LIGHT MICROSCOPY OF MEIOTIC ZOOSPOROGENESIS AND MITOTIC GAMETOGENESIS IN *ULVA MUTABILIS* FØYN

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SUMMARY

The light-microscopical cytology of synchronized meiotic zoosporogenesis and mitotic gametogenesis in the sea-lettuce *Ulva mutabilis* Føyn, is described. The similarity in time course and morphological events of the 2 processes is stressed and discussed with particular reference to the use of *Ulva* in the biochemical study of cellular preparation for meiosis and mitosis.

INTRODUCTION

Among the chlorophycean algae the members of the family Ulvaceae are characterized by a multicellular structure and a haplo-diplontic life-cycle that proceeds without formation of special generative organs. The transformation in these algae of ordinary vegetative cells into generative ones producing either gametes mitotically or zoospores meiotically was a major reason for introducing *Ulva mutabilis* Føyn as an object for the biochemical study of meiosis (Nordby & Hoxmark, 1972). In this connexion it was felt necessary to carry out a closer examination of the cytology of the 2 kinds of generative divisions mentioned.

In the literature, the generative divisions in *Ulva* species have been partly described by Carter (1926), Føyn (1934), Smith (1947) and Yabu & Park (1969).

The most detailed description of the nuclear events is given by Føyn, who also was the first to show that genetic segregation took place during meiosis in this alga.

For *Ulva mutabilis* specifically, short electron-microscopical descriptions have been given by Bråten (1971), Bråten & Løvlie (1968) and Bråten & Nordby (1973). Of studies on related algae, the most extensive report is probably that of Ramanathan (1939) on *Enteromorpha*.

The present report concerns only the gametophytes and diploid sporophytes. The parthenosporophytes are dealt with elsewhere (Hoxmark & Nordby, 1974).

MATERIALS AND METHODS

The alga

*Ulva mutabilis* Føyn was originally found on the coast of Portugal in 1952. Descendants from these plants have since been cultivated in unialgal culture in our laboratory. The organism has been used in a number of genetical, physiological/biochemical and electron-microscopical studies – a review has been given by Løvlie (1968).
The alga has a main life-cycle as shown in Fig. 1. Several mutants exist and are regularly held in culture. Among these, the undifferentiated fast growing 'Slender' (SI) has been found especially valuable also for our present studies on the biochemistry of meiosis, since its transition from vegetative to generative division pattern is fairly easy to control and synchronize (Nordby & Hoxmark, 1972). The pattern of generation shifts is the same in the mutant and the wild type. The mutant contains only one cell type, equivalent to the blade cells in the wild type (Lovlie, 1964).

![Diagram of Ulva mutabilis, Slender phenotype life cycle](image)

Fig. 1. Simplified life cycle of *Ulva mutabilis*, Slender phenotype. The fusion of 2 gametes of opposite mating types gives rise to a zygote which resorbs its flagella after settling down. The zygote germinates into a diploid sporophyte in the form of a flattened tube, where the tube wall consists of one cell layer. By meiosis and subsequent mitotic divisions the sporophyte thallus cells are transformed to sporangia. The 4-flagellated haploid zoospores that emerge are of mating types + and −, but do not fuse. Instead they settle and grow into haploid + and − gametophytes of the same general morphology as the sporophyte generation. From these plants + and − gametes are formed mitotically.

**Cultivation**

Haploid algae (gametophytes) were grown from unisexual zoospores obtained from parthenosporophytes. Diploid algae (sporophytes) were grown from zygotes prepared from gametes of mating types plus and minus as described by Foyn (1959). Both types of plants were first grown in Petri dishes in enriched seawater for about 2 weeks, then explanted into 10-l. aerated glass aquaria (Nordby & Hoxmark, 1972). A light-dark programme of 17 h light, 7 h dark was held throughout the growth period. The light period was from 04.00 to 21.00 hours.

