Ultrastructural and Immunofluorescence Studies of Zoosporogenesis in *Laminaria angustata* [1)]

By

TAIZO MOTOMURA

Introduction

Studies on zoosporogenesis in the Laminariales began with KYLIN's work (1918) in *Chorda filum* using a light microscopy. Afterward, Japanese investigators (ABE 1939, YABU 1957, 1958, 1964, YABU and TOKIDA 1963, INOH and NISHIBAYASHI 1954, NISHIBAYASHI and INOH 1956, 1957, 1958, 1960a, b, 1961, OHMORI and INOH 1963, OHMORI 1967) have observed zoosporogenesis in detail in many Japanese laminarean plants using a light microscopy. NISHIBAYASHI and INOH (1956) had investigated the zoosporogenesis in *Laminaria angustata*. These observations demonstrated that the first nuclear division in developing sporangia of macroscopic sporophyte was meiotic in the life cycle of the Laminariales.

Although many light microscopic observations have been carried out on zoosporogenesis in the Laminariales, little information was available using an electron microscopy. The ultrastructures of zoosporogenesis in the Laminariales was described for *Macrocystis pyrifera* (CHI and NEUSHUL 1972) and *Chorda tomentosa* (TOTH 1974). The ultrastructure of the synaptonemal complex during meiosis in sporangial parent cell was first reported by TOTH and MARKEY (1973) in the phaeophycean plants, *Chorda tomentosa* and *Pylaiella litoralis*.

I have been studying the key events in the life cycle of *Laminaria angustata* Kjellman, and already published on gametogenesis (MOTOMURA and SAKAI 1984), ultrastructure of egg (MOTOMURA and SAKAI 1988) and sperm (MOTOMURA 1989), and fertilization (MOTOUMURA 1990). In the present study, I observed in detail the zoospore formation in the unilocular sporangia of *Laminaria angustata* using electron microscopy, and also changes of microtubule (MT) arrangement during meiosis, mitosis, and flagellar elongation in the unilocular sporangia by anti-β tubulin immunofluorescence microscopy.

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1) This manuscript is a part of a thesis for a doctor's degree, Hokkaido University (1985).

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Materials and Methods

Electron microscopy

Mature sporophytes of *Laminaria angustata* KJELLMAN were collected at Charatsunai, Muroran, Hokkaido, Japan, in September-December of 1984, 1991 and 1992. Sorus tissue was cut into pieces (1 mm × 1 mm) with a razor and fixed immediately. Samples were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 2% NaCl, 0.1% $CaCl_2$ and 1% caffeine for 1.5 hr at 4°C. They were washed with 0.1 M cacodylate buffer containing 2% NaCl and 0.1% $CaCl_2$, and post-fixed with 2% OsO_4 for 2 hr at 4°C in 0.1 M cacodylate buffer (pH 7.2) containing 2% NaCl and 0.1% $CaCl_2$, or 1% OsO_4 in the same buffer composition overnight at 4°C. Liberated zoospores were fixed by adding 1 m*l* of 10% glutaraldehyde and 1 m*l* of 4% OsO_4 solution in 10 m*l* seawater containing numerous zoospores. After fixing for 10 min at room temperature, zoospores were collected by centrifugation. They were then washed with H_2O and *en-bloc* stained with 0.5% uranyl acetate for 15 min at 4°C. Samples were dehydrated in a graded series of acetone and embedded in SPURR's epoxy resin (SPURR 1969). Sections were cut using a diamond knife on a Porter-Blum MT-1 ultramicrotome and mounted on formvar-coated slot grids. Sections were stained with uranyl acetate and lead citrate (REYNOLDS 1963) and observed with a Hitachi H-300 electron microscope.

For SEM specimens, liberated zoospores which were fixed as above were placed on TOYO membrane filters (regenerated cellulose type, with 1.0 μm and 0.2 μm pore sizes), and dehydrated in an acetone series. They were critical point dried using dry ice (Eico DX-1), coated with gold and carbon, and observed with a Hitachi S-510 electron microscope.

Histochemistry

Several histochemical studies were conducted for detection and comparison of polysaccharide and lipid.

Periodic acid - thiocarbohydrazide - silver proteinate staining (PATAg staining) for polysaccharide: Sections were oxidated in 1% aqueous periodic acid for 30 min, washed with distilled water, floated on 0.2% thiocarbohidrazide in 20% acetic acid, washed with the acetic acid solution with decreasing concentration (30 min) and finally rinsed with distilled water (30

min). Then they were treated with 1% silver proteinate in darkness (30 min) and washed with distilled water. Controls of the PATAg staining test were prepared by omitting periodic acid or thiocarbohydrazide treatment.

Toluidine blue-O for polysaccharide: Semi-thin sections (0.5-1 μm) were stained with 1% toluidine blue-O in 1% sodium borate for 1-5 min at ca. 70°C on a hot plate and rinsed in distilled water.

Sudan black-B for lipid: Semi-thin sections were pretreated in 70% ethanol (2 min), then stained with 0.4% sudan black-B in 70% ethanol at 60°C for 1hr. They were rinsed in 70% ethanol for 1min, and washed in distilled water.

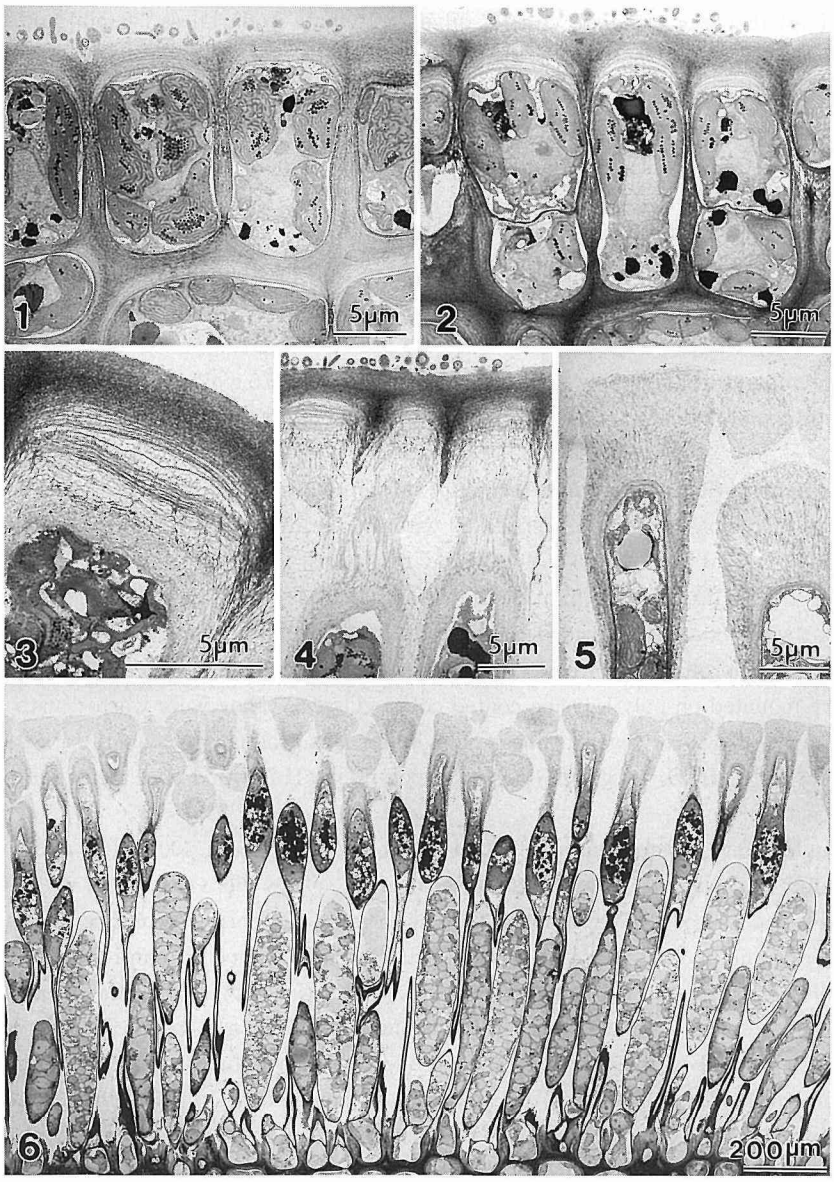
Oil red-O for lipid: Semi-thin sections were rinsed 60% isopropanol, and stained (10min at room temperature) in freshly filtrated oil red-O solution (0.5 g oil red-O is added to 100 m*l* 98% isopropanol and 6 m*l* of this stock solution is diluted with 4 m*l* of distilled water).

Indirect immunofluorescence microscopy of microtubules

Small pieces of sorus tissue were fixed with 3% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.5) containing 4% NaCl for 30min at 4°C. Through this fixation step using 10 mM EGTA, protoplasts of numerous unilocular sporangia was extruded from the tip of unilocular sporangia by softly pipetting. These liberated protoplasts of unilocular sporangia were collected by gentle centrifugation, and were mounted on poly-L-lysine coated cover glasses. They were washed with PHEM containing 4% NaCl for several times. The buffer was gradually changed to PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 0.7 g, KH_2PO_4 0.2 g per liter, pH 7.4) and specimens were finally washed with PBS.

Samples were treated with PBS containing 0.5% Triton X-100 for 20 min at room temperature and washed with PBS for several times. They were pretreated with blocking solution, composed of PBS containing 2.5% skim milk, 5% normal rabbit serum and 0.1% NaN_3, for 30 min at 35°C. Then they were incubated with monoclonal anti-β tubulin (Amersham, diluted 1:500 with PBS) for 30 min at 35°C. After rinsing with PBS, they were incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (Amersham, diluted 1:20 with PBA) for 30 min at 35°C. Following microtubules staining, the specimens were immersed in DAPI solution (4'-6-diamidino-2-phenylindole, 1 μg/m*l* PBS) for 10 min at room temperature. After washing with PBS, finally the samples were mounted with Mowiol 4-88 mounting medium (OSBORN and WEBER 1982) containing 0.2% p-phehylenediamine.

Observations were carried out with an Olympus epifluorescence microscope (BH2-RFK). G520 and B460 passing filters (Olympus Co. Ltd.) were used for omitting autofluorescence of chlorophyll and phenolics. Photographs were taken on Tri-X film (Eastman Kodak) at ASA 1600 and developed with Pandol (Fuji Photo Film Co. Ltd.).



Figs. 1–6. Development of paraphysis of the sorus. Fig. 1 Meristoderm cells of the thallus. Note a large number of plastoglobules in the chloroplasts. Fig. 2. Elongation and division of meristoderm cells. Note accumulated electron-dense material. Fig. 3. Accumulation of fibrous layer and amorphous matrix. Fig. 4. Cuticle is raised by accumulation of amorphous matrix. Fig. 5. Cuticle is detached by the completion of paraphysis cap. Fig. 6. Fully matured sorus part. The upper part of paraphyses takes a cylindrical shape with characteristic cap, whereas the lower part of them becomes slender.

