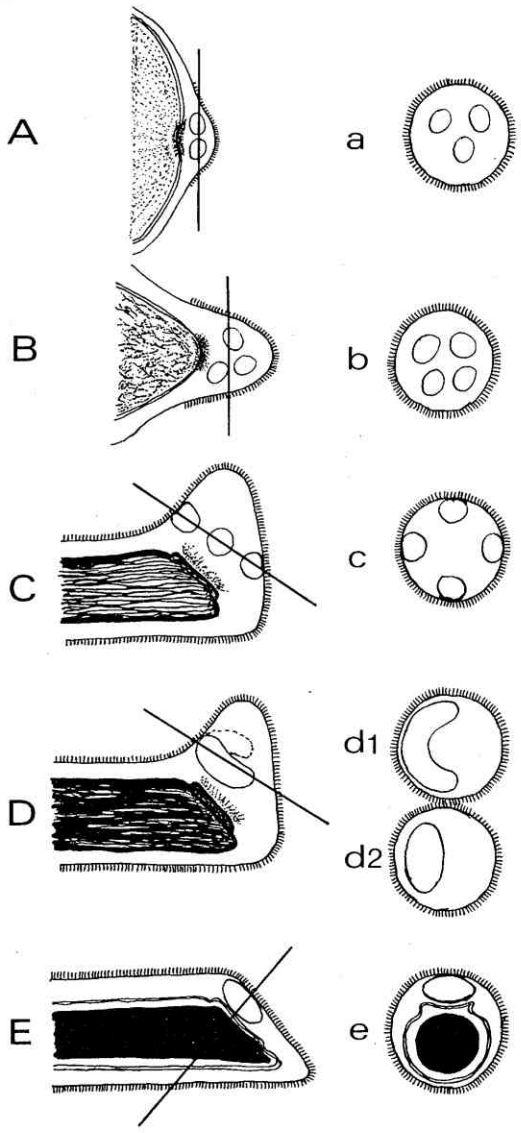

Fig. 3. Schematic illustration showing the differentiation of the acrosome in *M. manhattensis*. In early spermatids (A, B), the plasma membrane at the apex expands to form a blister which is covered with fuzzy extracellular material. Vesicles (50-60nm in diameter) are present in the blister. They are moderately electron-dense and at least three or four in number (a, b). Midway through spermiogenesis (C, D), these vesicles attach to the inner surface of the plasma membrane enclosing the blister (c). These vesicles, thereafter, fuse with each other along the inner surface of the plasma membrane and form a horseshoe-shaped acrosomal vesicle (d1) which transforms into a sphere (d2). In mature spermatozoa (E), the acrosome is a slightly depressed sphere positioned at the apex (e). The illustrations marked by small letters represent the transverse sections at the level indicated in those marked by capital letters, respectively.
[Fukumoto [43]].

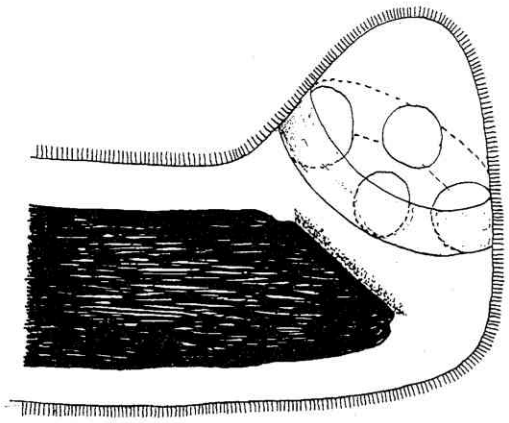

Fig. 4. Schematic illustration showing a hypothetical scaffold which facilitates vesicle contact and fusion. See details in text [Fukumoto [43]].

species, proacrosomal vesicles derived from the Golgi complex(es) coalesce to form one acrosomal vesicle [5, 18, 26, 30, 103].

In *H. roretzi*, usually one proacrosomal vesicle appears in a blister-like region under the cell membrane of early spermatids. It becomes larger during spermiogenesis and differentiates into an acrosome [52]. Two vesicles in *Styela plicata* and *Pyura haustor* [40] and in *Diplosoma macdonaldi* [49], three or four vesicles in *Molgula manhattensis* appear in the blister at the anterior region of spermatids and fuse to form an acrosome during spermiogenesis.

In *M. manhattensis*, the acrosome is horseshoe-shaped just after fusion and rounds up during further differentiation. The fully differentiated acrosome is approximately 80 x 80 x 40 nm and is positioned at the apex of the sperm head. The differentiation of the acrosome in *M. manhattensis* is schematically illustrated in Figure 3. At intermediate stages of spermiogenesis in *M. manhattensis*, the vesicle in the blister attach to the inner surface of the blister at essentially the same positional level [43]. This suggests that the inner surface of the blister functions as a scaffold for the vesicles. This area encircles the blister at a definite positional level as a band as shown in Figure 4. During intermediate stages of spermiogenesis, vesicles make contact with the inner surface of this band. This band confines the field of vesicle movement, making it easier for them to recognize each other, and/or this band generates a contractile force which causes the vesicle to move along the inner surface of the band. After contacts are made between these vesicles, they fuse with each other on the inner surface of this band resulting in the formation of a horseshoe-shaped acrosome which transforms into a sphere during subsequent development [43]. In view of the fact that early spermatids have a fairly well developed Golgi apparatus and Golgi derived vesicles, it seems safe to assume that the acrosomal vesicles are derived from Golgi vesicles [40, 43].

In *H. roretzi*, Kubo *et al.* [72] have reported that the acrosomal vesicle is formed from coalescing Golgi derived vesicles during spermiogenesis.

[V] The Acrosome Reaction

In mammalian spermatozoa, an acrosome reaction occurs by the vesiculation resulting in release of lysin(s) [1, 101, 124, 125].

In some marine invertebrates, the acrosome reaction consists of a calcium-dependent exocytotic process followed by the formation of one or more acrosomal process(es) which are enclosed by the inner acrosomal membrane and are responssible for the fusion of the sperm with the egg plasma membrane. Exocytosis occurs through an opening formed by the fusion between the acrosomal outer membrane and the plasma membrane of the sperm enclosing the acrosome [13, 16, 17]. During this process, lysin(s) stored in the acrosome is released to the extracellular space and assists the spermatozoon in moving through the oocyte vestments [59].

Calcium ionophore A23187 is known to induce the acrosome reaction in spermatozoa of various species of animals through the influx of Ca^{2+} [11, 105].

Usui *et al.* [112] suggested that the acrosome reaction induced by calcium ionophore A23187 in *C. intestinalis* spermatozoa was similar to that of mammalian spermatozoa, mainly because it seemed to involve just the exocytosis of acrosomal substance. However, this claim was not as convincing as it could be, because membrane fusion between the acrosome and the overlying plasma membrane was not demonstrated.