The Petri dishes were illuminated from one side with cool white fluorescent tubes (Philips TL 32) giving a light intensity of 10500 lux measured at dish centre. The aquaria were illuminated from above with Powergroove tubes giving about 4500 lux measured at bench level. The temperature was 17-18 °C.
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Induction of generative divisions

Four-week-old algae harvested at 14.00 hours were fragmented, washed and transferred to Petri dishes in the sporulation room as previously described (Nordby & Hoxmark, 1972). The sporulation room had a temperature of 21 °C, and its dark period in the 17 h/7 h light programme ran from 14.00 to 21.00 hours. The dishes were illuminated from the side with 10,500 lux. Under these conditions zooid formation was induced synchronously in nearly all cells. The resulting gametes or zoospores were released during the third light period after transfer to the sporulation room.

Cytological methods

Samples of algae or algal fragments were fixed in Bouin-Dubosque's solution (0.75 % picric acid in 80 % ethanol (150 ml), 40 % formalin (24 ml) and glacial acetic acid (15 ml)). After fixation or storage in the fixative, the samples were decolourized in 70 % ethanol, brought down to water and hydrolysed for 15 min at 60 °C in 1 N HCl. They were then rinsed in water and stained for 40 min at 60 °C in haematoxylin-chrome-alum prepared according to Schweizer (1942) as follows: 5 g of chrome alum are dissolved in 125 ml, 40 % ethanol and boiled until the solution turns completely green. Haematoxylin (0.5 g) is added by stirring into the still-hot solution. After cooling, 4 ml 10 % H2SO4 are added by stirring, and 10 ml 25 % K2Cr2O7 are added dropwise. The solution is rapidly brought to the boil for 2–3 min (until the colour turns violet and the solution shows increased tendency to adhere to glass). The stain is then allowed to cool and is filtered before use. Best results are obtained with freshly made batches of stain.

If necessary, further differentiation was performed in 45 % acetic acid (2–10 min) after staining. The preparations were then washed in water, blued in ammoniacal water, dehydrated and mounted in Eukitt.

Photographs were taken with a Zeiss photomicroscope II. Measurements were made with a Zeiss screw ocular micrometer, or a drawing apparatus was used to trace on paper the mutual contour of 100 cells or sporangia from a fragment. Ten to thirty such areas were drawn from each preparation and measured planimetrically.

Length measurements on whole algae were made either by using a simple sighting device, or by means of a cathetometer as described by Lovlie (1964). The algae were suspended in growth medium by a thin nylon thread tied to one end and stretched by a weight tied to the other end.

RESULTS

Induction pattern of generative divisions in whole plants and fragments

Since there appear to be no conspicuous differences between the patterns in mitotic gamete formation and meiotic zoospore formation these processes will here be treated together and called 'sporulation' or 'zooid formation'. When intact Ulva plants are cultivated in enriched seawater, mitotic growth may cease spontaneously and be succeeded by zooid formation (Fig. 2). Predominantly, this happens in old or over-dense cultures. More conspicuous is the burst of zooid formation that occurs 2–3 days after renewal of the growth medium.

In the wild-type plants only the blade cells are capable of forming zooids. The 2 other cell types present, the giant stem cells and the rhizoid cells have never been observedable to form zoospores or gametes (Lovlie, 1964). Maximally, all blade cells in the thallus may be synchronously transformed to sporangia or gametangia. More often, however, only parts of the blade will sporulate at one time. The 'induction
of zooid formation' then seems to spread successively for each period in the light–dark cycle from the first committed areas. Typically, the process starts at the blade rim where the two cell layers do not adhere as closely to each other as in the greater part of the wild-type blade. The division between sporulating and non-sporulating areas of the blade in healthy algae is usually sharp, also when seen in the microscope. A

\[ \text{Fig. 2. Length measurements during growth and spontaneous complete sporulation of a gametophyte (Sl G+, □) and a sporophyte (Sl/Sl, ■). Note cessation of thallus expansion about 1 day before either gametes (from Sl G+) or zoospores (from Sl/Sl) are released at times marked with (•). Black bars indicate dark periods.} \]

'diffuse sporulation' with thalli showing an interspersion of vegetative and generative cells is, on the other hand, characteristic of algae that have been grown under poor conditions or in overcrowded cultures, or of algae that have been stored in the cold room for long periods.