Results

Paraphysis formation

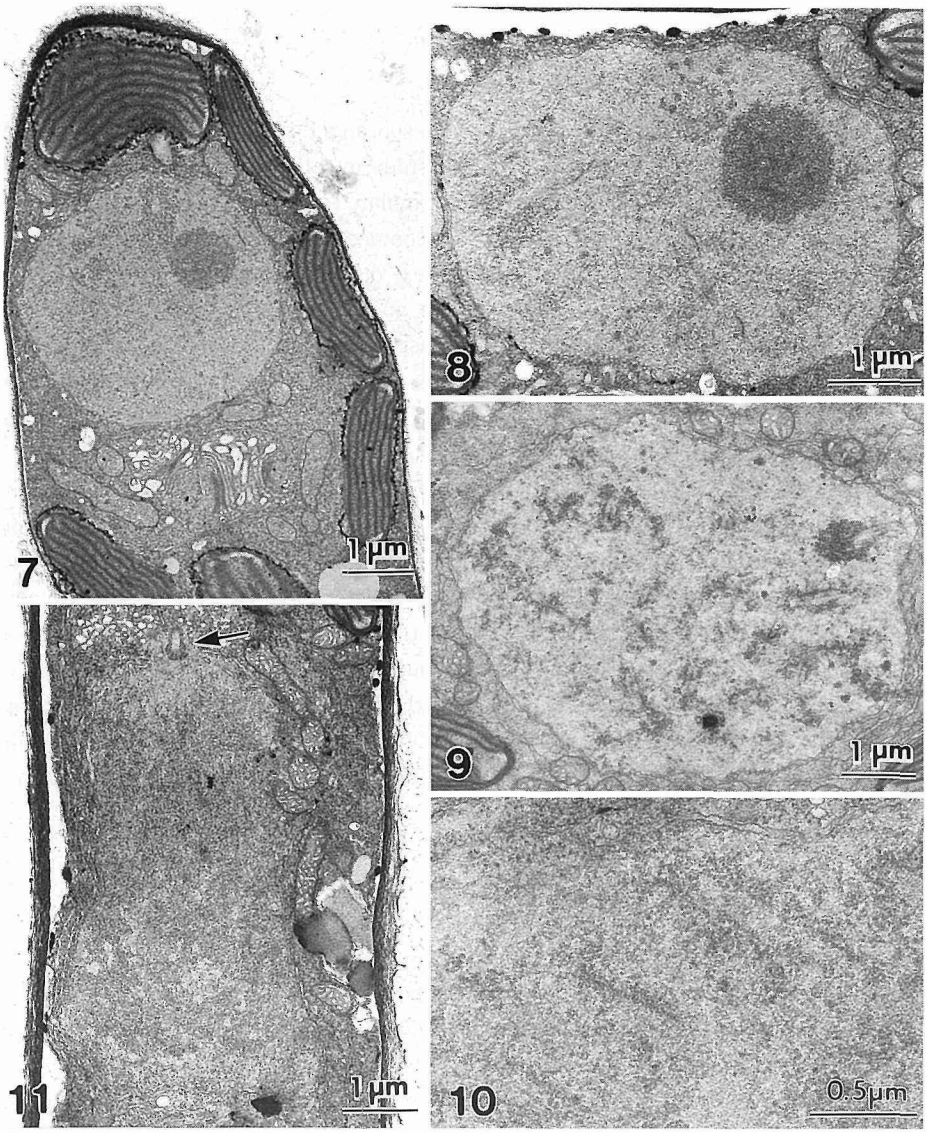
The very first change in the formation of the sporangial sorus occurred in meristoderm cells at the surface of the thallus. At first, meristoderm cells started to elongate longitudinally (Figs. 1, 2) and electron dense materials accumulated in the cytoplasm, especially at the surface portion. This material was probably composed of polyphenolic substances (= physode). A large number of plastglobules were detected in the chloroplasts. During elongation of meristoderm cells, fibrous layers and amorphous matrix were deposited between the original surface cell wall and plasmamembrane of the upper side of the meristoderm cells (Fig. 3). This portion was stained an intense pink color with toluidine blue -O. This layer was the initial of mucilage cap of the paraphysis cells, which was assumed to be composed of a sulfated polysaccharide (= fucoidan).

Afterwards, the elongated meristoderm cell divided transversely into a basal cell and a paraphysis primordium (Fig. 2). The paraphysis primordium elongated to form young paraphysis without cell division. Plastglobule-rich chloroplasts were present in paraphysis cytoplasm. The upper part of paraphysis took a characteristically cylindrical form whereas the lower part became slender, and almost all of the cytoplasm migrated to the upper part (Fig. 6). In the cytoplasm of paraphysis, a large number of physodes and active Golgi bodies were evident. Accompanying an elongation of the paraphysis, a sulfated polysaccharide layer became more thickened and started raising the cuticle (= original upper cell wall of meristoderm cells) (Fig. 4). The cuticle was detached by the thickness of sulfated polysaccharide on the mature sorus portion (Figs. 5, 6).

Sporangial parent cell formation

When the upper cell derived from the meristoderm cell differentiated into the paraphysis, the basal cell elongates upward as a ovoid-shaped sporangial parent cell between paraphyses (Fig. 7). There were several chloroplasts in the sporangial parent cell. In the chloroplasts, 6-12 of three-thylakoid lamellae extended longitudinally, and the number of plastoglobules was fewer than in the paraphysis chloroplasts. Accompanying the development of sporangial parent cell, endoplasmic reticulum (ER) developed gradually, and the Golgi bodies began producing large vesicles. Golgi bodies occasionally existed near ER, apart from the perinuclear region. Intracristal tubular structures which were found in the complete zoospores could not be detected yet in the tubular cisternae of mitochondria.

A nucleus (ca. 5 μm in diam.) in the sporangial parent cell ordinarily contained one nucleolus (Fig. 7). Occasionally, nucleolar vacuoles were observed. Meiosis occurred in the unilocular sporangial parent cell. Chromatin started to condense into chromosomes, which could be detected as electron dense masses under electron microscopy. In early prophase



Figs. 7–11. Sporangial parent cell and meiosis. Fig. 7. Sporangial parent cell. Fig. 8. Early prophase of meiosis I. Note that synaptonemal complexes are observed as linear structures. Fig. 9. Late prophase of meiosis I. Note synaptonemal complexes between chromosomes. Fig. 10. Details of synaptonemal complexes near nuclear membrane. Fig. 11. Anaphase of metaphase I. Note that one of a pair of centrioles locates at the pole (arrow).

(Fig. 8), a nucleolus existed, but began to vanish toward late prophase. In the prophase nucleus, synaptonemal complexes appeared as linear structures (Fig. 10). Figure 9 shows the

late prophase nucleus. Chromosomes were seen as electron dense areas, and synaptonemal complexes could be observed in the group of chromosomes. Figure 11 shows the late anaphase nucleus in meiosis. The centrioles existed at the pole, and the nuclear envelope almost intact except the pole. Not all stages of meiosis could be found in this experiment using electron microscopy.

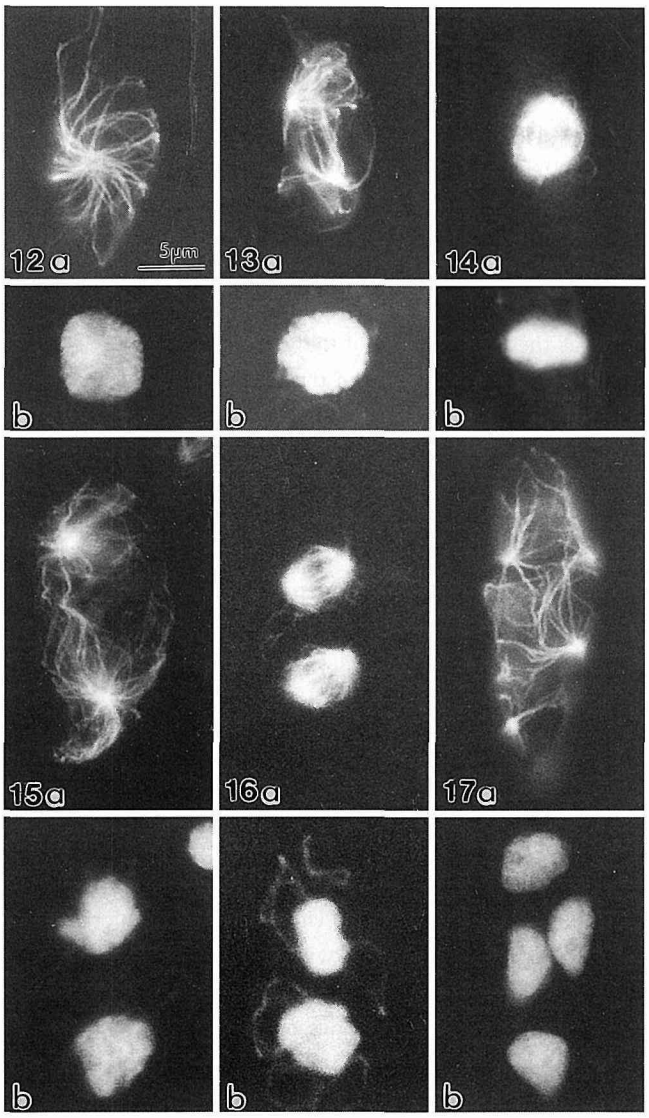
Fluorescence microscopy using DAPI and anti-β tubulin antibody showed that nuclear division and spindle formation in meiosis were conducted in the same manner as nuclear division in vegetative cells of the brown algae. A sporangial parent cell firstly had a nucleus with a centrosome (Fig. 12). Almost all MTs in the cell were radiating from the centrosome and were functioning as the MT skeleton of the whole cytoplasm. Therefore, there was no specialized cortical MT system. MTs from the centrosome reached the plasmamembrane and ran just beneath it. During chromosome condensation (prophase), the centrosome divided into two and migrated to both poles of following nuclear division (Fig. 13). In metaphase (Fig. 14), each chromosome became arranged at the nuclear equator, and a typical spindle was formed. No cytoplasmic MTs could be observed and all MTs participated into the metaphase spindle. After meiosis I, the two derivative nuclei each received one centrosome, and MTs radiated from them again (Fig. 15). In meiosis II, two derivative nuclei synchronously proceeded to the second nuclear division (Fig. 16). As the result of meiosis, four nuclei were formed in the unilocular sporangium, and each centrosome was positioned near plasmamembrane at this stage (Fig. 17).

Development of unilocular sporangium

After meiosis, sporangia developed further. Accompanying the development of sporangial parent cell, vacuoles which were filled with electron translucent material gradually became conspicuous (Fig. 18). This vacuole has been called as "type B vacuole" (RAWLENCE 1973, TOTH 1974, HENRY and COLE 1982). Under the light microscope, when semi-thin sections of unilocular sporangia were stained with sudan black-B or oil red-O, these vacuoles were stained blue-black (Fig. 20) or red, respectively. Therefore, these vacules were possibly filled with lipids. Such an accumulation of many lipid granules could only be observed in the sporangial parent cell and more developed unilocular sporangium, whereas lipid granules were hardly seen in the paraphyses and other vegetative cells. Also osmiophilic inclusions increased in the unilocular sporangium (Fig. 18).

In the sporangium cytoplasm, chloroplasts were ordinarily arranged in the periphery. ER became well developed and several layers of ER characteristically surrounded each nucleus (Fig. 18). ER connected with chloroplast ER. The Golgi bodies existed in the perinuclear region, and the most proximal cisternae of them faced the ER around the nucleus. The Golgi bodies were inactive when compared to those in the sporangial parent cell. Near each nucleus, a pair of centrioles, a Golgi body and a chloroplast were seen (Fig. 19).

