Electron Microscopical Studies on Reproductive Cells of Plants II.
Structure of Spermatozoids of *Marchantia polymorpha* and
***Isoetes japonica*¹**

by

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Recent studies on human and animal spermatozoa by means of the electron microscope have been carried on by several workers. The first work on human spermatozoa was that of Seymour and Benmosche (1941), followed by Baylor, Nalbandov and Clark (1943) on chick and bull spermatozoa; Bretschneider (1947) on bull spermatozoa; and Imagawa (1949) and Yasuzumi (1950) on human, bull and goat spermatozoa. These investigations threw some light upon the ultra-structure of spermatozoa which was not disclosed by means of the light microscope.

There are, however, no reports yet on studies of spermatozoids of plants by means of the electron microscope. In plants, some species in Chlorophyceae and Phaeophyceae, and all of Charophyta, Bryophyta, Pteridophyta, Cycadaceae and Ginkgoaceae are sexually reproduced by means of eggs and spermatozoids. The structure of these spermatozoids shows various types according to the species. The spermatozoids offer several problems in transporting the male genes during the reproductive process of the above indicated groups. Hitherto, there were many works on the spermatozoids of Bryophyta and Pteridophyta by means of the light microscope, but there were no observations reported on their ultra-structure.

We have recently reported upon the ultra-structure of the spermatozoids of *Marchantia polymorpha* as studied by means of the electron microscope (Sinotô, Yuasa and Satô 1949, Satô 1950). In this paper, details of the former results together with the new data of observation obtained on the spermatozoids of *Isoetes japonica* will be described.

Materials and Methods

The HU-4 type electron microscope of the Hitachi Company was used. The electron micrographs in this paper were taken by Dr. S. Okada. The spermatozoids of *Marchantia polymorpha* were placed directly on the collodion membrane supported by the single hole specimen

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holder, and in case of *Isoetes japonica* by the copper mesh holder. The spermatozoid samples of *Marchantia polymorpha* were obtained from the antheridium of male stock collected at Nakano in Tôkyô from July to September 1949, and those of *Isoetes japonica* drawn from the mature microspores collected in a stream beside the Syakuzii Pond in Tôkyô. The collodion membrane was produced in 45°C water by a microdrop of a dilute solution of collodion in amyl acetate (1.5 per cent).

The specimen was prepared as follows:

Spermatozoids of *Marchantia polymorpha*.

1. A drop of distilled water is allowed to fall on the male receptacle, with the result that many groups of spermatozoids rise up from the antheridia within this drop. Then from this drop a microdrop is drawn up by a pipette, and is placed on a slide glass. A microdrop of suspension is placed on the collodion membrane with a platin loop under the light microscope (unfixed specimen).

At the same time, a specimen fixed with the vapour of a 2 per cent solution of osmic acid for a short time is also used (fixed specimen).

2. The specimen is then dried in the air.

3. The dried specimen is examined under the light microscope.

4. When the specimen is suitable for further study, it is washed by the distilled water, and dried in the air again.

5. Lastly a shadow-cast with chromium after Williams and Wyckoff (1946) was used.

A second method was also used.

The specimen was treated with 0.05 per cent solution of pepsin. Hydrochloric acid was used as a buffer, and the pH was adjusted at about 3.0. The temperature was maintained at 37°C.

Spermatozoids of *Isoetes japonica*.

1. A drop of suspension of the microspores in which the spermatogenesis had been completed, is placed upon a slide glass, and coal-gas is blown on it and then a great quantity of spermatozoids are at once drawn off (Fujii (1925), Wada (1930)).

2. A microdrop of the suspension of the spermatozoids is transferred to a collodion membrane by a micropipette and allowed to dry in the air (unfixed specimen).

At the same time, the fixed specimen is prepared in the same way as used in *Marchantia polymorpha* (fixed specimen).

3. The dry specimen is selected under the light microscope, and a suitable specimen for study is washed in distilled water to remove excess debris, and then was dried in the air.

4. The other specimen was fixed with 2 per cent osmic acid solution after ammonium vapour was applied for several seconds.

Figs. 1-8. Electron micrographs of spermatozooids of *Marchantia polymorpha*. Osmic acid fixation and shadow-cast with chromium except Fig. 6. 1. Spermatozoid consisting of body (composed of blepharoplast, spermatozoid-nucleus and plasmic region) and two flagella. $\times 5,000$. 2. Ends of two flagella. Showing two types, brush-like form and hook-like form. $\times 10,000$. 3. Showing connection at top of blepharoplast and bases of two flagella. Showing plasmic region. $\times 5,000$. 4. End of flagellum. $\times 5,000$. 5. Flagellum consisting of several fine fibrils. $\times 35,000$. 6. Spermatozoid after treatment with 0.05 per cent pepsin in pH 3.0 at 37°C for 25 hours. It appears as a band-like structure in spermatozoid-nucleus. $\times 5,000$. 7. Spermatozoid after treatment with 0.05 per cent pepsin in pH 3.0 at 37°C for 37 hours. Showing spermatozoid-nucleus and flagellum. $\times 4,600$. 8. Enlargement of a portion of the flagellum of Fig. 7. $\times 30,000$.