In mutants where the 2 cell layers in the thallus show much less adherence to each other, such as 'Bubble' (bu) and 'Slender' (Sl) the synchronously sporulating areas are relatively bigger. In these mutants that lack the giant stem cells and the rhizoid cells, it sometimes happens that more than 90% of thallus cells virtually explode synchronously into gametes or zoospores. As indicated from cell counts (Nordby & Hoxmark, 1972), the transformation to generative divisions is, at least in Slender, preceded by a block of ordinary somatic mitoses and later also by a cessation of thallus expansion (Fig. 2). Other more precise cathetometer measurements on diploids show that the stop in thallus expansion is a rather late and abrupt event occurring in the
light period at a time that probably coincides with onset of the prophase of the first meiotic division (Fig. 3).

It has been known and utilized for years that tearing of the thalli with needles before transfer to fresh growth medium stimulates greater parts of the thalli to form zooids within the first 2–3 days after transfer. We have used this principle by fragmenting thalli in a razor-blade blender as described elsewhere (Nordby & Hoxmark, 1972). With the selected light–dark programme zooid formation may then regularly start in more than 90% of the cells within 48 h after transfer. Typically, this coincides with having nearly all fragments single-layered. Double-layered fragments tend to form zooids the next (third) day, or may continue vegetative growth for some time.

Also in fragments typical sharp borders are observed between sporulating and non-sporulating areas.

**Cytology of meiotic zoosporogenesis in diploids**

**In vivo observations.** The vegetative cells in mature diploid Slender (SI/SI) thalli have a free outer cell area of $147 \pm 21 \, \mu m^2$, and a height, i.e. thickness of cell layer, of approximately 20 $\mu m$. The chloroplast is situated within the cell more or less as indicated in Fig. 4, with the nucleus in a nearby position. As described by Bråten &
Løvlie (1968) the nucleus is spherical and bears a prominent nucleolus. The interphase nuclear diameter is $2.9 \pm 0.4 \mu m$ in fixed and stained preparations.

Even if vegetative mitoses seem to be blocked at transfer to fresh medium (Nordby & Hoxmark, 1972), the cells continue to grow for about 40 h, and in meiotic prophase have an average outer free surface area of $226 \pm 27 \mu m^2$.

![Fig. 4](image-url)

Fig. 4. Schematic view of nuclear orientation and movement during somatic mitosis and meiotic zoospore formation. Left: A somatic interphase cell seen in 2 projections, where abcd designates cell walls against neighbour cells, o and i in the outer and inner thallus surfaces respectively. In the cell are shown the nucleus (black) and the chloroplast (hatched). To the right are shown stages in somatic mitosis (upper half) and in meiosis (lower half) as seen in the same projections. Note differences in the position of the nucleus and movement of daughter cells after meiosis. (From Bråten & Nordby, 1973.)

During the first light period after transfer, starch granules accumulate in the chloroplast forming a pattern that is characteristic for pre-sporogenic cells. In the first part of the second light period, the future exit pore for the zoospores is formed as a protruding bulge of the outer cell wall. Also in single-layered fragments the future pores, with very few exceptions (approximately once in 10000 cases), are formed on the former outer thallus surface. Thereafter the cells apparently lose their turgor, become more rotund and reorient within the cell wall envelope, as shown in Fig. 4. During the meiotic prophase the nucleus, now rotating at 3-4 rev/min, wanders from its position near the chloroplast to the centre of the opposite cell wall, i.e. a cell wall against a neighbouring cell. Very often hundreds of neighbouring cells show a coordinated orientation, and this also applies to fragments from a suspension culture. Any directional influence of light on this orientation can thus be eliminated. As shown in Fig. 4, this nuclear orientation is different from that found during somatic mitosis (Løvlie & Bråten, 1970), where the dividing nucleus lies close to the cell wall that constitutes the inner thallus surface.
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The chloroplast is seen to be stretched around the cell, and the use of appropriate filters reveals that the chromogenic granules that will later form the eyespot of the zoospores (Braten & Lovlie, 1968) are already aggregating.