Recently, I have shown, using calcium ionophore A23187 on spermatozoa of *C. intestinalis* and *H. roretzi* that the acrosome reaction occurs by fusion

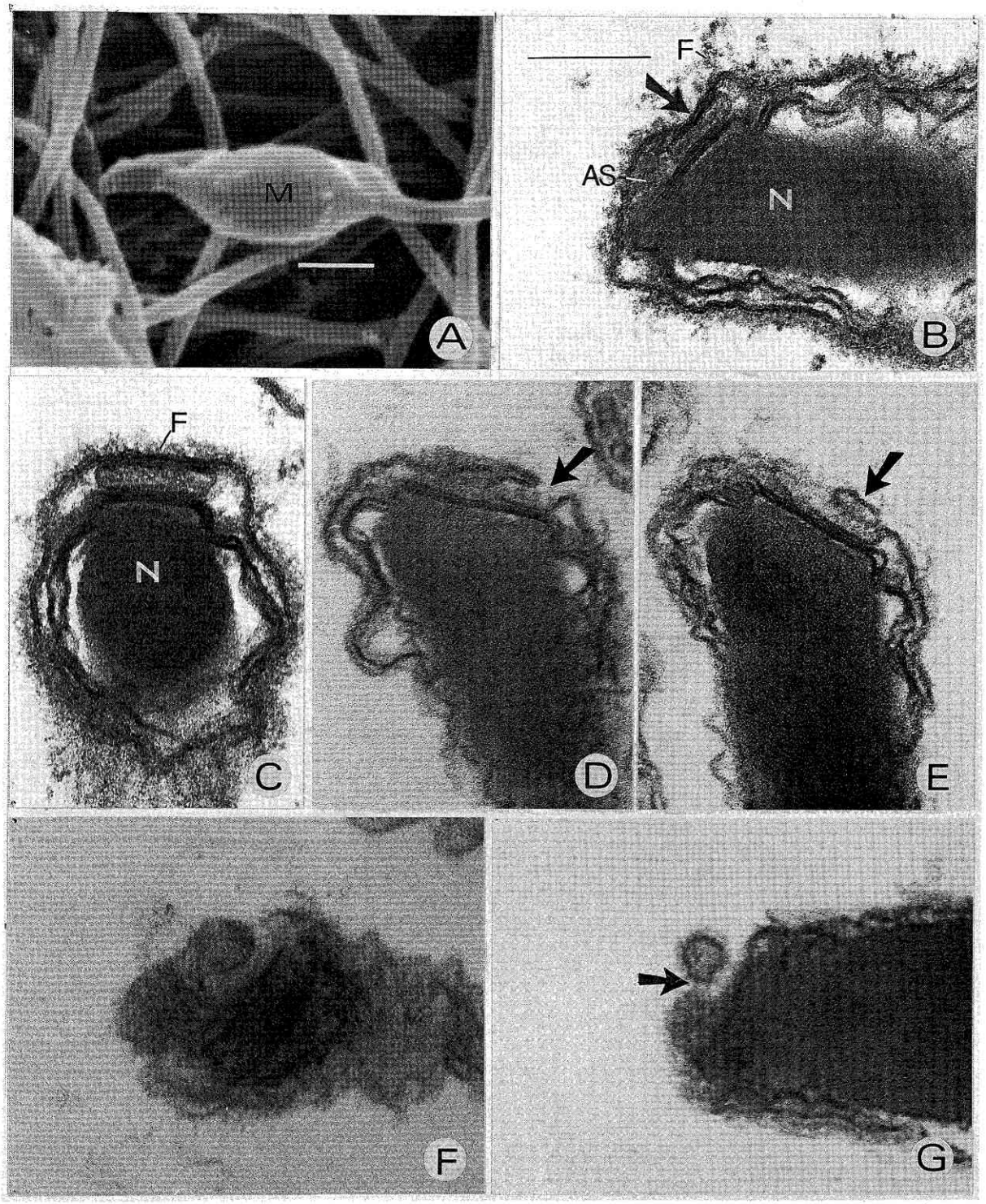

Fig. 5. Acrosome reaction in *C. intestinalis*.
A: Scanning electron microscope image of a *C. intestinalis* spermatozoon. Bar, 1 μm. **B** and **C**: Sagittal and transverse (at the level of the acrosome) sections through the anterior region of the sperm head, respectively. An acrosome (arrow) is present. The acrosomal outer membrane and the overlying plasma membrane are in close contact with each other. An electron-dense plate in the acrosome is obvious in B. Bar, 200 nm (this scale is also applicable to C-G). **D**: Longitudinal slightly oblique section through the apex of the sperm head. Membrane fusion between the acrosomal outer membrane at its peripheral margin and the overlying plasma membrane has occured (arrow). **E**: Longitudinal slightly oblique section through the apex of the sperm head. The hybrid membrane sac made up of the acrosomal outer membrane and the overlying plasma membrane is present (arrow). **F**: Longitudinal slightly oblique section through the apex of the sperm head. Fusion between the acrosomal outer membrane and the overlying plasma membrane seems to occur along the peripheral margin of the acrosome. **G**: Sagittal section through the anterior region of the sperm head. Shedding of a hybrid vesicle bounded by fused membrane between the acrosomal outer membrane and the overlying plasma membrane is probably about to occur. A thin connection between the hybrid vesicle and the anterior tip of the sperm head is still present (arrow). AS, apical substance; F, fuzzy material; M, mitochondrion; N, nucleus. [Fukumoto [51]].