Results

Spermatozoids of *Marchantia polymorpha*

The spermatozoid of this plant is formed by the body and two flagella attached at the anterior portion which is called the "blepharoplast" (Fig. 1). The end of the flagella as shown in Figs. 1 and 2 is of two types, the brush-like form and the hook-like form. Each flagellum consists of a bundle of several fibrils (Fig. 5). At the end of the flagella, especially in the brush-like region, four fibrils can be seen (Figs. 1 and 2). The diameter of one fibril (in the brush region) lies between 300 Å and 500 Å. In the hook-like region, the fibrils show a network (Figs. 1, 2 and 3). In the hook-like region also, the plasmic substance or thin membrane (Figs. 1 to 4) can be seen, and the semi-transparent and thin membrane such as a sheath at the circumference of the main axis in the flagellum is also seen (Fig. 1).

The body of the spermatozoid consists of the blepharoplast, the spermatozoid-nucleus and the plasmic region, and these parts are too great in density and thickness for penetration by the electron beam (50 KV). In Fig. 3, however, the connection of the flagella and the blepharoplast can be seen; the roots of the two flagella and the top of the blepharoplast are separated. Fig. 7 is the electron micrograph of the specimen which has been treated with 0.05 per cent pepsin in pH 3.0 at 37°C for 37 hours. This shows a difference in the structure between the spermatozoid-nucleus and the blepharoplast which shows conspicuous expansion and discontinuity in some places.

The spermatozoid-nucleus is too thick for the electron beam, because the width is ca. $0.4\ \mu$ at its middle portion, therefore its interior cannot be observed. An analysis of the interior structure of the spermatozoid-nucleus was tried by means of enzyme digestion in some instances, and by a destruction through chemicals in others. The result of these experiments is shown in Fig. 6, the subject of which has been treated with 0.05 per cent pepsin in pH 3.0 at 37°C for 25 hours. In one side of this spermatozoid-nucleus the regular bands are shown, but the other side is covered by a flagellum. It is thought that the outside matrix has been desolved away from the surface of the spermatozoid-nucleus by pepsin and the inside bands (chromonema?) have been exposed. In Fig. 7, it seems that the spermatozoid-nucleus is spiral or is twisted. From Figs. 6 and 7, however, it may not directly be determined whether the structure of spermatozoid-nucleus is a band or spiral in nature.

The middle piece which lies between the spermatozoid-nucleus and the plasmic region was never distinguished from others in any of the electron micrographs. Although those parts possess differences in both

density and thickness, these cannot be observed in the electron micrographs, because it seems that there is no "electron difference" sufficient enough to react to the electron beam. However, in many photographs, the spermatozoids are usually apt to bend at the middle piece, which seems to suggest that there is a peculiarity in their structure. The plasmic region is larger than the spermatozoid-nucleus in width, and appears to show lower density (Fig. 3).

Spermatozoids of *Isoetes japonica*

The body length of the extended spermatozoid of this plant is ca. $33\ \mu$. In the anterior portion of the body, there is a blepharoplast bearing eleven cilia. This spermatozoid like that of *Marchantia poly-*

Fig. 9. Electron micrographs of spermatozoid of *Isoetes japonica*. Cilium consists of many delicate fibrils, some of which are bundled together in a ring-like formation. $\times 2,500$.

morpha is also too large and thick for penetration of the electron beam. However, by the electron micrographs several results worthy to note were obtained.

Each cilium consists of many fibrils (about twenty in number) which

Figs. 10-13. Electron micrographs of spermatozoid of *Isoetes japonica*. 10, Plasmic portions like fins running along the main axis. Unfixed specimen. $\times 2,700$. 11, Nuclear portion and plasmic portion after treatment with ammonium. Expansion occurring and bands observable. $\times 2,200$. 12, Nuclear portion and plasmic portion after treatment with ammonium. Fibrous structure appearing. $\times 3,500$. 13, Nuclear portion and plasmic portion after treatment with ammonium. Expansion and break occurring, and fibrous structure appearing. $\times 3,100$.

are connected with the blepharoplast. Sometimes most of the fibrils forming a bundle are seen separated at their bases, and moreover, a structure like a ring can be seen by which many fibrils are bundled together (Fig. 9). The diameter of one fibril lies between 200 Å and 500 Å.