The first cytokinetic plane lies perpendicular to the cell layer plane, but already during anaphase the cell rotates within the cell wall envelope. Thus, when the first meiotic division is completed, one finds the daughter cells lying on top of each other as indicated in Fig. 4. The same divisional rotation pattern is now repeated for the second meiotic division and for the two mitoses that follow. As published elsewhere (Braten & Nordby, 1973), the resulting 16 haploid daughter cells form a hollow sphere within each sporangium. Electron micrographs show that the flagella grow into the lumen of this sphere. Later the daughter cells differentiate into zoospores, thereby becoming oblong in shape.

All meiotic and postmeiotic divisions, and the differentiation of the spores, can take place in the dark. With the 17/7 h light–dark programme, the first meiotic cytokinesis usually takes place at the beginning of the dark period while the release of the mature zoospores takes place in the middle of the following light period. If matured in an extended dark period the zoospore release is triggered by illumination within a couple of minutes. As reported for Enteromorpha (Ramanathan, 1939), the Ulva zoospores escape rear end first. The hole formed in the exit protuberance is narrow enough to give the zoospore an hour-glass shape as it passes through. For some seconds the zoospore now lies nearly motionless on the thallus surface, then its cytoplasm seems to stiffen and the flagella start to beat. As reported by Feyn (1958), the zoospores, each of which bears 4 flagella, are first positively phototactic, but soon change their reaction and settle in the dark part of the culture dish. The morphology and settling behaviour of the zoospores have been described by Feyn (1958) and by Braten & Lovlie (1968).

Sometimes, especially when old algae are used as starting material, abnormal spores with 2 or more apices and sets of flagella are formed and released. Division of such abnormal spores may occasionally be followed in the microscope.

The nuclear division. Haematoxylin-chrome-alum stained preparations make it easy to follow the stages in nuclear division (Figs. 5, 6–14). Counter-staining with eosin visualizes the pyrenoid in the chloroplast especially well. After transfer to fresh growth medium, the nucleus steadily increases in diameter from 2.9 to 4.5 ± 0.7 μm. The final size refers to the first discernible stage, synizesis, in the meiotic prophase where the nucleus is still fairly spherical.

This first stage in the meiotic prophase starts while the nucleus still lies close to the chloroplast, and lasts until the nucleus has wandered into division position. In stained preparations, the stage is characterized by having the chromatin condensed into a tight synizetic knot (Fig. 7). As reported elsewhere (Braten & Nordby, 1972), no signs of such chromatin condensation are seen in specimens fixed in glutaraldehyde for electron microscopy.