Figures 21-23 respectively show MT arrangement in interphase of 8-, 16-, and 32-



Figs. 12-17. Anti-β tubulin immunofluorescence staining (a) and DAPI staining (b) on meiosis of unilocular sporangial parent cell. Scale in Fig. 12 also applies Figs. 13-17. Fig. 12. MT cytoskeleton in interphase of sporangial parent cell. Note MTs radiated from one centrosome near a nucleus. Fig. 13. Early prophase in meiosis I. A centrosome has divided into two with chromosome condensation. Fig. 14. Metaphase in meiosis I. Note that cytoplasmic MTs can not be detected. Fig. 15. After meiosis I, two daughter nuclei receive one centrosome, and MTs radiate from them. Fig. 16. Metaphase in meiosis II. Fig. 17. Four-nuclei stage in interphase after meiosis. Note 4 centrosomes at the periphery of the cytoplasm and a basket-like arrangement of MTs.

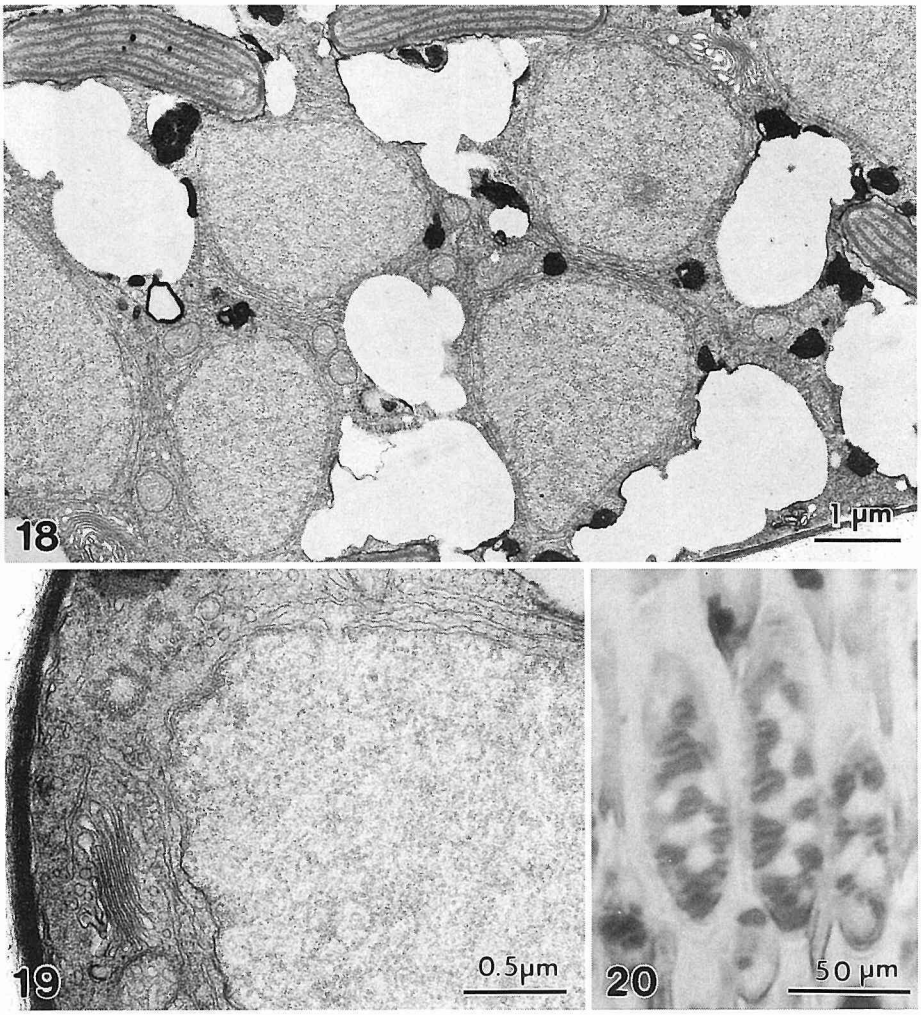
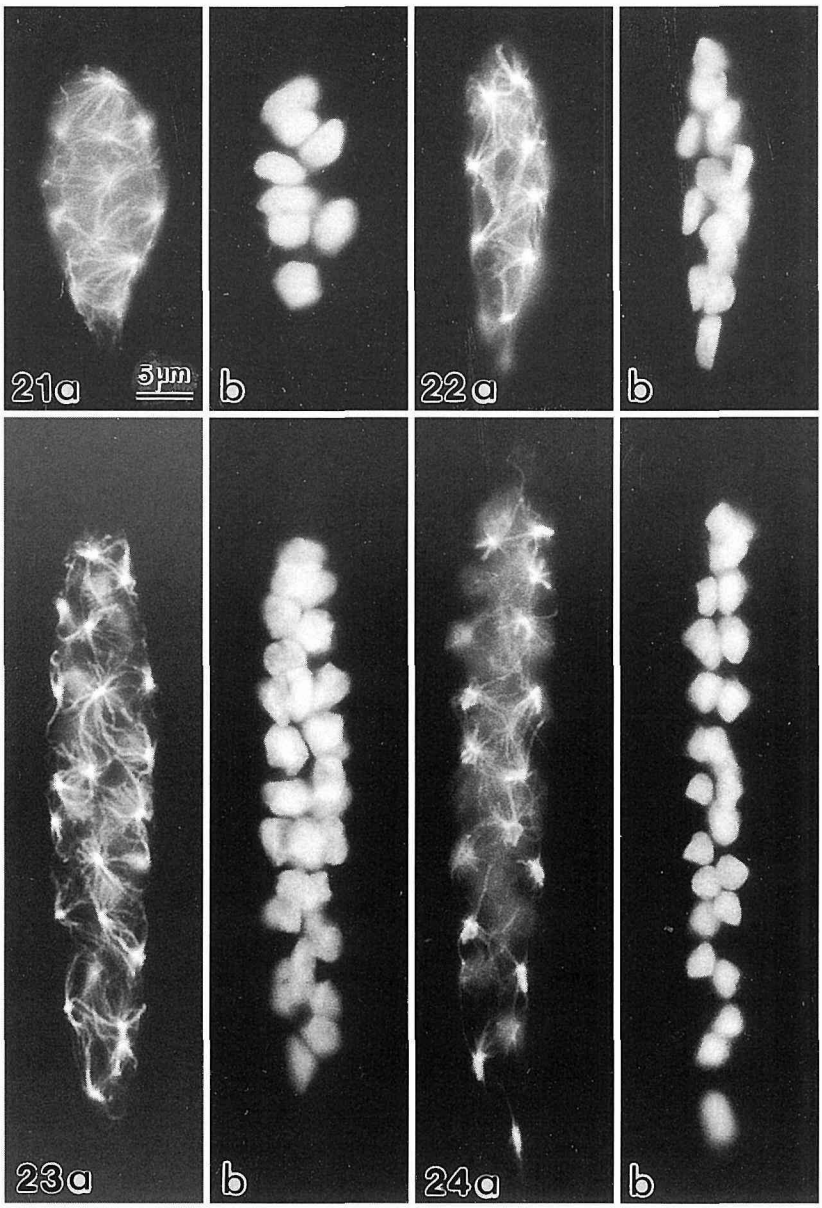


Fig. 18. Probably 8-nucleate stage of the unilocular sporangium. Electron-light staining lipid granules are scattered in the cytoplasm. Note peri-nuclear ER and inactive feature of Golgi bodies. **Fig. 19.** A pair of centrioles locate at the periphery of the unilocular sporangium. **Fig. 20.** Sudan black-B staining. Lipid granules in the unilocular sporangium is reacted positively.

nucleate stages of unilocular sporangia. Centrosomes were near nuclei in the periphery of cytoplasm, and MTs radiated from them. These MTs from each centrosome appeared to be interconnected with each other. Therefore, as a whole, MTs were arranged as a basket-like organization in the interphase stage of unilocular sporangia. These basket-like MT arrangement in interphase were observed in all the mitoses following meiosis (Fig. 17). As mentioned above, pairs of centrioles, one component of the centrosome in the brown algae,



Figs. 21–24. Anti-β tubulin immunofluorescence staining (a) and DAPI staining (b) on various developmental stages of unilocular sporangia. Scale in Fig. 21 also applies Figs. 22–24. Fig. 21. Eight-nucleate stage. Fig. 22. Sixteen-nucleate stage. Fig. 23. Thirty two-nucleate stage. Fig. 24. Initiation of flagellar elongation in the unilocular sporangium. Note that MTs radiate from each centrosome nearby nuclei, and form a basket-like arrangement in the unilocular sporangium.

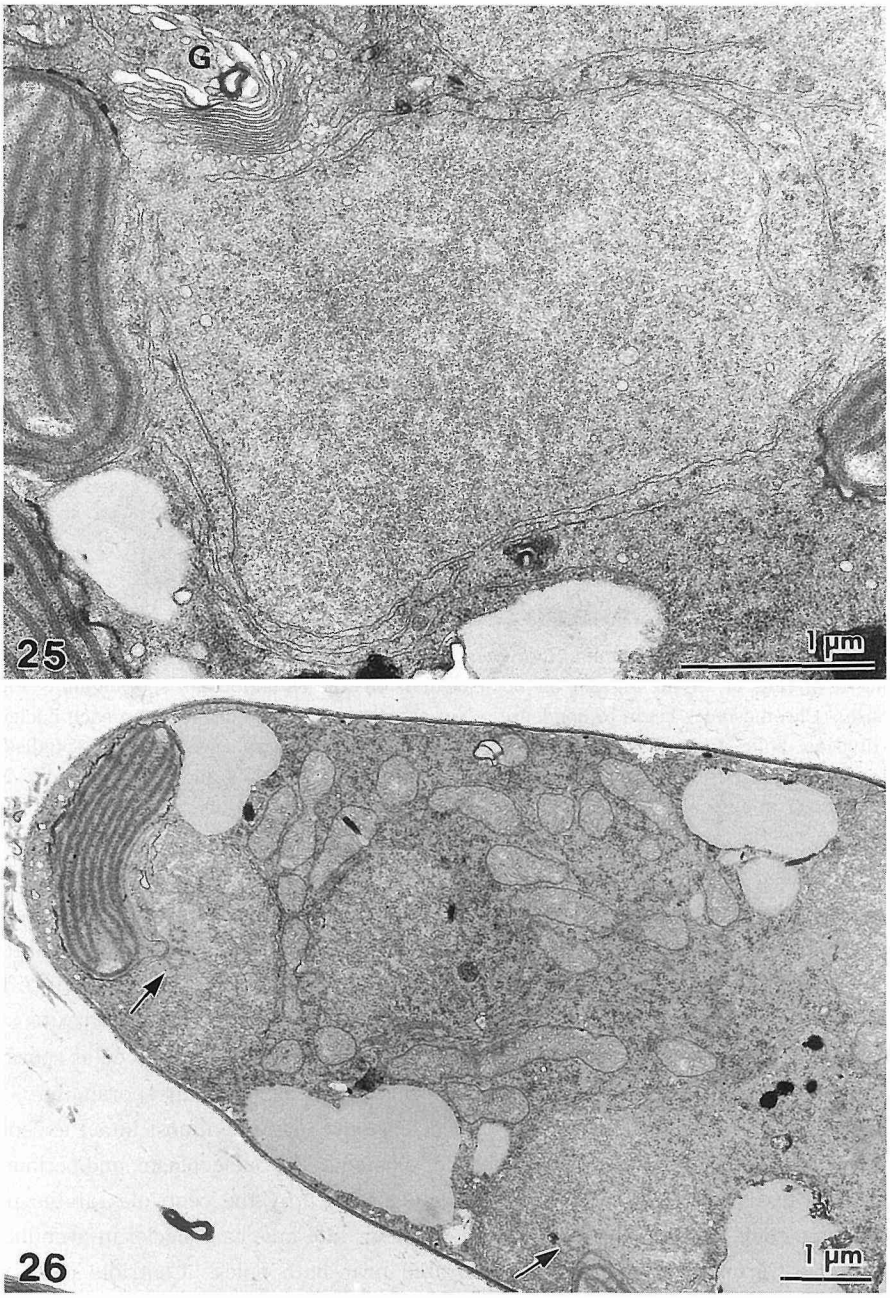
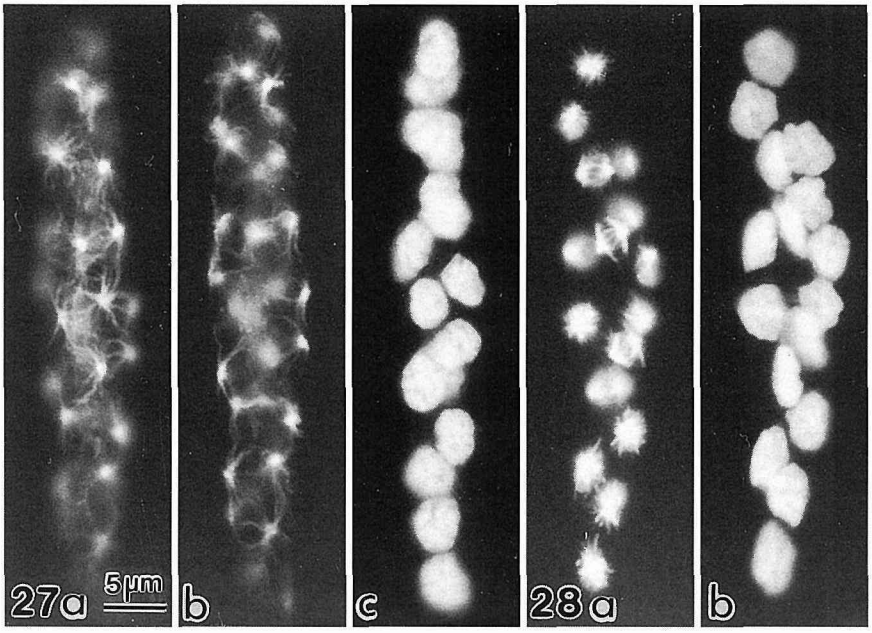