The blepharoplast is almost triangular, bearing cilium at one side, the other side being the border-brim which shows high density. The connecting-portion between the head portion and the posterior portion is the neck-portion which is somewhat narrow and appears to show lower density than the others (Fig. 9). It is clear in the stained specimen under the light microscope that the nuclear portion runs from the neck-portion as far as the posterior end of the spermatozoid, accompanying the plasmic band. In the electron micrographs the plasmic band could not be distinguished from the nuclear portion, because it was too large in density and thick. However, in the unfixed specimen (Fig. 10), the plasmic portion was observed to resemble fins which run along the main axis in various places. In the specimen treated with ammonium vapour, Yuasa (1940) stated that the nuclear portion was expanded, and that it was the mass of innumerable extended chromonemata under the light microscope. By the same method, the hanging water-drop containing many fresh spermatozoids was exposed with ammonium vapour for several seconds and fixed with 2 per cent osmic acid solution vapour, and was observed by means of the electron microscope. Then the fibrous structure was seen in both nuclear portion and plasmic band. It seems most likely that this structure appeared due to expansion and partial breakage of proteins affected by ammonium vapour. In Figs. 11 and 12, the bands (or spiral structure?) appeared, and in Fig. 12, beside the region of the bands the fibers are seen. Also in Fig. 13, the expansion and the breakage in two places occurred, and elongated fibers can be observed.

Discussion

The studies on the spermatozoids of *Marchantia polymorpha* by means of the light microscope were reported by Ikeno (1903), Lindforss (1905), Miduno (1934) and Yuasa (1941). Ikeno observed two flagella, blepharoplast, spermatozoid-nucleus and plasmic region staining with iodine-green-fuchsin mixture. Miduno reported as follows: in the materials stained with gentian-violet, carbol-fuchsin and Heidenhain's iron-haematoxylin, there is a lightly stained globular vacuole, which contains deeply stained granules situated between the spermatozoid-nucleus and the plasmic region. The two flagella are joined side by side at their proximal ends for a short distance. This region of union is then followed by another structure which is termed the "Stammsubstanz".

This "Stammsubstanz" is like a link between the united proximal ends of the flagella and the spermatozoid-nucleus. The distal ends of the flagella terminate in a thick fan-shaped structure. This terminal structure contains the plasmic substance. Yuasa observed that the spermatozoid-nucleus was composed of matrix (outside portion) and chromonema (inside portion), and that there was "border-brim" lying along the blepharoplast.

Sinotô, Yuasa and Satô (1949) and Satô (1950) have also made investigations on the *Marchantia* spermatozoid by means of the light microscope in addition to studies by means of the electron microscope. By staining with carbol-fuchsin and gentian-violet, and by Feulgen's staining, the following results were obtained: the difference in the structure of the blepharoplast and the spermatozoid-nucleus is shown by the fact that the blepharoplast does not react to Feulgen's staining, but the spermatozoid-nucleus does. There is a middle piece (ca 0.4 μ in length), unstained with carbol-fuchsin or gentian-violet, between the spermatozoid-nucleus and the plasmic region which is stained lightly. The end of flagellum is hook-like in form in which the plasmic substance is contained. When untreated, the structure of the spermatozoid-nucleus was homogeneous, while, when it was treated with 1 per cent trypsin for one hour, tetra-ethyl-ammonium hydroxide and ammonium chloride being used as a buffer, and with the pH about 8.0, the spiral structure of the spermatozoid-nucleus could be observed by Feulgen's staining, and the number of coils were between 3 and 3.5. It might be considered that the matrix in the surface of the spermatozoid-nucleus was digested by trypsin.

Some coincidence in the morphology of the spermatozooids as observed by means of the electron microscope with that seen by means of the light microscopic studies suggests that additional informations may be obtained by means of the electron microscope. It had also been observed by means of the light microscope that the flagellum consists of several fibrils, and that the end of the flagellum is either hook-like in form containing the plasmic substance, or brush-like. It may be a problem whether the flagellum consists of a main axis which is composed of several fibrils forming a bundle, encased in a semitransparent plasmic membrane which surrounds the main axis, or whether the flagellum is only a bundle of several fibrils. Is the semitransparent plasmic membrane an artifact? Such a membrane which can be seen in the electron micrographs was only obtained by a shadow-cast with chromium. Is this an artifact introduced by this method? We have considered that this membrane is so thin and of such lower density that contrasts do not appear in unshadowed specimens. On the spermatozoa of the bull, Yasuzumi (1950) observed that