At the end of this stage, which may last for some 1.5 h, the synizetic knot loosens up and the nuclear volume is filled with fine chromosomal threads (Fig. 8). Doubleness of the fibres is not discernible nor is it possible to sort out conventional meiotic
Fig. 5. Time/percentage plot of stages in the synchronized formation of zoospores (upper curves) and of gametes (lower curves). ○, pre-generative interphase; ●, synizesis in meiosis or first generative prophase in gametogenesis; □, spiremal stage in meiosis; △, diakinesis-anaphase I, respectively metaphase-anaphase; ■, 2-cell stage; □, 3-4 cell stage; △, 4-8 cell stage; ▲, stage with more than 8 cells per sporangium or gametangium. Dotted curves in lower diagram show expected course of the 8-16 and 16-32 cell stages in gametophytes. Black bar indicates the dark period. The distribution at each time point was derived from classifying 200 randomly chosen sporangia or gametangia in the fixed and stained preparations.
prophase stages. The loose term ‘spiremal stage’ is thus used for this stage. Probably it would be equivalent to pachytene–diplotene stages in other organisms. A subsequent diakinetic stage with highly contracted chromosomes maximally spread in the nucleus does exist however (Fig. 9). Similar figures have been reported by Foyn (1934) on *Ulva lactuca* and by Ramanathan (1939) on *Enteromorpha*. It must be emphasized that with our cytological techniques it is only with some imagination possible to observe that the diakinetic chromosomes are bivalents. Also chromosomal condensation in *Ulva* starts on several loci of each chromosome, and is not completed before the end of diakinesis. Thus actual bivalent counts are difficult to perform at the diakinetic stage. Usually the numbers obtained from such counts vary between 7 and 14 ‘bivalents’. Careful focusing may reveal connexions between some of the supernumerary bivalents. Therefore chromosome counting during meiosis is also possible only at the subsequent stage, the prometaphase, where the chromosomes appear fully contracted, but not yet assembled in the metaphase plate. Eight or possibly nine chromosomal pairs are then observed. Metaphase itself is not useful because the plane of division practically always gives a side view of the chromosomal plate. In the few exceptional cases where the division plane is tilted, one can observe that the 8–9 chromosome pairs form a ring. Between the chromosomal groups in early anaphase one can often observe bridges that may indicate chiasmata (Linskens & Vennegoor, 1967).

The second meiotic division and the 2 following divisions are typically mitotic in appearance and it may be noted that between each division, at least at the 2- and 4-cell stage, definite interphase nuclei with decondensed chromatin appear. However, after reaching the final 16-cell stage the chromatin stays condensed, forming small dark zoospore nuclei. The number of zoospore nuclei per sporangium has never been observed to exceed 16.

**Cytology of mitotic gamete formation in haploids**

The vegetative cells of the haploid gametophytes are, as reported by Foyn (1958), distinctly smaller than diploid cells. *In vivo* their outer free surface area is 92 ± 15 μm² and the cell layer thickness is about 18 μm. Probably the cell volume (minus cell wall) is about half that of diploid cells, a relation that is also reflected in dry weight and content of DNA, RNA and protein (S. Aaneby, in preparation). Also in haploid thalli the transfer of fragments to fresh growth medium inhibits ordinary somatic cell divisions, and apparently commits the cells to zooid production. Prior to the gametogenic divisions the mother cell nucleus increases from 2.4 ± 0.4 in diameter to 3.7 ± 0.8 μm. The same time scheme of cell growth, exit pore formation and rounding of the cells is also observed before gametogenesis. In fact, from *in vivo* observations no distinct differences, apart from cell size, are observed between diploid cells preparing for meiosis and haploid cells preparing for mitotic gamete formation. The same division and cell rotation pattern is also observed, but 1 more cell division takes place so that 32 gametes appear to be produced from each gametangial mother cell. The figure is conjecture, since a reliable count is difficult to perform.

The first gametogenic division in particular is very like an ordinary vegetative
mitosis in nuclear appearance (apart from nuclear location) (Figs. 15–20). During
the following divisions the nuclei become progressively smaller, but at least at the
2- and 4-cell stages definite interphase nuclei with decondensed chromatin appear.
The interphases last longer than in the post-meiotic divisions (Fig. 5). It is clear from
the best-stained preparations that the final number of gametes per mother cell must
be considerably higher than 20, but, as mentioned, exact counts are difficult.

In the usual light–dark programme the formation and differentiation of the
gametes is often completed within the dark period, and the gametes start to swarm
out in the first part of the following light period. They escape through the exit pore
rear end first and swim away immediately without the delay displayed by zoospores.
The gametes are phototactic positive, but samples transferred to fresh medium may
begin to lose this orientation after some 30 min. Typically, they settle all over the
bottom of a laterally illuminated culture dish, sometimes more concentrated in the
light side of the dish. They then grow into parthenosporophytes of the corresponding
mating type (Föyn, 1958), or occasionally into new gametophytes (R. C. Hoxmark, in
preparation).