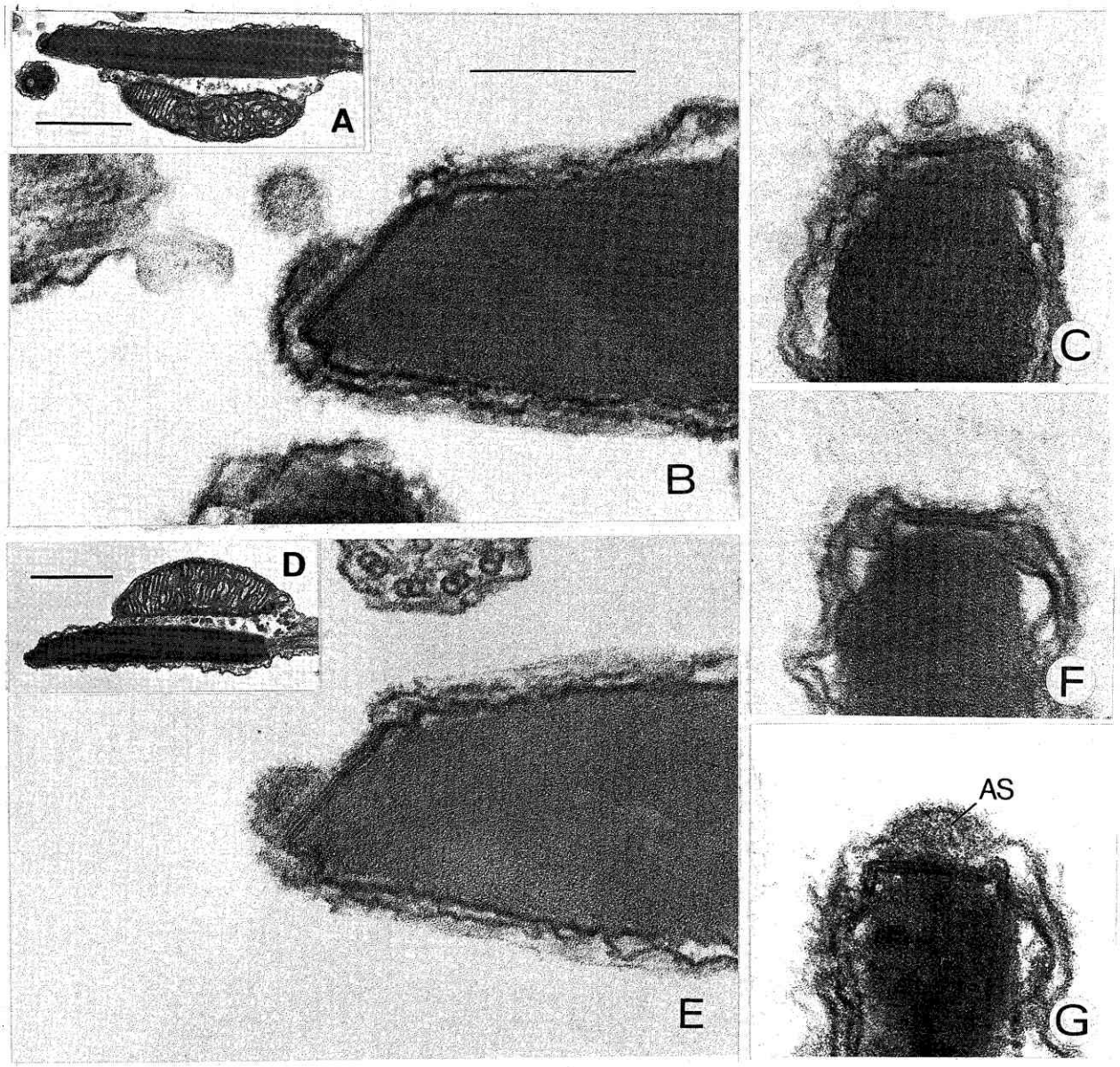

Fig. 6. Acrosome reaction in *C. intestinalis*.
A: Sagittal section through the head. Although the acrosome reaction is almost completed, the mitochondrion is still applied to the nucleus. Bar, 1 μm. **B**: Enlargement of the anterior tip of the head in A. A hybrid vesicle of fused membrane is probably being shed. Bar, 200nm (this is also applicable to C, E-G). **C**: Horizontal section through the anterior region of the head. A hybrid vesicle is seen at the anterior most region. **D**: Sagittal section through the head. The acrosome reaction is completed. Bar, 1 μm. **E**: Enlargement of the anterior region of the head following the acrosome reaction. The acrosome has disappeared from its proper location. **F** and **G**: Serial horizontal section through the head apex, following the completion of the acrosome reaction. AS, apical substance. [Fukumoto [51]].

between the plasma membrane overlying the acrosome and the outer membrane of the acrosome at its peripheral margin [51]. A small flat hybrid sac consisting of the acrosomal outer membrane and the overlying plasma membrane forms at the sperm tip. Membrane fusion proceed along the peripheral margin of the acrosome. The hybrid flat sac transforms into a vesicle that eventually detaches from the sperm tip. The acrosomal inner membrane is exposed and becomes a part of the plasma membrane enclosing the

anterior region of the sperm head. During this process, the acrosomal contents are externalized (Figs. 5 and 6). Figure 7 provides a diagramatic explanation how the acrosome reaction is thought to occur in *C. intestinalis* spermatozoa [51].

A similar acrosome reaction has been induced in *S. plicata* and *M. manhattensis* spermatozoa (Fukumoto, unpublished). These facts suggest that this type of acrosome reaction is not confined only to *C. intestinalis* and *H. roretzi*. The acrosome reaction described here has been actually observed in the perivitelline space during normal fertilization of *H. roretzi* [53]. ***This is a new type of acrosome reaction which has not been described previously*** [53].

Caffeine induces the same morphological changes as the calcium ionophore A23187 in the acrosome of *C. intestinalis* spermatozoa [47]. Caffeine probably operates by causing Ca^{2+} release from internal stores [123].

Two claims have been made about an acrosome reaction; (1) The acrosome reaction consists of the break down of the plasma membrane at the tip of the head, the opening of the acrosomal vesicle, blebbing of the acrosomal inner membrane and the formation of tubules which make contact with the fibrillar network of the chorion via species-specific binding molecule on the chorion.[15, 20, 23, 83, 86, 89, 92]. (2) The acrosome reaction is triggered *in vitro* by a fucosyl-containing glycoprotein extracted from the chorion [21] and from the ovary [22]. These claims are unreliable, because they were never backed up by convincing pictures documenting the acrosome reaction.

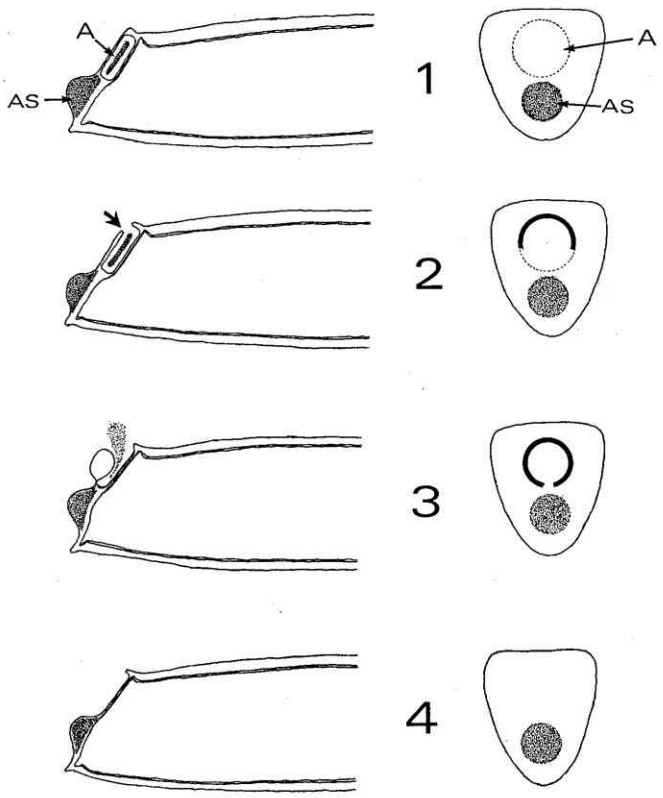

Fig. 7. A possible explanation of the morphological changes during the acrosome reaction in *C. intestinalis* spermatozoa. Left: sagittal views. Right: frontal views. **1**: Left, an intact acrosome is present at the apex of the head. The acrosomal outer membrane is tightly apposed to the overlying plasma membrane. Apical substance (AS) is located at the anterior-most tip of the head. Right, the peripheral margin of the acrosome is indicated by the dotted line. **2**: Left, the acrosome reaction occurs through the fusion between the acrosomal outer membrane at its peripheral margin and the overlying plasma membrane, resulting in the formation of hybrid membrane sac. Right, a solid line represents the region where membrane fusion between the acrosomal outer membrane and the overlying plasma membrane is occuring (arrow). **3**: Left, membrane fusion proceeds along the peripheral margin of the acrosome, resulting in the formation of small hybrid vesicle. During this process, the acrosomal contents might be released. A thin connection at which membrane fusion does not occur yet temporally remains between the hybrid vesicle and the plasma membrane overlying the acrosome. Right, the membrane fusion along the peripheral margin is almost completed except for a small region where thin connection is temporally present. **4**: The hybrid vesicle has come off. The acrosomal inner membrane is exposed and is contiguous with the plasma membrane enclosing the apex. [Fukumoto [51]].