Fig. 25. Metaphase nucleus. Note that nuclear membrane persists except for both polar fenestrations, and a Golgi body (G) and a chloroplast locates near the pole. **Fig. 26.** Anaphase nuclei. A pair of centrioles can be detected near the chloroplast (arrow).



Figs. 27, 28. Anti-β tubulin immunofluorescence staining (a, b in Fig. 27, a in Fig. 28) and DAPI staining (c in Fig. 27. b in Fig. 28) on nuclear division in the unilocular sporangium. Fig. 27. Prophase. Chromosomes begin to condense. Note that two centrosomes exist on each nucleus as both division poles (a and b, different focusing). Fig. 28. Metaphase. Typical spindles are synchronously formed and note that there are interzonal spindle MTs and no cytoplasmic MTs. Scale in Fig. 27 applies also in Fig. 28.

were visible near the cell wall of unilocular sporangia (Fig. 19).

Nuclear division occurred synchronously producing 32-nuclei in each unilocular sporangium. The process of the nuclear division in the unilocular sporangium was identical to the results obtained by other investigators of phaeophycean mitosis (MARKEY and WILCE 1975, BERKALOFF and ROUSSEAU 1979, LA CLAIRE 1982, KATSARAOS *et al.* 1983, MOTOMURA and SAKAI 1985). Therefore, detailed description of nuclear division in the unilocular sporangia is unnecessary. Figure 25 shows a metaphase nucleus in a unilocular sporangium. The nucleus took a typical spindle form, and the nuclear envelope was almost intact except for both polar fenestrations. Several small vesicles existed in the nucleoplasm, and perinuclear ER surrounded the nucleus. The Golgi body located nearby the centrioles at the poles. Kinetochores could not be detected. Figure 26 shows late anaphase nuclei in a unilocular sporangium. Chromosome masses had migrated near both poles. Centriole pairs were observed near the chloroplasts.

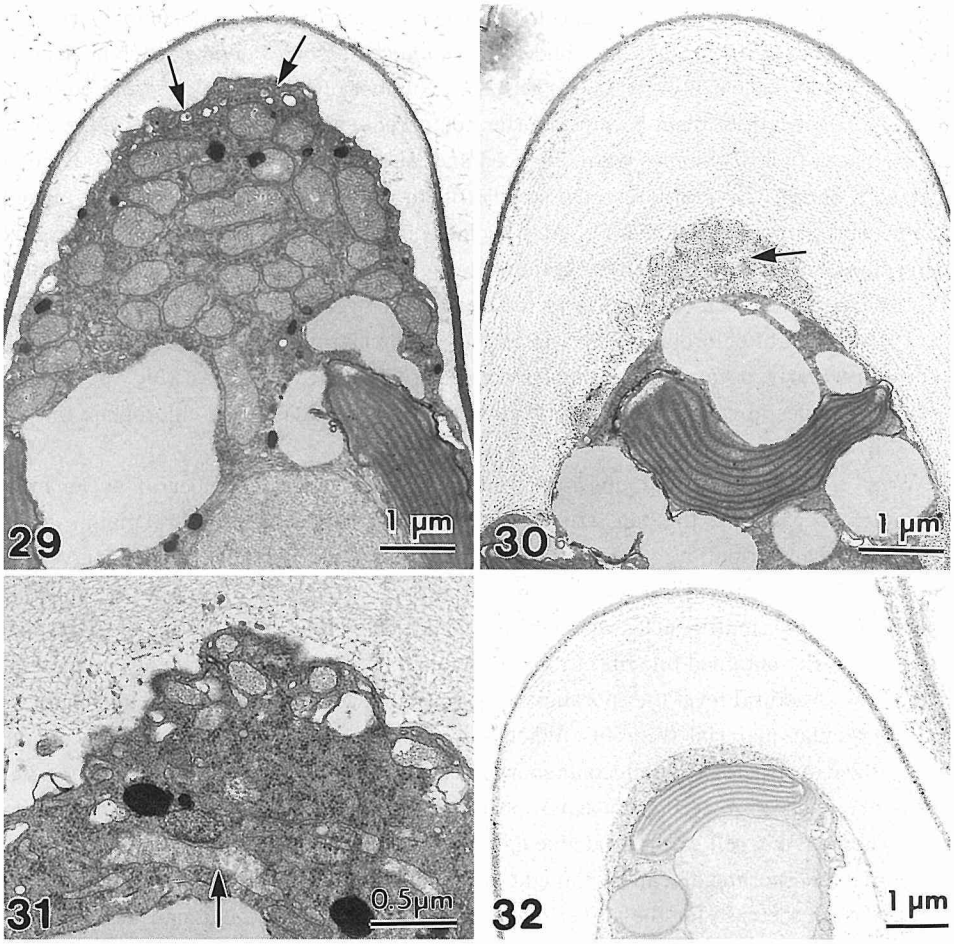
Immunofluorescence microscopy using anti-β tubulin antibody also showed clearly that nuclear division in the unilocular sporangium occurred synchronously. Figures 27 and 28

show mitosis from 16-nucleate stage to 32-nucleate stage in the unilocular sporangium. During prophase with chromosome condensation and centrosomes divided, each nucleus had two MT foci at nuclear division poles (Fig. 27). Number of MTs radiating from both poles increased but length of them became shorter compared with interphase stages (Figs. 21–23). In metaphase, typical spindles were observed and, at that time, cytoplasmic MTs were not detected (Fig. 28). Several interzonal spindle fibers were also detected. Division plane of each nucleus arranged in random. After nuclear division, each derivative nucleus received one centrosome, each centrosome migrated to the periphery of the cytoplasm, and finally MTs radiated from each centrosome as the basket-like organization in the interphase stage.

The division of chloroplast could not be observed clearly in this study, but it must have occurred regularly, because each completed zoospore had one chloroplast (Fig. 49). Since a pair of centrioles always existed near the chloroplast, the division of chloroplast must be related to the nuclear division.

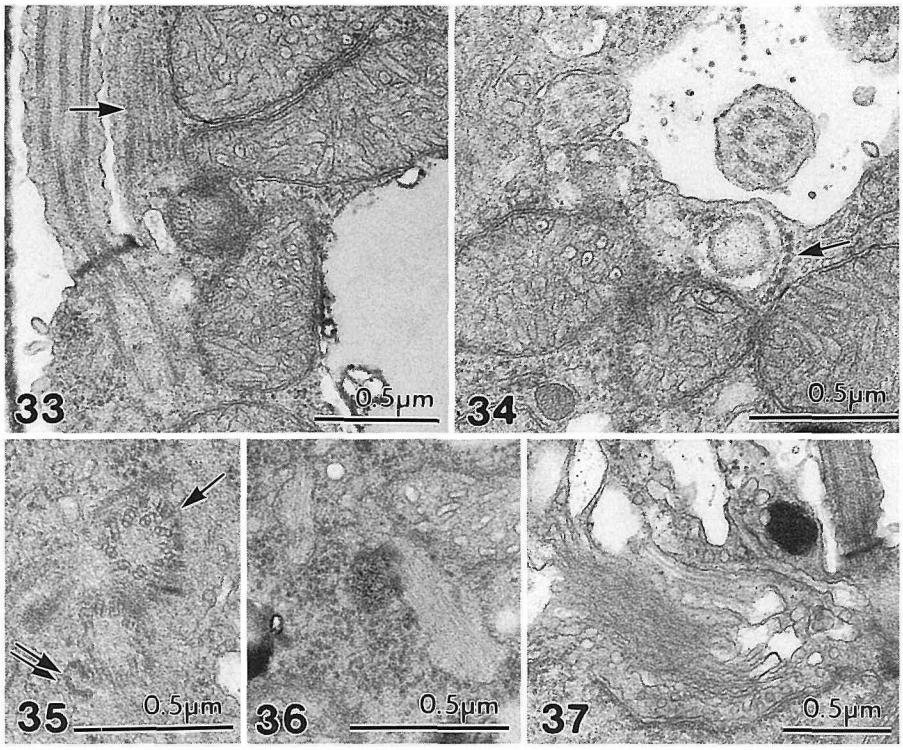
When a series of nuclear divisions were completed and the number of nuclei in the sporangium became 32, the cap structure was formed at the tip of sporangium. At the initiation of cap formation, numerous mitochondria and small vesicles were observed just below the plasmamembrane at the apex of the sporangium (Figs. 29, 31). A little swollen ER existed near these small vesicles which would be formed by the fragmentation of ER. These vesicles and ER contained fine fibrous material and they were deposited as the cap material. At the ultrastructural level the sporangial cap was homogeneous in structure and composed of fine granular material (Fig. 30). When semi-thin sections were stained with toluidine blue-O, the cap structure of unilocular sporangium was recognized as pink part under a light microscope. The cap structure was not positively reacted by PATAg for electron microscopy, whereas the cell wall of unilocular sporangium was positively stained (Fig. 32). Therefore, the sporangial cap is thought to be composed of a sulfated polysaccharide, fucoidan, as in the case for the antheridial and oogonial cap of the Laminariales.