the tail consists of a main axis and a homogeneous membrane surrounding it. And in the same materials, Yasuzumi reported that the matrix of the tail showed the fine spiral structure after it was treated with ribonuclease for three days, and that the spiral structure was broken away and several (from five to ten) fibrils were formed when the main axis was treated with ribonuclease for a long time. The author is of the opinion in the case of spermatozooids of *Marchantia polymorpha* that the flagellum showed a spiral structure due to pepsin treatment (Figs. 7 and 8). By means of the light microscope, the author observed that the flagellum was separated into several fibrils (four in number), after treatment with 0.01 per cent ribonuclease¹ at 64.5°C for 24 hours and then staining with carbol-fuchsin. The end piece of the tail of spermatozoa of the human, bull and goat separates into several fibrils (Yasuzumi 1950). In the spermatozoa of the chicken and bull, Baylor, Nalbandov and Clark (1943) reported that the end of the tail-like brush consists of many free and very long filaments, and that breaks occurring in the main or end pieces of the tail have shown flared brushes which make it seems likely that the axial filament consists of a bundle of five fibers.

The spermatozoid-nucleus, the plasmic region and the middle piece could not be observed perfectly, because these were too large in density and thick for the electron beam. However, in the specimen treated with pepsin, it seems that the spermatozoid-nucleus shows the band (spiral?) structure.

In the studies of the spermatozooids of *Isoetes* plants by means of the light microscope, there were *Isoetes* sp. (Belajeff 1885, Dracinschi 1932), *Isoetes lacustre* (Liebig 1931) and *Isoetes japonica* (Yuasa 1933, 1937, 1940). Yuasa (1933) observed that the head (blepharoplast) of the spermatozoid was almost triangular, bearing about eleven cilia, and that this head was bordered by the border-brim. Fig. 9 shows that an end of the border-brim of the blepharoplast-portion separates from the nuclear portion and extends straight out. As Yuasa (1933) observed, besides the plasmic band and the nuclear portion, there are the fin-like appendages whose density was low (Fig. 10). These appendages seem to be plasmic substances. On the other hand, in the fixed specimen (Fig. 9) the plasmic parts cannot be seen. Furthermore by means of the light microscope it was never observed that the cilium consists of about twenty fibrils. In Fig. 9 it is interesting to note that the ring-like arrangements binding many fibrils are seen, though the presence of the ring nature must yet be confirmed.

Liebig (1931) reported that the structure of the nuclear portion

¹ Kindly offered by Dr. A. Sibatani, Laboratory of Microbiology, Ôsaka University.

consists of the matrix and the granules. Yuasa (1931) observed the structure of the nucleus and the plasmic portion by means of the following methods. The suspension of fresh spermatozoids is exposed to ammonium vapour for several seconds. Then, it is fixed with osmic acid vapour, dried, and washed by hydrogen peroxide solution. Finally it is stained with gentian-violet or carbol-fuchsin. He observed that the nuclear portion was made up of masses of many stretched and extended chromonemata, and that the plasmic band and the blepharoplast were also conspicuously expanded, and that the border-brim was almost unbroken. He also showed that the chromonemata composing the chromosome were extended by ammonium vapour (cf. Kuwada and Nakamura 1934). In the electron micrographs obtained according to the same methods (except for the staining), the expansion, the break, the band structure and the fibrous structure were observed to some degree. It may be thought that the chromonema consists of the fibers.

Summary

The spermatozoids of *Marchantia polymorpha* and *Isoetes japonica* have been studied by means of the electron microscope.

1. Spermatozoids of *Marchantia polymorpha* :

The flagellum consists of a bundle of several fine fibrils, and the semitransparent plasmic membrane surrounding this bundle was observed. The end of the flagella showed two types, a brush-like or a hook-like forms. In the hook-form, the network of the fibrils and plasmic substance were observed.

The blepharoplast, the spermatozoid-nucleus, the middle piece and the plasmic region are seen homogeneous by reason of the thickness and high density for the electron beam.

In the specimen treated with pepsin, it seems that the blepharoplast, the spermatozoid-nucleus and the plasmic region differ in their structure.

2. Spermatozoids of *Isoetes japonica* :

Each of cilia consists of many delicate fibrils (about twenty in number), and the arrangements binding these fibrils near the blepharoplast were observed.

The nuclear portion and the plasmic bands are homogeneous in the electron micrographs, while in the specimens treated with ammonium an expansion was produced, and the band structure and the fibrous structure which forms the bands were observed.

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POSTSCRIPT

While preparing this proof, Manton, I. and B. Clarke (1950) have reported "Electron microscope observations on the spermatozoid of *Fucus*" (Nature, 1950, 166, 4232: 973-974).