[VI] Fertilization in Ascidians

The ascidian egg is enclosed by a relatively thick and tough chorion (vitelline coat) which is covered by a single layer of vacuolated follicle cells. After passage through the space between the follicle cells, spermatozoa bind to the chorion in a species-specific manner as a prerequisite step for fertilization [2, 20, 89]. The binding of the spermatozoa to the chorion involves the tip of the sperm head [10, 20, 48, 50, 89, 90] or the fuzzy material on the plasma membrane of the sperm apex [45, 48, 50] and the outer fibrous structures of the chorion.[20, 48] It has been suggested that sperm binding might be mediated by an enzyme-substrate complex established between a sperm surface glycosidase and

corresponding glycoside on the chorion. In *C. intestinalis*, α-L-fucosidase on the sperm surface is thought to interact with fucose residues on the chorion [67]. These fucose-containing glycoproteins which act as sperm receptors on the chorion are synthesized by oocyte itself, but not by test cells and follicle cells [91]. In *P. mammillata* [56, 61, 63], in *A. callosa, A. paratropa* and *A. ceratodes* [73, 74], *N*-acetylglucosamidase on the sperm corresponds to *N*-acetylglucosamine residues on the chorion. Recently, Takizawa *et al.* [104] have shown that a sperm chymotrypsin-like protease, most probably the chymotrypsin-like protease in the proteasome (multicatalytic protease complex) plays a key role in binding sperm to the chorion in *H. roretzi*. Furthermore, it has been proposed that β-D-*N*-acetylglucosamidase (GlcNAcase) inhibitor from the vitelline coat (chorion) functions as a receptor for sperm binding [80].

In self-sterile ascidians, a self-nonself recognition site is supposed to be associated with sperm receptors on the chorion. Kawamura *et al.* [70] have proposed that an acid seawater extract of *C. intestinalis* eggs contains a glucose-enriched nonspecific inhibitor of sperm-egg binding, which could be the primary effector of self-incompatibility, and glutamine-enriched modulators, which serve as receptors of allo-sperm. The cooperative interaction of these components may be responsible for the diversity of allo-recognition in *Ciona* gametes. Self-nonself recognition activity first appears in the chorion during late stage of oogenesis in *C. intestinalis* [24] and in *H. roretzi* [37].

Follicle cells are thought to play multiple roles in fertilization. They have been implicated in sperm chemoattractant production in *C. intestinalis* [81]. They are necessary for the sperm penetration through the chorion in *H. roretzi* [35, 36]. They facilitates sperm-chorion interactions in *A. nigra* [76], *P. mammillata* [63] and in *C. intestinalis* [69]. They play a role in the interspecific block to fertilization in *Ascidia aspersa* and *A. malaca* [84, 85, 119]. There is also evidence that follicle cells might participate in the establishment of the self-nonself recognition activity in the chorion in both *C. intestinalis* [24] and *H. roretzi* [37].

Test cells are located in the perivitelline space between the egg surface and the chorion. Any role of these cells in fertilization is still enigmatic.

With the electron microscope, it is difficult to detect morphological changes associated with gamete fusion , especially, in spermatozoa during the process of fertilization, because of the difficulty of getting polyspermy, even when inseminating with a high concentration of sperm. It has been found that polyspermy can be induced in *C. intestinalis* and *P. nigra*, when caffeine- or theophylline-treated eggs are inseminated by fairly high concentrations of spermatozoa [45, 48, 50].

Recently, in *H, roretzi*, the pH of the sperm suspension was increased to pH 9.4 by the addition of 0.1N NaOH, causing sperm to move vigorously [35]. Once activated, they continue to move vigorously even after the pH was readjusted to 8.0. When eggs were inseminated with a high concentration of these activated spermatozoa (approximately 10^8 sperm/ml), about 100% of eggs were polyspermic (Fukumoto, unpublished), resulting in the formation of a multipolar first cleavage. This procedure has provided us with useful material for observing how gamete fusion occurs and any structural changes in ascidian spermatozoa prior to gamete fusion.

A number of studies which used specific inhibitors for trypsin and for chymotrypsin have revealed that *H. roretzi* spermatozoa have three kinds of lysins; two trypsin-like enzymes, spermosin and ascidian acrosin, and one chymotrypsin-like enzyme, sperm proteasome [66, 93, 94, 95, 96, 97, 99, 126] and that *C. intestinalis* spermatozoa have chymotrypsin-like enzymes involved in sperm penetration through the chorion [79, 87].

There have been several papers which insist that chorion (vitelline coat) lysin(s) are stored in an acrosome(s) and are released via an acrosome reaction on the chorion for assisting the passage of sperm through the chorion in *C. intestinalis* [15, 20, 83, 92].

In *H. roretzi*, spermatozoa can pass through the chorion with an intact acrosome, leaving a distinct hole in the chrorion [53]. It is reasonable to presume that in this species the chorion lysins are nondiffusible and located on the surface of the sperm head. This assumption is also suported by the recent findings of a sperm surface chymotrypsin-like enzyme in *Ascidia ceratodes, A. callosa* and *A. paratropa* [71].

The idea that lysins are present on the surface of the sperm head was originally proposed by Woollacott [121]. He was able to extract proteases (lysins) from the

sperm of *C. intestinalis*. He suggested that these lysins were closely associated with the ridge-like surface ornamentation on the sperm head, mainly because he tried to find an acrosome but failed to detect one. Hoshi *et al.*[66] suggested that lysins from *H. roretzi* were ectoenzymes, because the inhibitors used for both trypsin and chymotrypsin were membrane-impermeable proteins. Subsequently, Hoshi [64,65] suggested that at least acrosin-like enzyme was stored in an acrosome as a chorion lysin in *H. roretzi* spermatozoa.

Because of the small size of the acrosome and the paucity of its contents, it is unlikely that the acrosome in ascidians contains a significant amount of chorion lysin(s). On the other hand, a well-developed Golgi apparatus and many Golgi vesicles of various sizes are found in the cytoplasm of spermatids in both *P. haustor* and *S. plicata*. It was hypothesized that ***"the ascidian spermatozoa contain a poorly developed acrosome which might react at an appropriate step in fertilization and participate mainly in the fusion of gamete plasma membranes and that the chorion lysin(s) are intercalated into the plasmalemma enclosing the sperm head"*** [40].