DISCUSSION

As expected from the similarity in cell structure and life history among ulvacean
algae (Fritsch, 1935), the cytology of the zooid formation in Ulva mutabilis closely
resembles that found in Enteromorpha (Ramanathan, 1939; Sarma & Suryanarayana,
1968; Sarma, 1970), Monostroma (Dube, 1967), and other species of the genus Ulva
(Carter, 1926; Föyn, 1934; Smith, 1947; Sarma, 1964; Yabu & Park, 1969).

For Ulva specifically, the present observations on U. mutabilis fully agree with
those published by Föyn (1934) on U. lactuca, except for the number of chromosomes
which we believe is \( n = 8 \) or possibly \( n = 9 \) (see Hoxmark & Nordby, 1974). In U.
lactuca, Föyn found \( n = 13 \), while Carter (1926) and Sarma (1964) reported \( n = 10 \)
for the same species. \( U. \) linza has, according to Levan & Levring (1942), \( 2n = 24 - 25 \),
while in \( U. \) pertusa Yabu & Park (1969) reported \( n = 9 \). The number \( n = 13 \) for \( U.
mutabilis \) as given by Linskens & Vennegoor (1967) is certainly incorrect.

Of special interest for our current studies on the biochemistry of meiosis and
mitosis in Ulva (Nordby & Hoxmark, 1972) is the pronounced similarity between
meiotic zoosporogenesis and mitotic gametogenesis in this alga. The 2 processes are
not only almost indistinguishable cytokinetically, the same time of preparation also
elapses from induction by fragmentation and renewal of the growth medium, until
the cells go into the first synchronous prophase of the zooid-forming divisions.

This means that if the biochemical preparations for meiotic and mitotic cell
divisions differ significantly, Ulva would seem to be a most promising object for the
study of these differences. We feel there is a good chance that any qualitative, quan-
titative or time-course differences found within the preparational periods could be
directly related to the type (meiotic or mitotic) of the coming nuclear division. To
be sure, other differences exist between gametogenesis and zoosporogenesis in
Ulva. Notably, 32 gametes, but only 16 zoospores, are formed per mother cell. The
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Biflagellated gametes show positive phototaxis and can mate, while the zoospores bear 4 flagella each, show negative phototaxis and do not mate. Also there are some differences in the duration of division stages in the zooid-forming periods. For example, Fig. 5 shows that at least the 4- and 8-cell stages last longer in the gametophyte than in the sporophyte. Probably this means longer duration of the corresponding interphases, and it is then interesting to note that relatively more DNA appears to be synthesized at this time in the gametophyte than in the sporophyte (Hoxmark & Nordby, 1974).

Compared with differences that exist, for instance, between mitotically dividing tissue cells and developing egg or sperm cells, these differences in Ulva are small. One might say that, as a system for the study of meiosis versus mitosis, Ulva has an exceptionally low differentiatational ‘background noise’. It must be emphasized, however, that these merits primarily concern the preparations for meiosis or mitosis. When it comes to the study of biochemical events within the meiotic process as such, Ulva offers fewer advantages, since its meiotic prophase is extremely short. Therefore one cannot in synchrony, i.e. stage purity of a given sample, approach anything like the marvellous system found in lily anthers (Stern & Hotta, 1969).

As pointed out elsewhere (Hoxmark & Nordby, 1974), there is evidence that the committing differences, with respect to meiosis or mitosis between sporophytes and gametophytes, might actually exist in the plants before they are induced to either form of zooid formation, and that these differences might be cytoplasmic and independent of nuclear degree of ploidy. We are currently investigating this possibility.

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Figs. 6–14. Meiotic zoosporogenesis. All micrographs are at the same magnification, and the scale line represents 5 μm. In numerical order the figures show: late pre-meiotic interphase; synizetic stage in prophase I; spiremal stage in prophase I; diakinesis; prometaphase I; metaphase I; anaphase I; 2-cell stage; 16-cell stage.

Figs. 15–20. Mitotic gametogenesis. In numerical order the figures show: late pre-gametogenic interphase; first gametogenic prophase; metaphase; anaphase, telophase; 32-cell stage.