At the same time as cap formation, flagellar elongation was initiated. A pair of centrioles migrated to the plasmamembrane, and differentiated into the flagellar basal bodies which had basal plates at the distal end of each centriole (Fig. 33). Flagellar basal bodies became arranged parallel with each other in unilocular sporangia (Figs. 34, 35). This arrangement was different from that in the liberated zoospore (Fig. 48). Accompanying the development of flagella, mastigonemes fused along one side of the anterior flagellum (Fig. 43). The mastigoneme elements were observed in ER (Figs. 36, 46) and Golgi bodies (Fig. 37). Several components of flagellar apparatus of zoospores could be found already in the early stage of flagellar development; the striated band (Fig. 35) connecting both basal bodies (O'KELLEY and FLOYD 1984) clearly existed on one third around a centriole and a major anterior rootlet (MAR, O'KELLY and FLOYD 1984) consisting of seven microtubules ran along a flagellum, probably anterior flagellum (Figs. 33, 34). Accompanying the development of flagella, intracristal tubules developed in mitochondrial cristae (Figs. 33, 34).



Figs. 29–32. Cap formation of unilocular sporangia. Fig. 29. Immature cap structure. Many mitochondria are gathering at the head of the unilocular sporangia. Small vesicles are observed at the beneath of the cap structure (arrow). Electron-dense outer layer is the original cell wall of the unilocular sporangium. Fig. 30. Full-matured cap structure. Small vesicles do not exist beneath the plasmamembrane at the cap. Granular material, which is stained more densely than the cap material, is filled between the cap and plasmamembrane (arrow). Fig. 31. Numerous small vesicles containing fine fibrous and granular material exist just beneath the plasmamembrane facing to the cap structure. Also, similar material is observed in swollen ER (arrow). Fig. 32. PATAg staining. The cap structure is stained negatively, while the original cell wall of the unilocular sporangium stained positively.

ER gradually swelled a little, and fine fibrous material was observed in ER cisternae, especially in the central region of unilocular sporangium (Fig. 39). The Golgi bodies in perinuclear region began producing large vesicles containing fibrous material again. Large



Figs. 33-37. Flagellar structure before the formation of individual zoospore. Fig. 33. Longitudinal section of a flagellum. Note a basal plate, a major anterior rootlet (MAR, arrow) composed of seven microtubules and intracristal tubules in mitochondria. Fig. 34. Transverse section. Note a MAR composed of seven microtubules (arrow) and intracristal tubules in mitochondria. Fig. 35. Striated band around one third of a basal body (arrow) and probably a bypassing rootlet composed of five microtubules (double arrows). Fig. 36. Mastigonemes in distended ER. Fig. 37. Mastigonemes in Golgi body.

lipid granules started to divide into several small ones. Electron dense, membraneous structures were formed gradually from the periphery of lipid granules (Fig. 39), which were stained pink metachromatically with toluidine blue-O under the light microscope. Highly magnified figures suggested that these structures were composed of electron dense granules and thin layers which were regularly arranged (Fig. 42).

Cytoplasm of the unilocular sporangium became highly vesiculated with the division of large lipid granules into small ones, and degradation from the periphery of lipid granules. Each of the chloroplasts at the periphery of cytoplasm (Fig. 38) started to migrate toward the inner part to surround a nucleus. As a result, the protoplasm of unilocular sporangium separated from the cell wall (Fig. 40). Fibrous and granular material which seemed to be derived from Golgi bodies, ER, and lipid granules filled in the cavity between the plasmamem-

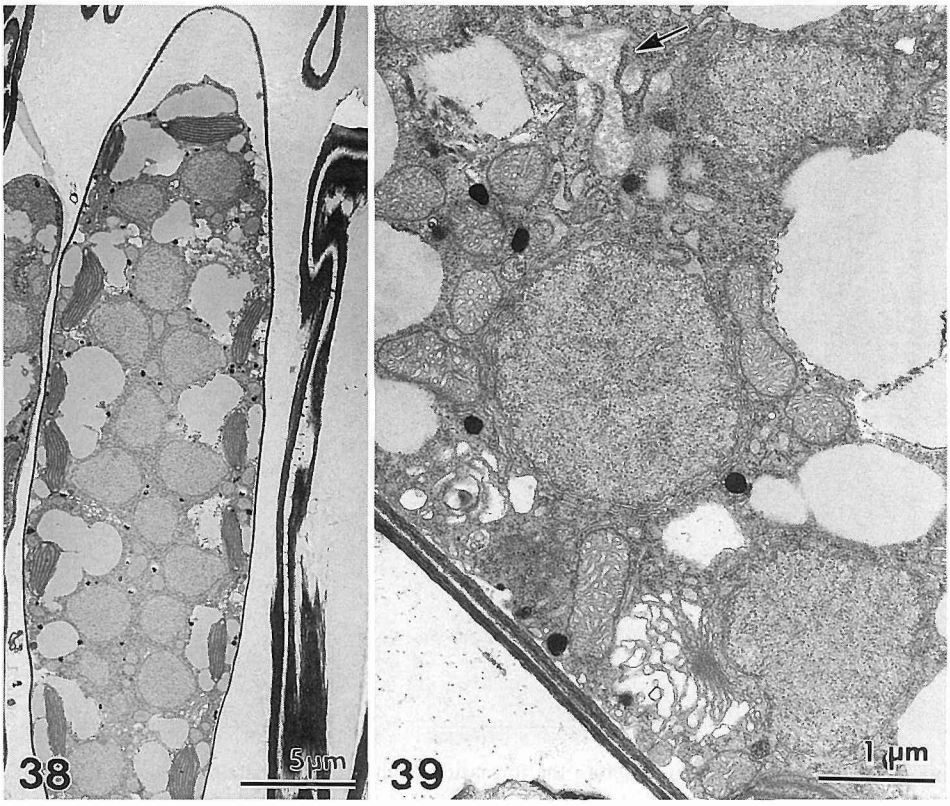


Fig. 38. Unilocular sporangium containing 32 nuclei after final mitosis. Nuclei locate at the middle part, while chloroplasts exist at the periphery. Lipid granules exist between nuclei and chloroplasts. Flagellar initiation started in this unilocular sporangium. **Fig. 39.** Peri-nuclear ER swelled partly and is filled with fine fibrous material (arrow). Note active Golgi bodies and flagella. Tubular structures can not be detected in the cristae of mitochondria.

brane and the cell wall. Nuclei contained heterochromatin-rich area (Fig. 41).

Individual zoospores developed in the unilocular sporangium when the plasmamembrane infurrowed and ER fragments fused forming large vesicles containing fibrous and granular material (Fig. 41, 43). Microtubules did not participate in the cleavage. Occasionally, the flagellar rootlet microtubules, a MAR, ran along the cleavage plane (Fig. 43). After cleavage, each unit containing a nucleus, a Golgi body, a chloroplast, a pair of flagella, several mitochondria and small lipid granules, could be distinguished as an individual zoospore (Fig. 44).

The cell surface of each zoospore unit was irregular at this stage. Several lipid granules remained in the cavity of unilocular sporangia, but they gradually degenerated. Two to three layered ER around the nucleus became swollen and vesiculated, and the cisternae of ER