In this context, we should pay attention to the fact that the plasma membrane enclosing the sperm head is externally decorated by a fuzzy extracellular material or surface ornamentation [40, 41, 43, 44, 45, 54, 121]. It is the fuzzy extracellular material at the apex of spermatozoa, which first makes contact with the chorion at fertilization [42, 45, 48]. In *C. intestinalis* spermatozoa, the region responsible for the binding to the chorion is a Con A binding site which is exclusively restricted to the plasma membrane at the tip of the sperm head [9, 10, 20, 89]. This suggests that the fuzzy material at the tip of the sperm head is chemically different from that ornamenting other part of the head. In *Perophora annectens* spermatozoa, the fuzzy extracellular material is restricted exclusively to the plasma membrane enclosing the anterior quarter of the apical structure (Fig. 8). This coincides with the region where the fragmented proacrosomal vesicles probably were incorporated during the process of spermiogenesis [41]. It has been proposed that the fuzzy extracellular material (surface ornamentation) of the sperm head in ascidians is the site where the lysin(s) are found and that it plays an important role in

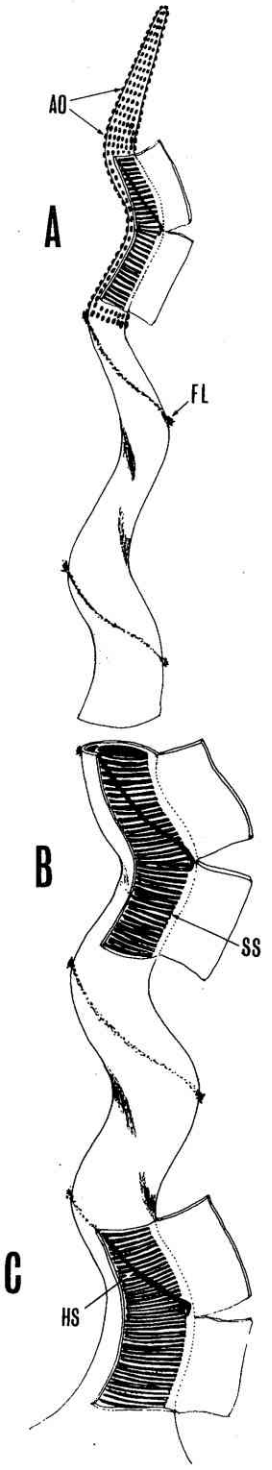

Fig. 8. Schematic illustration of the apical structure in *Perophora annectens*. The plasma membrane enclosing the anterior quarter of the apical structure is decorated by the anterior ornaments (AO). A helical string (HS) can be observed running inside along the ridge of the helix. Fluff (FL) is present on the plasma membrane just outside the region corresponding to the helical string. A, B and C are views inside the anterior, middle and posterior regions, respectively. [Fukumoto [41]].

sperm-chorion interactions at fertilization [39, 40, 44, 46].

In the perivitelline space, several processes protrude from the apex of the acrosome-reacted sperm head in normal and polyspermic fertilization [45, 48, 50, 53]. These processes have never been observed at the apex of spermatozoa in which acrosome reaction has been experimentally induced by caffeine [47] or by calcium ionophore A23187 [51]. It seems safe to assume that the factor(s) that elicit(s) the acrosome reaction and process formation exist(s) in the perivitelline space of *C. intestinalis* [45, 46] and *H. roretzi* [53]. In *C. intestinalis*, these processes (up to 10 in number) typically are about 100 nm in length and 40 nm in diameter [45, 48].

In some marine invertebrates, the plasma membrane enclosing the acrosomal process(es) are derived from the acrosomal inner membrane [13]. However, it is not clear that the plasma membrane enclosing the process(es) in ascidians is derived from the acrosomal inner membrane, because many process(es) protrude from the peripheral margin at the anterior tip of the sperm head which is a fair distance from the location of the acrosome. For this reason, these process(es) have been designated as "***apical process***" [45, 48]. Figure 9 shows the apical processes of *Ciona intestinalis* spermatozoa. Gamete fusion between the sperm and the egg plasma membrane occurs by means of the apical process(es), resulting in the incorporation of the spermatozoon into the egg cytoplasm begining at the anterior tip of the sperm head [48, 50, 53]. This suggests that ***the apical process is functionally homologous to the acrosomal process of some other marine invertebrates*** [45, 48]. Xie and Honegger [122] reported that these processes were not observed in ultrastructural studies on fertilization in *P. mammillata* and *B. villosa*. They claimed that sperm possesing apical processes and the mode of sperm-egg fusion observed in *C. intestinalis* were not widespread within ascidians and reflected secondary modifications of sperm structure. However, the occurence of apical processes and their membrane fusion with egg plasma membrane in *H. roretzi* and in *P. nigra* suggests that this type of fertilization is not a special variant confined only to *C. intestinalis* [53].

In some marine invertebrates, the formation of an acrosomal process is due to the rapid polymerization of actin molecules which are stored as a subacrosomal or periacrosomal substances [13, 17, 102, 107] or by rapid

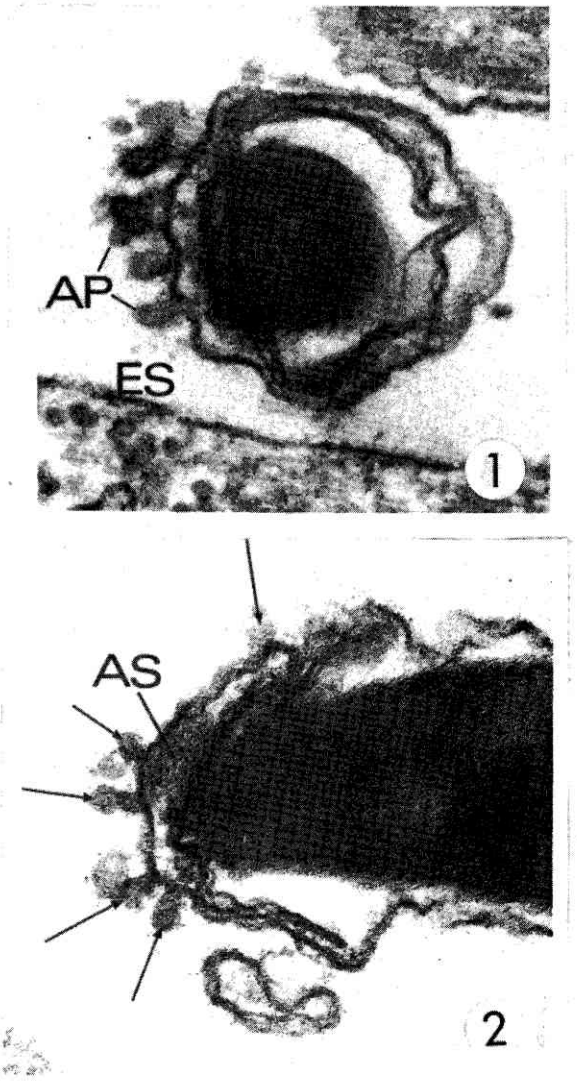

Fig.9.A, Transverse section through the apex of a spermatozoon in the perivitelline space. Apical processes protrude from the apex of the head. Connections are recognized between the base of apical processes and the unclear membranes.
B, Sagittal, slightly oblique, section through the apex of a spermatozoon in the perivitelline space. Apical processes (arrows) protrude from a region which is fairly distant from the place where the acrosome was.
AP, apical process ; AP, apical substance. ; ES, egg surface. Bar : 200 nm.

projection of previously polymerized actin filaments [19, 106, 108]. With respect to apical process formation, it is worth mentioning that actin is probably present in the apex of the sperm head in *B. villosa* and *Cnemidocarpa finmarkiensis* [77], although negative results have been obtained in *A. ceratodes* [77] and in *P. mammillata* [62, 63]. In *C. intestinalis* spermatozoa, an accumulation of

electron-dense granular material, about 5-7 nm in diameter, is observed at the anterior-most tip of the head, which is referred to as ***"apical substance"***[45]. Such an accumulation of electron-dense material was first reported at the apex of the differentiated spermatozoa in *A. callosa*, where it was assumed to correspond to the periacrosomal substance found in some animal species [12]. However, the apical substance in *C. intestinalis* still remains in its proper location after the acrosome reaction and apical process formation [45, 48]. Further morphological and immunohistochemical studies are needed to clarify the precise role of the apical substance.

In *P. mammillata*, several vesicles (up to eight) have been found at the apex of the sperm head [62, 63]. It has been suggested that some of these vesicles fuse with the overlying plasma membrane and release their contents prior to passage of the sperm through the

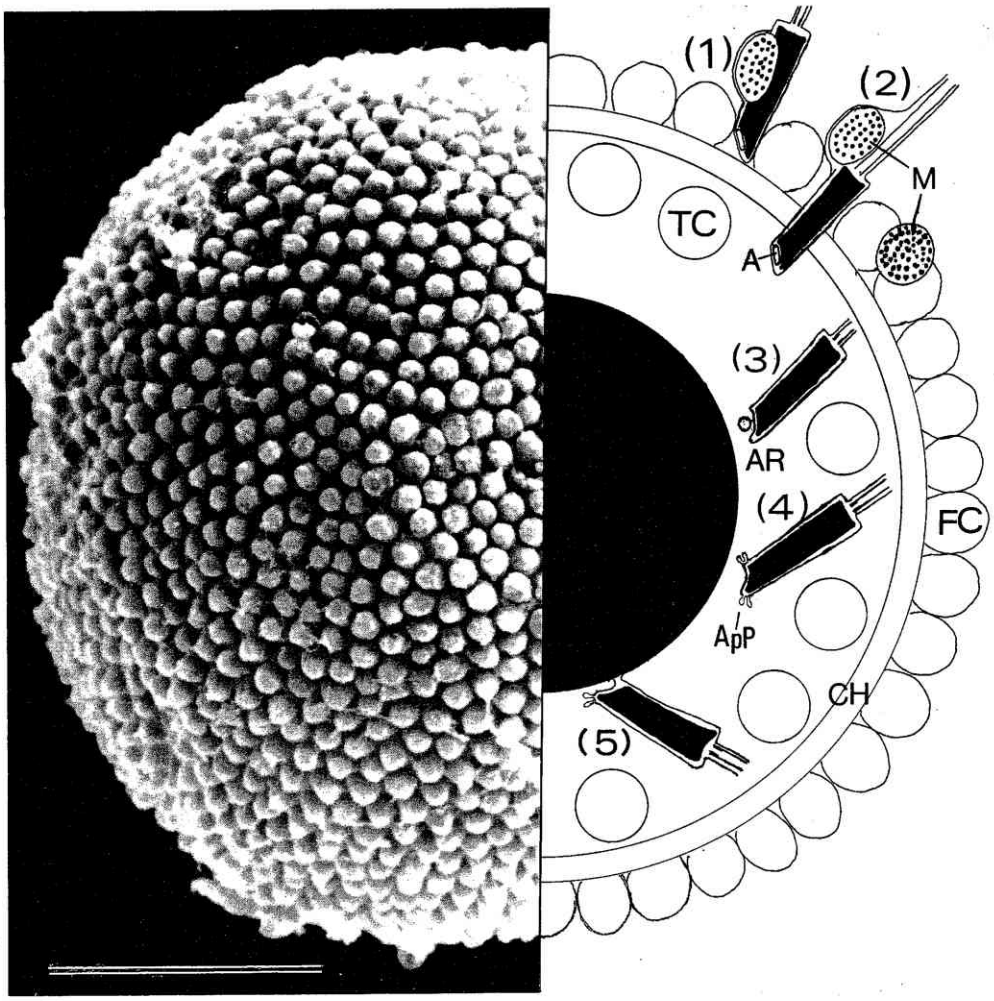

Fig. 10. A half SEM (bar, 50 μm) and half-drawing representing the morphological steps of fertilization in *H. roretzi*. The egg is enclosed by a chorion (CH). A single layer of highly vacuolated follicle cells (FC) adheres to the outside of the chorion. Test cells (TC) are located in the perivitelline space. (1) The spermatozoon binds to the surface of the chorion by the anterior tip of its head. (2) The spermatozoon passes through the chorion with an intact acrosome (A) while its mitochondrion (M) is trapped by the stick-like surface ornamentation which decorates the outer surface of the follicle cells. (3) The acrosome reaction (AR) occurs in the perivitelline space. (4) In the perivitelline space, apical proceses (ApP) protrude only from the apex of the acrosome -reacted sperm head. (5) Gamete fusion takes place between some of the apical processes and egg plasma membrane, resulting in the incorporation of the sperm into the egg from the anterior tip of its head. [Fukumoto and Numakunai [53]].

chorion and their remains could be recognized in the spermatozoa in the perivitelline space. Honegger [63] speculated that these vesicles contained chorion lysin(s) and/or additional enzymes that participated in the process of sperm-egg fusion. It has been suggested that sperm-egg fusion in *P. mammillata* occurs between the plasma membrane of the post-acrosomal region of the sperm head and the egg membrane, as observed in mammalian fertilization [62,63]. In *C. intestinalis* and *H. roretzi*, in contrast to the findings in *P. mammillata*, the acrosome reaction occurs via "fenestration" or "vesiculation" and gamete fusion takes place between the apical processes of the sperm head and egg plasma membrane, resulting in the incorporation of the spermatozoa into the egg from the anterior tip of the sperm head, in the same way that it does in other marine invertebrates [45,48]. In *B. villosa* and *P. mammillata*, Xie and Honegger [122] insisted that the apical vesicles might play a role in sperm-egg fusion in addition to their role in vitelline coat (chorion) penetration, because of their persistence in late stages of vitelline coat (chorion) penetration. Unfortunately, their photographs are not convincing, because they don't show an acrosomal vesicle during passage of sperm through the chorion.