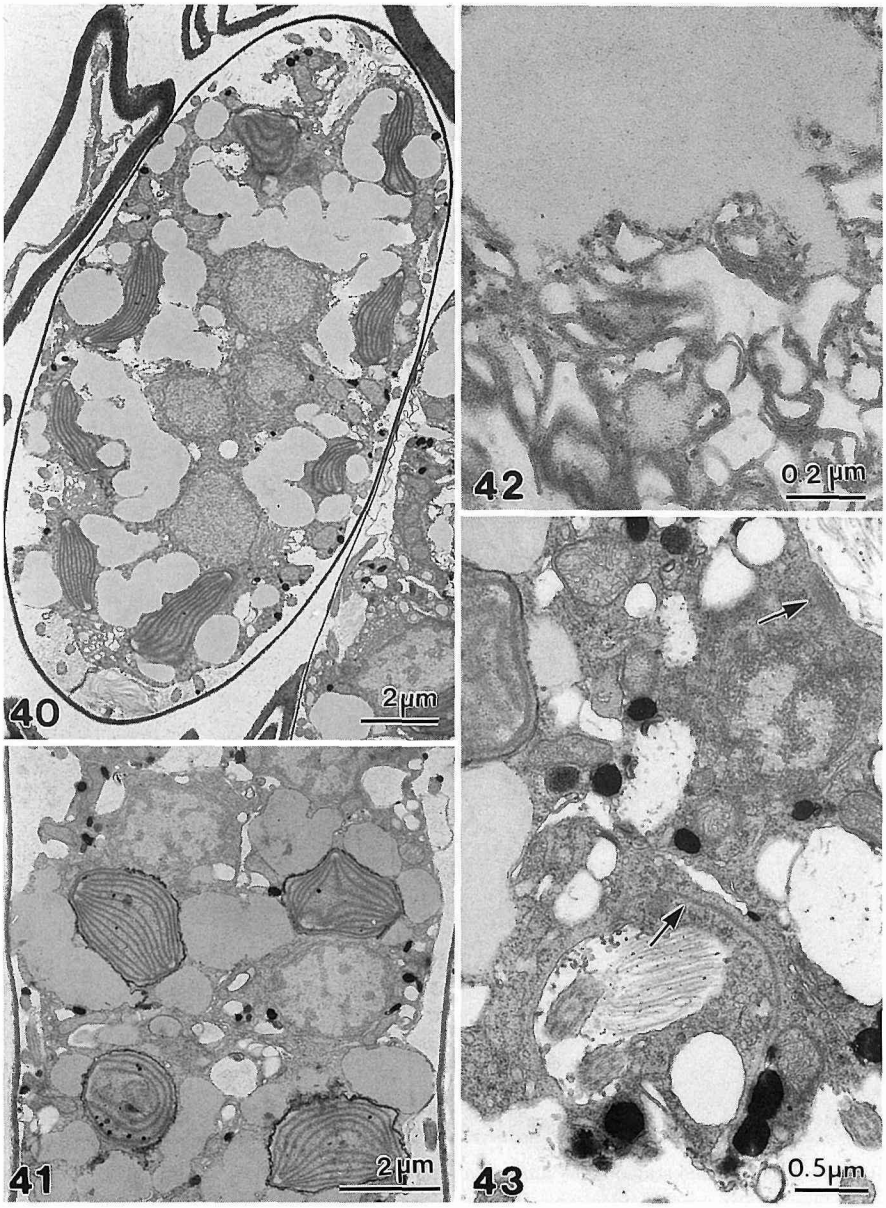
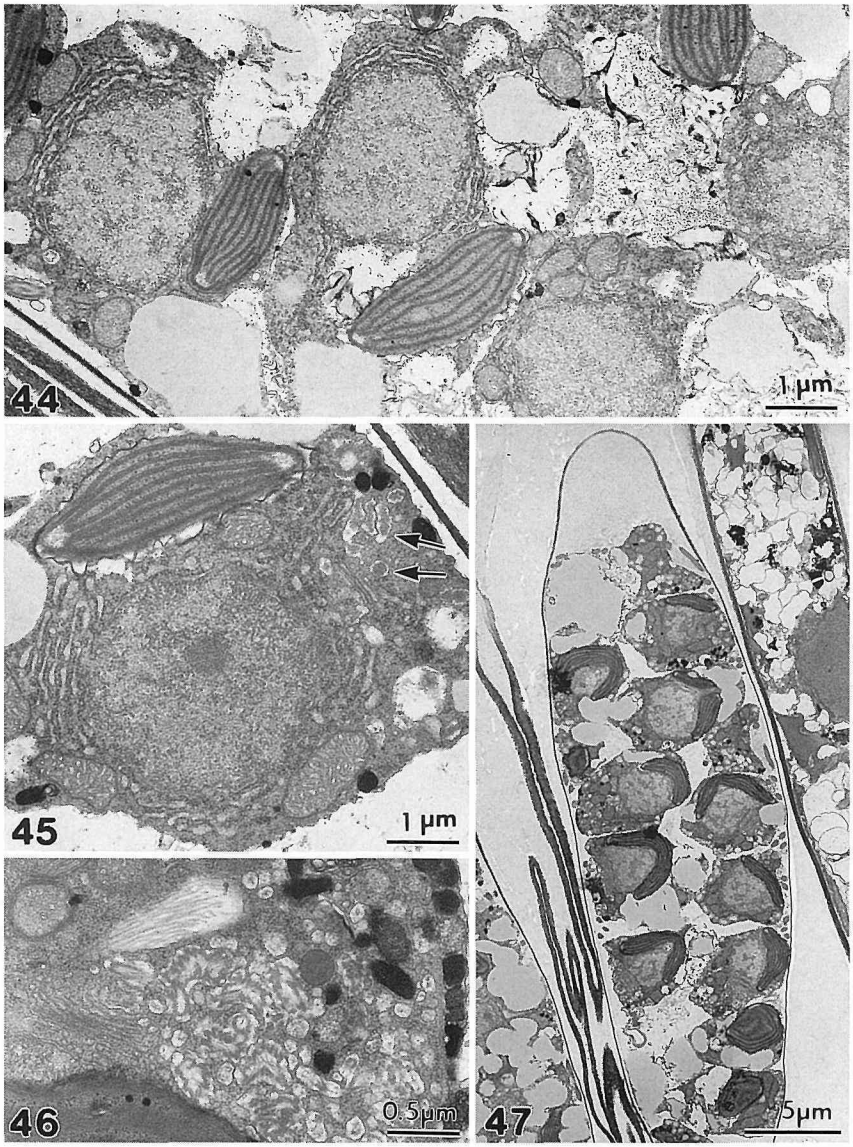
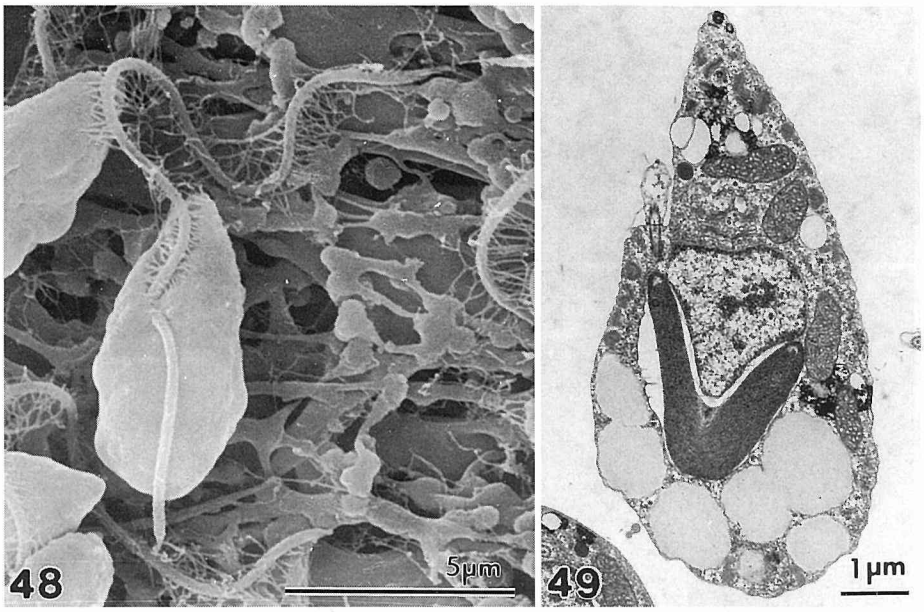


Fig. 40. Cytoplasmic cleavage in the unilocular sporangium. The cytoplasm begins to separate from the cell wall and lipid granules are dividing into smaller ones. Note that lipid granules are degrading at the periphery and the cytoplasm becoming vacuolated. **Fig. 41.** Initiation of cleavage. Small and large vesicles are coalescing for cytoplasmic cleavage. **Fig. 42.** Lipid granules are degrading at the periphery and thin layered structures can be observed. **Fig. 43.** Major anterior rootlets (arrow) run along cytoplasmic cleavage.



Figs. 44–47. Pre-matured stage of the unilocular sporangium. Fig. 44. A chloroplast exists near a nucleus. The cavity among zoospores in the unilocular sporangium is filled with fibrous and granular material accompanying with degradation of lipid granules. Note the perinuclear ER swelling. Fig. 45. Premature zoospore. Note swollen perinuclear ER and the formation of adhesive vesicles (arrow). Fig. 46. Golgi body and adhesive vesicles. The content of adhesive vesicles is composed of electron-light core and electron-dense periphery. The surface of adhesive vesicles in this stage is very rough and notched. Note mastigonemes in the cisternae of swollen ER. Fig. 47. Longitudinal section of a fully-matured unilocular sporangium. Each zoospore is completed.

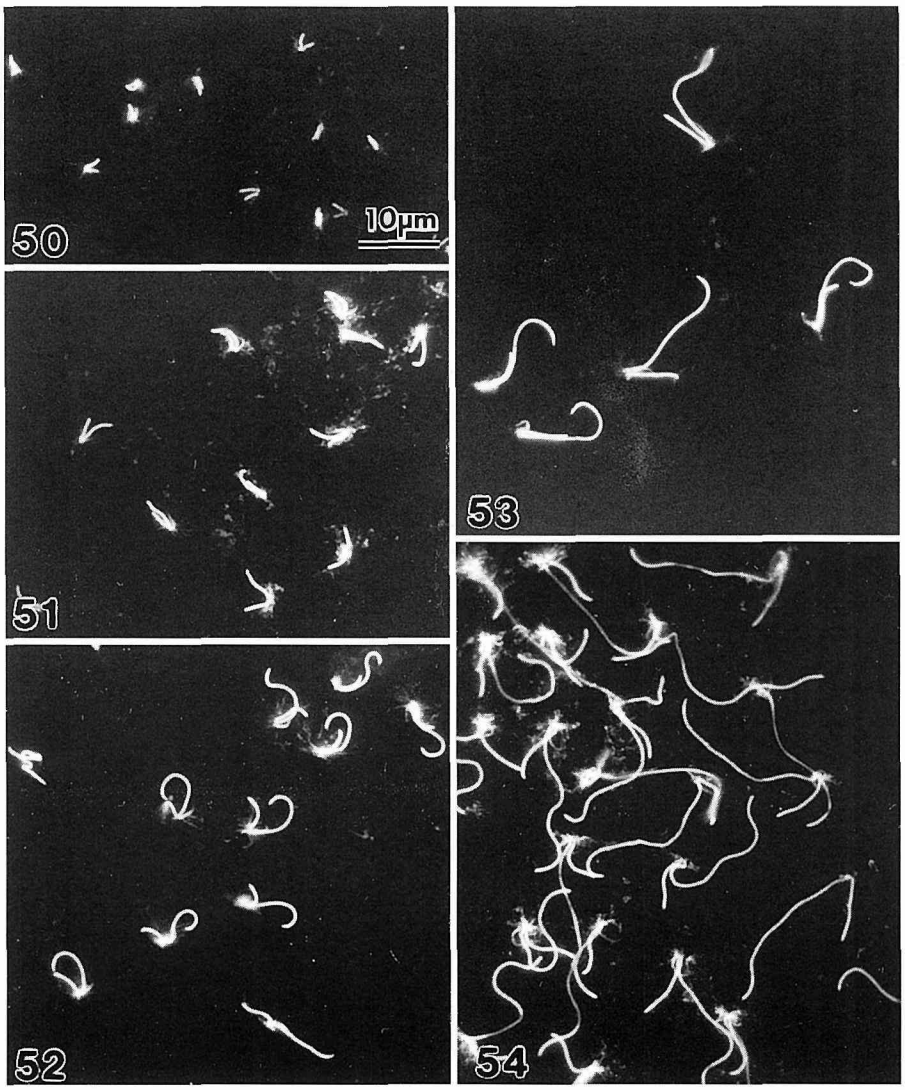


Figs. 48, 49. A liberated zoospore of *Laminaria angustata* observed under scanning (Fig. 48) and transmission electron microscope (Fig. 49).

was filled with fine fibrous material (Fig. 45). When zoospore formation was completed, these perinuclear ER layers were no longer visible (Fig. 49). Adhesive vesicles were usually differentiated into electron-dense periphery and electron-light core containing fine fibrous material. When adhesive vesicles were initially produced, their surfaces were very rough and notched (Fig. 46). However, in the cytoplasm of liberated zoospore, adhesive vesicles were elongated and curved, containing granular electron-dense material (Fig. 49). The cup-shaped chloroplast enclosed the nucleus. Afterwards, the chloroplast started to divide into two. Completed zoospores in the unilocular sporangium were ellipitcal in form and ER surrounding the nucleus could not be observed (Fig. 47).