It has been suggested that mitochondrion is left outside the chorion during fertilization [28,111]. In *A. ceratodes* spermatozoa, Lambert and Epel [75] found that mitochondria translocated along the tail during fertilization and also in vitro. It has been proposed that sperm first bound to the chorion by their apex and then by their mitochondria by means of the plasma membrane overlying the mitochondria so that the energy generated by mitochondrial translocation along the tail would drive the sperm through the perivitelline space to the egg surface [71,78]. Lambert [74] showed that *N*-acetylglucosaminidase activity was originally located at the tip of the sperm head but subsequently remained with the surface of the plasma membrane overlying the mitochondrion during translocation in *Ascidia paratropa*. In *P. mammillata*, the localization of this enzyme not only at the sperm tip but also on the sperm membrane overlying the mitochondrion was confirmed histochemically at the EM level [55]. However, Xie and Honegger [122] never found the mitochondrion anchored to the chorion at early stages of sperm penetration in *B. villosa* and *P. mammillata*. We also never found mitochondria anchored to the chorion at any time during sperm penetration through the chorion and passage through the perivitelline space in *H. roretzi* [53]. In this context, it is of particular interest that stick-like structures decorate the outer surface of the follicle cells. High-magnification EM pictures suggest that the mitochondrion may be trapped by these structures. I propose that the follicle cells instead of the chorion anchor the mitochondrion by means of stick-like structures, at least in *H. roretzi* [53]. This assumption would explain why follicle cells seemed to be completely necessary for fertilization in *H. roretzi* [35,36]. Surface ornamentation of follicle cells was first reported by Villa and Ptricolo [119] and Patricolo and Villa [84,85]. They suggested that the honeycomb-shaped structures on these cells played a role in preventing interspecific fertilization.

Figure 10 is a diagramatic representation explaining the morphological aspects of fertilization in *H. roretzi* [53].

[Ⅶ] Block to Polyspermy

Lambert [74] has reported that the eggs of *A. callosa*, *A. ceratodes*, *A. nigra* and *P. mammillata* release *N*-acetylglucosaminidase into the seawater at fertilization. This enzyme has been shown to modify GlcNAc residues on the chorion (VC), which causes a rapid decline in the number of sperm binding to the chorion, resulting in a block to polyspermy. The enzyme activity is found in the supernatant SW by 15s after fertilization, which suggests that it is stored very near the egg surface [74]. In mammals [58] and sea urchins [113,114], the egg releases proteases from cortical granules which result in the loss of sperm binding. Similarly, amphibian eggs release a glycosidase from cortical granules which results in depressed sperm binding [57]. In *H. roretzi*, successful fertilization can be recognized by the expansion of the chorion. This expansion in normal conditions seems to be caused by a trypsin-like enzyme released from the egg at fertilization [66,98]. The fact that this elevation was induced by treatment with calcium ionophore suggests that calcium-dependent exocytosis might occur at the surface of the egg. After elevation of the chorion by calcium ionophore, fertilization becomes impossible

[127]. The elevation of the chorion might be one of the mechanisms for blocking polyspermy. It is not yet known whether this enzyme is located in the putative cortical granules in *H. roretzi* eggs, which exocytose after fertilization [53]. In *C. intestinalis* eggs, it has been proposed that subcortical granules are released after fertilization [88].

[Ⅷ] Fertilization in Compound Ascidians

Although several papers have been published describing spermiogenesis and mature sperm of compound ascidians with internal fertilization [6, 34, 39, 41, 109, 110], studies on the fertilization have not been done mainly because technical and physiological difficulties.

Recently, two interesting papers on the internal fertilization in *Diplosoma listelianum* have been published. Prior to fertilization, the sperm transform dramatically in shape. In non-modified sperm, a groove runs spirally all along the head, while in those in the fertilization canal, it coils in the anterior half of the head, pressing back the long mitochondrion and endoplasmic tubules [7]. At fertilization, the sperm head is incorporated into the oocyte by a process recalling phagocytosis, with the formation of an engulfing pocket. Fusion of plasma membranes takes place immediately after gametes contact. Expulsion of numerous cortical granules was observed in the egg penetrated by the sperm [8]. It has been demonstrated that exogenous sperm can be stored in the lumen of the ovary in each zooid of *D. listelianum*, making heterologous fertilization possible [4].

[Ⅸ] Some Speculations on Ascidian Fertilization

In *H. roretzi*, the acrosome reaction occurs in the perivitelline space after the passage through the chorion with an intact acrosome [53]. It is reasonable to presume that acrosome reaction inducing factor(s) might exist in the viscous fluid contained in the perivitelline space. The acrosome reaction occurs on the surface of the chorion or after passage through the chorion in the perivitelline space in *C. intestinalis* [48] and during passage through the chorion in *P. nigra* [50]. These discrepancies in the site where the acrosome reaction takes place during fertilization in different species of ascidians might reflect some secondary modifications of the chorion. Among these species, *C. intestinalis* has the thinnest chorion (about 100 nm thick) which consists of loosely wound fibers [20, 45, 48] so that the acrosome reaction inducing factor(s) in the perivitelline space can diffuse out through the chorion and elicit the acrosome reaction on the outer surface of the chorion in some cases. In *P. nigra*, the chorion is the thickest (about 6 μm thick) but composed of the thinnest fibers of the lowest density among these three species and reaches to the egg surface in some places, which makes the perivitelline space very narrow. In this situation, acrosome reaction inducing factor(s) might exist not only in the perivitelline space but also might reside in the chorion, which induces the acrosome reaction in spermatozoa during passage through the chorion [50]. The chorion of *H. roretzi* has the thickest fibers. The integrity of the chorion (about 2 μm thick) may keep the acrosome reaction inducing factor(s) exclusively in the perivitelline space [53].

The assumption that factor(s) which induces the acrosome reaction and/or elicits apical process formation exist in the viscous fluid of the perivitelline space may explain the following puzzling observations. In *H. roretzi*, fertilization of intact eggs occurs within a few minutes after insemination and first cleavage takes place synchronously within 2 hr. However, naked eggs dechorionated by needles cleaved asynchronously at various times up to 4 hr after insemination [66]. In *C. intestinalis*, Monroy and Rosati [83] firmly believed that the acrosome reaction occurred on the chorion only when precisely "molecular match" existed between the receptors on the chorion and those at the sperm head. As dechorionated eggs could be fertilized, they could not explain how sperm-egg fusion occurred in the absence of acrosome reaction. The acrosome reaction and apical process formation might occur in the perivitelline viscous fluid remaining around the dechorionated eggs, making fertilization possible. The asynchrony in cleavage initiation might be explained by differences in the amount of the perivitelline viscous fluid remaining around individual eggs after dechorionation by the needles. If the quantity of remaining fluid surrounding the egg is small, factor(s) in the fluid might be less effective in inducing an acrosome reaction and apical process formation which would delay fertilization resulting in