Flagellar elongation in the unilocular sporangium

After 32 nuclei were formed in the unilocular sporagium after meiosis followed by 3 nuclear divisions, anterior and posterior flagella grew from the basal bodies. Anti-β tubulin immunofluorescence microscopy showed that the flagella elongated from MT foci (= centrosomes; centrioles under electron microscopy). Initiation of flagellar formation occurred simultaneously in the unilocular sporangium (Fig. 24). As the flagellar elongation progressed, MTs radiating from each centrosome became sparse (compare Fig. 23 and Fig. 24). Figures 50-54 show various developmental stages of flagellar elongation. These samples were prepared by air-drying after fixation for easy observation on flagellar elongation. It



Figs. 50–54. Various developmental stages of anterior and posterior flagella in the unilocular sporangia. Anti-β tubulin immunofluorescence staining on air-drying samples. Note that anterior (long) and posterior (short) flagella can be distinguished from the very early stage of flagellar elongation. Scale in Fig. 50. applies also Figs. 51–54.

became clear that anterior and posterior flagellar elongation occurred synchronously in one unilocular sporangium. Careful observation revealed that the anterior and posterior flagella (= long and short flagella) can be distinctive in very early stages of flagellar elongation. In Figure 55, each dot shows the relationship between the length of anterior (= long) flagellum

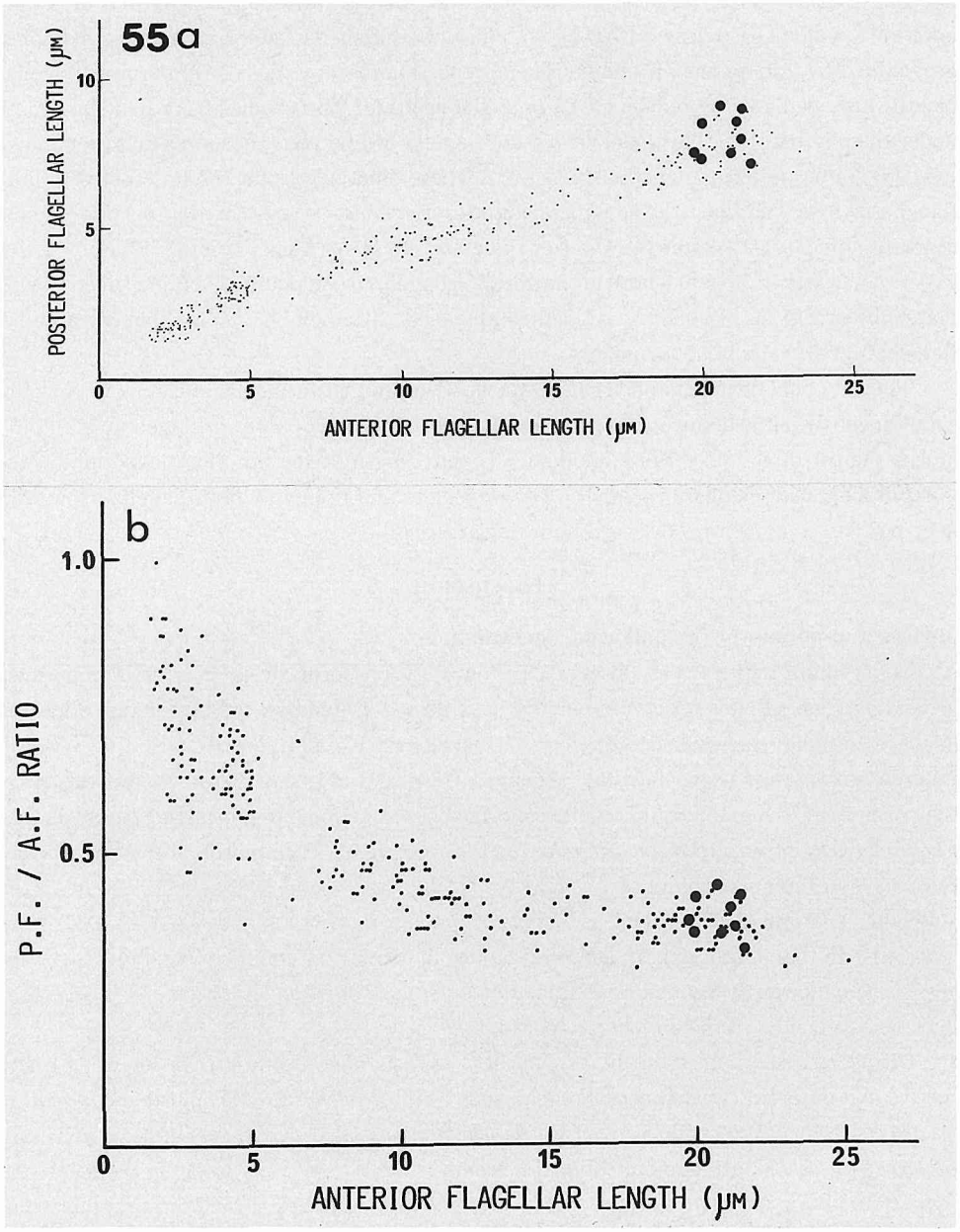


Fig. 55. Flagellar length relationships during flagellar elongation in the unilocular sporangia. Each dot shows the relationship between the anterior flagellar length and the posterior flagellar length (a) or between the anterior flagellar length and the ratio between the posterior and anterior flagellar length (b). Large dots show the case of liberated zoospores.

and the length of posterior (= short) flagellum (a), or the ratio between the posterior and anterior flagellum (b, redrawn from a) of a pair of both flagella. Centrioles (= basal bodies) are stained by anti-β tubulin immunofluorescence microscopy, therefore, the real flagellar length was calculated by reducing 0.8 μm as the length of basal bodies from flagellar length under the microscope. Large dots show the flagella of liberated zoospores (anterior flagellum: 20-22 μm, posterior flagellum: 7-9 μm). These data prove that the ratio between both flagella is over 0.5 when the length of anterior flagellum is less than 5 μm, but become constant value (0.3-0.4) after the anterior flagellum elongates longer from 12-13 μm. In this experiment, data in 5-7 μm length of the anterior flagellum could not be obtained even though many observations of flagellar developmental stages. It might be possible that the anterior flagellum grow rapidly at this stage.

Thus, it could be understood that, (1) the anterior and posterior flagella start to elongate simultaneously in the unilocular sporangia; (2) the growth rate of anterior flagellum is slightly higher than it of the posterior flagellum till 5 μm length of the anterior flagellum; (3) the anterior flagellum elongate rapidly at 5-7 μm lenght; and finally (4) both flagella grow with keeping the constant ratio between both flagllar length.

Discussion

Meiosis and mitosis in the unilocular sporangium

Many light microscopic observations on the zoosporogenesis in the Laminariales originated from KYLIN's observation (1918) have demonstrated that the first nuclear division in the sporangial parent cell is meiotic. OHMORI (1967) reported the zoosporogenesis in *Laminaria angustata* using light microscopy. TOTH and MARKEY (1973) showed the ultrastructure of the synaptonemal complexes in meiosis of unilocular sporangial parent cell of *Chorda tomentosa* and *Pylaiella littoralis* for the first time. Afterwards, the synaptonemal complexes in the antheridium of *Fucus serratus* (BERKALOFF and ROUSSEAU 1979) and in the unilocular sporangial parent cell of *Halopteris filicina* (KATSAROS and GALATIS 1986) were reported. In the present experiment, synaptonemal complexes could be found ultrastructurally in the unilocular sporangial parent cell of *Laminaria angustata*. Therefore, it is evident that meiosis occurs in the unilocular sporangia.

Ultrastructural studies on the brown algal mitosis have been carried out using many species and different developmental stages, such as in: *Pylaiella littoralis* plurilocular sporangia (MARKEY and WILCE 1975), *Sphacelaria tribuloides* apical cell (KATSAROS *et al.* 1983), *Cutleria hancockii* male gametangium (LA CLAIRE and WEST 1979), *Cutleria cylindrica* trichothallic meristem (LA CLAIRE 1982), *Carpomitra cabrerae* trichothallic meristem (MOTOMURA and SAKAI 1985), *Fucus vesiculosus* antheridium (LEEDALE 1970), *F. vesiculosus* embryo (BRAWLEY *et al.* 1977), *F. serratus* antheridium (BERKALOFF and ROUSSEAU 1979), and *Hormosira banksii* embryo (FORBES and HALLUM 1979). Recently, the process of nuclear division of *Laminaria angustata* zygotes was observed by anti-β tubulin immunofluorescence

microscopy (MOTOMURA 1991). Although the degree of nuclear membrane breakdown is different among species, these observations show that there are no significant deviations in the process of the brown algal nuclear division. Established processes of nuclear division in the brown algae can be summarized as follows; 1) In vegetative cells, MTs radiate from one centrosome, which is composed of a pair of centrioles, similar to that in animal cells. This MT array is the core of MT cytoskeleton in the brown algae. 2) Accompanying chromosome condensation, a centrosome divided into two and each of them migrates to both poles. Several ultrastructural studies suggested that the duplication and migration of centrioles occurred in early prophase (MARKEY and WILCE 1975, LA CLAIRE 1982, MOTOMURA and SAKAI 1985). 3) In prophase, numerous MTs radiate toward the polar depression of nuclear membrane from pericentriolar region at both poles, and finally polar fenestrations of nuclear membrane are formed. MTs which elongate toward the cytoplasm gradually become shortened. 4) In metaphase, typical spindle is completed, and almost all MTs participate to the spindle MTs. Kinetochores have been reported only in *Sphacelaria tribuloides* (KATSAROS *et al*. 1983). 5) In anaphase and telophase, chromosomes separate to both poles and are surrounded by nuclear membrane. Interzonal spridle MTs can be observed. Derivative nuclei receive one centrosome (a pair of centrioles) and MTs start to radiate toward cytoplasm like the interphase stage. Meiosis and mitosis in the unilocular sporangium in *Laminaria angustata* proceed in the above-mentioned manner. Nuclear division occurs synchronously as in other multinucleate algae, for example *Griffithsia* (LEWIS 1909), *Blastophysa* (SEARS 1967) and *Valonia* (STAVES and LA CLAIRE 1985),*etc*.

Different from vegetative cells, cytokinesis and cell wall formation do not occur after nuclear division in the unilocular sporangium. MT systems, like phycoplast and phragmoplast, have not been observed along a furrowing plane in the brown algal cytokinesis (RAWLENCE 1973, MARKEY and WILCE 1975, LA CLAIRE 1981, KATSAROS *et, al*. 1983). BRAWLEY and ROBINSON (1985) reported that cytokinesis of *Fucus* and *Pelvetia* proceeds with a system of actin microfilaments. Unfortunately, detailed observations have not been carried out on the behavior of actin microfilaments systems in the brown algal cell cycle. Microfilament dynamics through mitosis in the unilocular sporangium might be different from the case of vegetative cells.

Mt cytoskeleton in the unilocular sporangium

Recently MOTOMURA (1991) reported the MT cytoskeleton in the development of *Laminaria angustata* zygotes and parthenogenetic sporophytes. It became clear that almost all MTs elongate from the centrosome which is constituted of a pair of centrioles. The unilocular sporangium of the brown algae possesses a multinucleate cell in the process of zoospore formation. SHIHIRA-ISHIKAWA (1987) reported on MT systems in coenocytic green alga *Ventricaria ventricosa* (as *Valonia ventricosa*, OLSEN and WEST 1988) in detail. She reported two types of MT systems; cortical MTs and nuclear associated MTs. In this alga,

nuclear associated MTs extend radially from peri-nuclear region of each nucleus and the ends of these MTs adhere to the chloroplasts surface. She suggested that nuclear associated MTs support an even distribution of nuclei and the positional relationships between nuclei and chloroplasts in the coenocytic condition. In the present study, centrosomes near each nucleus located at periphery and MTs radiate from each centrosome in interphase stage of the unilocular sporangium in *Laminaria angustata*. As a whole, MT cytoskeleton possessed a basket-like arrangement, and nuclei are evenly spaced in this "basket". Thus, in the multinucleate stage of the unilocular sporangium of brown algae, MTs radiating from the centrosome near each nucleus would have the same function on organelle spacing as occurs in the coenocytic green alga *Ventricaria*. This MT arrangement must have a critical role for zoosporogenesis in unilocular sporangium, because zoospores must have a nucleus, a chloroplast, a Golgi body and other cellular organella.

Chloroplast

NISHIBAYASHI and INOH (1956) and OHMORI (1967) reported 4-7 chloroplasts in the sporangial parent cell of *Laminaria angustata*. TOTH (1974) reported 2-3 chloroplasts in *Chorda tomentosa*, and CHI and NEUSHUL (1972) reported 4 chloroplasts in *Macrocystis angustifolia*. These observations in the Laminariales show that the number of chloroplasts in the sporangial parent cell is random. Zoospores have a nucleus, a chloroplasts, a Golgi body and several mitochondria, and therefore, a ratio between the number of nuclei and chloroplasts must become 1:1 at a certain stage in the process of unilocular sporangium formation. After that, the division of chloroplasts must be coordinated with mitosis. In this study, exact timing of close association between a nucleus and a chloroplast could not be determined. In the unilocular sporangial parent cell, the close contact between nucleus and chloroplast could not be detected and polar centrioles did not exist near the chloroplast at the first anaphase of meiosis. After meiosis I, centrosomes did not exist at the periphery, but after meiosis II, each centrosome of four nuclei existed at the periphery and MTs from these centrosomes possessed a basket-like arrangement accompanying with chloroplast migration to the periphery. Adding to the immunofluorescence study, ultrastructural observations showed that a pair of centrioles on each nucleus located near the chloroplast and Golgi body in interphase and mitotic period at the multinucleate stage after meiosis. Therefore, it would be proper to consider that a correlation between a nucleus and a chloroplast might start after meiosis. It is well known that nuclei and chloroplasts become to be in close contact during the zoosporogenesis in the unilocular sporangium (BAKER and EVANS 1973, TOTH 1974, MARKEY and WILCE 1976 a). MARKEY and WILCE (1976 a) reported that chloroplasts were distributed throughout the cytoplasm at the first mitosis after meiosis in *Pylaiella littoralis* unilocular sporangium, but after that, the nuclei and chloroplasts were close in contact when they were present at the sporangium periphery.

Cap structure of unilocular sporangium

At the initiation of cap formation of the unilocular sporangium, a large number of mitochondria were observed at the tip of unilocular sporangium which had become to contain 32 nuclei. This gathering of mitochondria at the tip of sporangium had not been reported previously. Although the significance of this phenomenon is obscure, it seems to be related to the cap formation.

TOTH (1974) found numerous small vesicles just below the plasmamembrane in the region of the cap of the unilocular sporangium of *Chorda tomentosa*, and indicated that these vesicles may be involved in the cap formation. These results can be also confirmed in *Laminaria angustata*. In this case these vesicles seem to be derived from ER, not from Golgi bodies. The material comprising the sporangial cap had been thought to be a sulfated polysaccharide (TOTH 1974, EVANS *et al*. 1973). The results obtained in the present experiment of staining test with toluidine blue-O and PATAg also suggested that the sporangial cap is comprised of the sulfated polysaccharide, which is the same in oogonial and antheridial caps of *Laminaria* (MAIER 1982).

Mitochondria

The tubular intracristal inclusions are found in mitochondria in the motile cell of the brown algae (MARKEY and WILCE 1976 a, POLLOCK and CASSELL 1977, CASSELL and POLLOCK 1979, LA CLAIRE and WEST 1979, HENRY and COLE 1982). In the present study, the tubular intracristal inclusions became apparent in the mitochondria accompanying flagellar formation, but were not evident in mitochondria in the sporangia before flagellar differentiation. Their presence would be correlated with the functional requirements of free-swimming cell.

Flagellar formation

In *Laminaria angustata*, flagellar formation is intiated before the cytoplasmic cleavage like in the unilocular sporangium formation in other species of the brown algae. On the other hand, it is noteworthy that flagellar formation is intiated after the completion of cytoplasmic cleavage in the *Fucus serratus* antheridium (BERKALOFF and ROUSSEAU 1979). Centrioles migrate to the plasmamembrane to function as flagellar basal bodies and both anterior and posterior flagella elongate in the space between plasmamembrane and the cell wall as described for other laminarean plants (CHI and NEUSHUL 1972, TOTH 1974, HENRY and COLE 1982). Whereas, in several brown algal species of the Ectocarpales (LOISEAUX 1973, MARKEY and WILCE 1976 a) and the Sphacelariales (KATSAROS and GALATIS 1986), flagellar vesicles are formed initially, and subsequently flagella develop in the flagellar vesicles in the cytoplasm. When all nuclei in the unilocular sporangium can migrate toward cell periphery like the Laminariales, both flagella elongate between the plasmamembrane and the cell wall. But if there are much more nuclei in the unilocular sporangium, flagellar vesicles would be needed for flagellar elongation especially at the central part. Probably, these flagellar

vesicles formation might be related to the number of zoospores which will be formed in the unilocular sporangium. As mentioned by MARKEY and WILCE (1976 a), these different patterns of flagellum development would be worthwhile on whether this characteristic has any phylogenetic significance or not within the Phaeophyta.

Anti-β tubulin immunofluorescence observation shows that anterior and posterior flagella elongate simultaneously from each centrosome in the unilocular sporangium, and both flagella can be distinguished by their length from initial stage of flagellar elongation. It means that differentiation of each centriole into anterior or posterior flagellar basal body would be occurred in very early stage, including a possibility that centrioles already have this potential even in vegetative cell stage of the brown algae.

Mastigoneme

Mastigoneme elements have been observed in various cellular parts: vesicles from the nuclear envelope in *Fucus* and *Ascophyllum* spermatogenesis (BOUCK 1969, BERKALOFF and ROUSSEAU 1979), Golgi cisternae in several species of the Ectocarpales (LOISEAUX 1973), and Golgi cisternae and ER in *Pylaiella* (MARKEY and WILCE 1976 a, b), *Cutleria* (LA CLAIRE and WEST 1978) and *Halopteris* (KATSAROS and GALATIS 1986). In *Laminaria angustata*, mastigoneme elements were found in Golgi cisternae and ER, but not in the nuclear envelope.

Cytoplasmic cleavage

In the present study, the cytoplasm of sporangial parent cell begins to divide into individual zoospores after flagellar formation. In most species of the brown algae, the cytoplasmic cleavage in zoosporogenesis has been reported to start after flagellar formation (LOISEAUX 1973, TOTH 1974, MARKEY and WILCE 1976 a), except for *Fucus serratus* antheridia (BERKALOFF and ROUSSEAU 1979).

In *Laminaria angustata* unilocular sporangium, the cytoplasmic cleavage is mainly initiated by fusion of several vacuoles and ER. These vacuoles were probably produced by Golgi bodies and by the degeneration of peripheral parts of lipid granules. The cytoplasmic cleavage was proceeded without the involvement of microtubule systems as in the process of zoidogenesis of other brown algae (LOISEAUX 1973, TOTH 1974, MARKEY and WILCE 1976 a, BERKALOFF and ROUSSEAU 1979), although a major anterior rootlet runs along the cytoplasmic cleavage in the present observation. As mentioned above, BRAWLEY and ROBINSON (1985) using NBD-phallacidin labeling, observed that cytokinesis of *Fucus* and *Pelvetia* proceeds with the system of actin microfilaments using NBD-phallacidin. Therefore, it would be proper to consider that the cytoplasmic cleavage into each zoospore is proceeded by microfilament system, as in vegetative cell cytokinesis in the brown algae.

Golgi body and ER

The features of Golgi bodies and ER changed drastically during the course of zoosporo-

genesis. ER developed well from the sporangial parent cell till the completion of individual zoospore, and ER predominantly surrounded the each nucleus. ER swelled before and after the cytoplasmic cleavage, and its lumen was filled with fine fibrous material. Golgi bodies seemed to be inactive at the multinucleate stage until flagellar formation, and then they begin to produce large vesicles containing fibrous and granular material at the same time as swelling of ER. The active Golgi bodies and swollen ER had been reported in the secretory cell of *Laminaria* spp. (EVANS *et al.* 1973). TOTH (1974, 1976) suggested that the mass of mucilaginous carbohydrate was accumulated around the zoospores in the unilocular sporangium. These active features of Golgi bodies and ER could be related to the production of mucilage in the unilocular sporangium. This mucilage composed of alginic acid and sulfated polysaccharide may be useful for the protection and release of zoospores (TOTH 1976).

OLIVEIRA *et al.* (1980) reported that adhesive vesicles are produced in the dictyosome of the zoospores prior to their release from zoosporangia in *Laminaria saccharina* and *Nereocystis luetkeana*. In *Laminaria angustata*, the formation of adhesive vesicles was initiated immediately following the cytoplasmic cleavage into the individual zoospore. Adding to the Golgi bodies, ER also appear to be related to the production of adhesive vesicles. On the basis of their cytochemical work, OLIVEIRA *et al.* (1980) suggested that the contents of adhesive vesicles are glycoprotein in nature.

Lipid granules

One of the characteristic features in the unilocular sporangium formation is an accumulation of a large number of lipid granules. CHI and NEUSHUL (1972) reported the accumulation of the gray-colored food vacuoles during the zoidogenesis of *Macrocystis angustifolia*. TOTH (1974) and HENRY and COLE (1982) observed numerous "type B" vacuoles in the unilocular sporangia and zoospores of the Laminariales. Here, "type B" vacuoles was named by RAWLENCE (1973), which was uniformly filled with less dense material in cells of *Ascophyllum nodosum*. In the present experiment, vacuoles corresponding to gray-colored food vacuoles and "type B" vacuoles are stained positively with oil red-O and sudan black-B, therefore, they are considered to be lipid granules. KATSAROS and GALATIS (1986) also reported that numerous granules containing lipophilic material accumulated in zoosporangium of *Halopteris filicina*. Since these lipid granules are also observed in liberated zoospores, they would be consumed as storage material during the germination of zoospores.

Large lipid granules divide into several small granules toward the maturation of unilocular sporangium after a series of nuclear division. These lipid granules degrade gradually from the periphery by the formation of electron dense granules and thin layers which are arranged regularly. These structures around lipid granules are stained pink color with toluidine blue-O, therefore, they seem to be composed of acidic polysaccharides. Probably the metabolic change from lipid material to acidic polysaccharides would have an important role for the accumulation of mucilage in the unilocular sporangium, adding to the

Golgi bodies and ER function. Further studies concerning the metabolic relationship between lipid material and acidic polysaccharides in the brown algae are necessary.

Summary

Zoosporogenesis in the unilocular sporagia of *Laminaria angustata* KJELLMAN was studied using electron microscopy and anti-β tubulin immunofluorescence microscopy. The initial change for zoosporogenesis in *Laminaria angustata* is elongation and transverse division of meristoderm cells, and the upper cell derived from a cell division differentiates into a paraphysis. The sporangial parent cell is developed from the lower cell among paraphyses. Meiosis occurs in the sporangial parent cell. This is supported by observations on synaptonemel complexes in prophase nucleus. After meiosis, the unilocular sporangium increases in size with synchronous nuclear divisions. Meiosis and mitosis in the unilocular sporangium proceed as the typical manner in the brown algal mitosis. MTs radiate from centrosome near each nucleus and possess a basket-like arrangement in interphase stage of the unilocular sporangium. Chloroplasts migrate toward the periphery and each pair of centrioles (= centrosome) exists near a nucleus and a chloroplast. Accompanying the development of unilocular sporangium, ER develops especially well around each nucleus. Large amounts of lipid granules are accumulated. When a sporangium reaches the 32-nucleate stage, a sporangial cap is formed at the tip. Numerous mitochondria are observed at the initial stage of cap formation. During the cap formation, flagellar formation is initiated. Anterior and posterior flagella elongate simultaneously and both flagella can be distinguished at the initial stage of flagellar elongation by their length. In the unilocular sporangium, both flagellar basal bodies arrange parallel. Before the cytoplasmic cleavage into individual zoospores, large lipid granules divide into small ones, and acidic polysaccharides are formed around lipid granules. Also, Golgi bodies appear active and ER becomes swollen. The cytoplasmic cleavage into each zoospore does not involve the MT system.

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