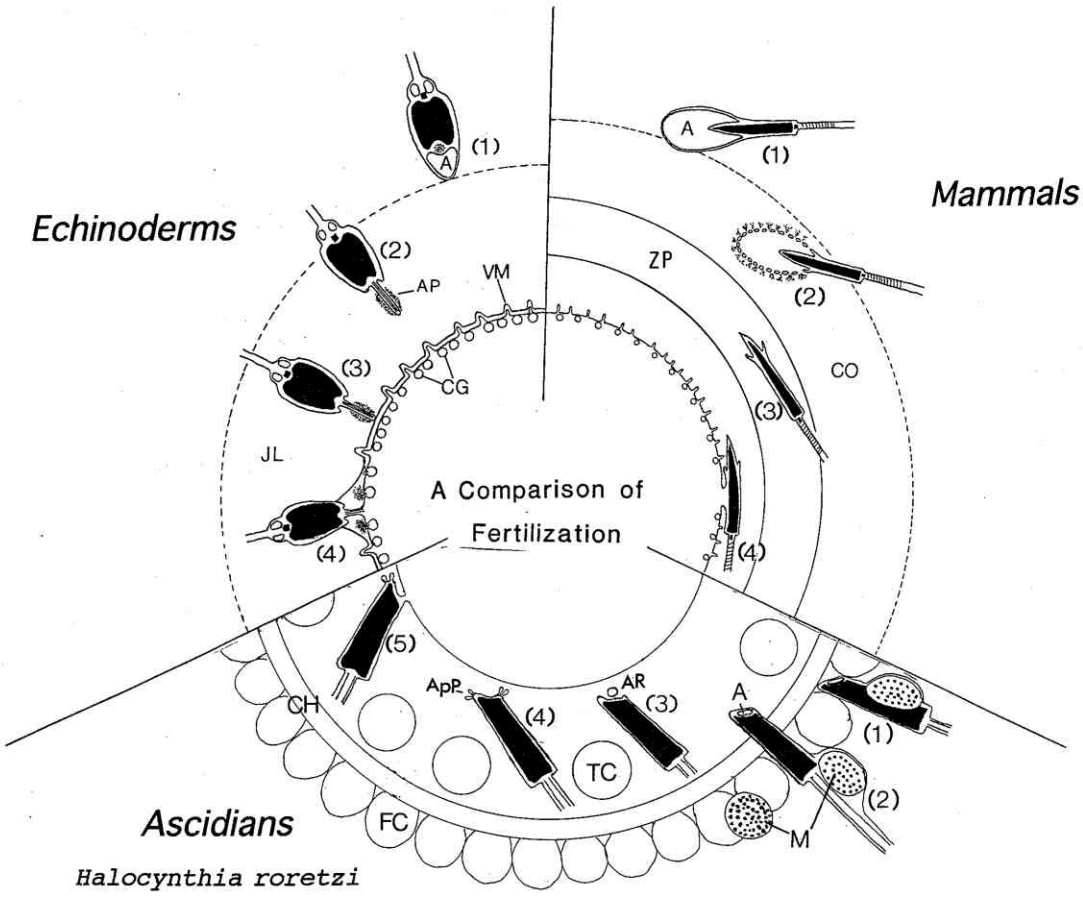

Fig. 11. Diagrammatic representation of a comparison of the sequential events during ascidian fertilization with those of mammals and echinoderms.

Mammals:

(1) A spermatozoon attaches to the cumulus oopholus (CO) which consists of several layers of cells. A, acrosome.

(2) A spermatozoon binds to the zona pelucida (ZP) in which ZP3 functions as a sperm receptor and induces an acrosome reaction. The acrosome reaction occurs via vesiculation.

(3) The spermatozoon passes through the zona pelucida (ZP) with the assistance of lysin (s) associated with the surface of the inner acrosomal membrane.

(4) Gamete fusion occurs between the membrane of the subacrosomal region and the egg plasma membrane.

Echinoderms:

(1) A spermatozoon attaches to the surface of the jelly layer (JL). A,acrosome.

(2) An acrosome reaction occurs via exocytosis. Acrosomal process (AP) enclosed by the inner acrosomal membrane protrudes out following the polymerization of subacrosomal substance (actin). Some lysin (s) and bindin are exposed and associated with the acrosomal process.

(3) An acrosomal process binds to the vitelline membrane (VM) via bindin. The vitelline membrane may be dissolved by the lysin.

(4) Gamete fusion occurs between the membrane of the acrosomal process at the tip and the egg plasma membrane. Cortical granules (CG) exocytose their contents into the space between vitelline membrane and the egg surface, resulting in the formation of a perivitelline space and a fertilization envelope.

Ascidians:

(1) The spermatozoon binds to the surface of the chorion (CH) by the anterior tip of its head.

(2) The spermatozoon passes through the chorion with an intact acrosome (A), while its mitochondrion (M) is trapped by the stick-like surface ornamentation which decorates the outer surface of the follicle cells.

(3) The acrosome reaction (AR) occurs in the perivitelline space.

(4) In the perivitelline space, apical processes (ApP) protrude only from the apex of the acrosome-reacted sperm head.

(5) Gamete fusion takes place between some of the apical processes and egg plasma membrane, resulting in the incorporation of the sperm into the egg from the anterior tip of its head.

asynchronous cleavage.

In dechorionated "naked eggs", interspecific fertilization is successful in most crosses between many ascidian species belonging even to different families [82]. This suggests that the factor(s) inducing the acrosome reaction and/or apical process formation in the perivetelline space might be common and "non-species specific".

Although the chemical nature and the precise role of the acrosomal substance remain to be elucidated, the fact that the acrosome reaction occurs in the perivitelline space and releases a relatively small amount of acrosome substance leads us to the working hypothesis that this substance might be enzyme responsible for membrane fusion between the apical processes and the egg plasma membrane in ascidians. In this context, it is of particular interest that a metalloendoprotease might induce membrane fusion between sperm and egg plasma membrane in *C. intestinalis* [25].

[X] Comparison of Events during Ascidian Fertilization with Those of Mammals and Echinoderms

Among animals, biochemical and morphological studies on the fertilization have been made intensively in mammals [120, 124, 125] and echinoderms [27, 113, 114]. We can compare the events during ascidian fertilization [48, 50, 53] with those of mammals and echinoderms. This will make the differencies and the similarities of fertilization of these animals clear. Figure 11 shows a diagramatic representation of the events associated with fertilization of these animals.

The acrosome reaction in *C. intestinalis* and *H. roretzi* occurs through vesiculation or fenestration without the formation of acrosomal process, in fundamentally the same way that has been observed in mammalian spermatozoa [47, 51]

In echinoderms, the acrosome reaction occurs via exocytosis of acrosomal substance follwed by the formation of acrosomal process which is responsible for the fusion of the sperm with the egg plasmalemma [16, 17, 18, 102, 103] . In *C. intestinalis* and *H. roretzi*, the apical processes protrude mainly from the peripheral region of the apex of the sperm head in the perivitelline space [45, 48, 53] . These processes are instrumental in gamete fusion between the sperm and the egg plasmalemma, resulting in the incorporation of the sperm into the egg at the anterior tip of its head [48, 53]. As the apical processes protrude from a region which is fairly distant from the proper location of the acrosom, they are analogous to but functionally homologous to the acrosomal processe of echinoderms. In this respect, gamete fusion between spermatozoa and eggs of ascidians at least with external fertilization takes place in the same way that it does in echinoderms.

It has been proposed that fertilization in ascidians has characteristics of both mammals and marine invertebrates [47].

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