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Calcification of *Chara braunii* (Charophyta) caused by alkaline band formation coupled with photosynthesis

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OKAZAKI, M. and TOKITA, M. Calcification of *Chara braunii* (Charophyta) caused by alkaline band formation coupled with photosynthesis. Jpn. J. Phycol. 36: 193-201.

A fresh water calcareous alga *Chara braunii* (Charophyta) formed one or two definite bands of $CaCO_3$ deposits on the surface of the young internodal cells when the alga was grown in aquaria containing Ca^{2+}-enriched pond water. The alkalinization of the culture media was always accompanied by $CaCO_3$ deposition on the cells. Many hexagonal and foliated crystals were observed with scanning electron microscope and they were identified as calcite by X-ray diffraction. The alga was embedded in 0.5% low gelling-temperature agarose gel prepared with artificial pond water containing HCO_3^- and pH indicator, phenol red, to study OH^- accumulation and its role in calcification. One or two reddish alkaline bands were detected on the internodal cells by illumination of the alga, and this formation was strongly but reversibly inhibited with Diamox, an inhibitor of carbonic anhydrase and DCMU, an inhibitor of photosynthesis. $CaCO_3$ bands were always associated with alkaline bands on the internodal cells but the reverse was not true. The alkaline bands often induced $CaCO_3$ crystals on uncalcified cell surfaces when the agarose gel contained sufficient HCO_3^- and Ca^{2+}. These results strongly suggest a close relationship between $CaCO_3$ deposition and alkaline band formation resulting from HCO_3^- utilization by photosynthesis.

Key Index Words: *$CaCO_3$ deposition—calcification*—Chara braunii—*OH^- efflux.*

It has been known that the fresh water algae *Chara* and *Nitella* deposit calcitic $CaCO_3$ in bands on the outer surface of their internodal cells (LEWIN 1962, BOROWITZKA 1982). SPEAR *et al.* (1969) and LUCAS and SMITH (1973) showed the formation of alkaline and acid bands on the surface of *Nitella* and *Chara* cells and suggested that this phenomenum was intimately coupled with algal photosynthesis. The base bands seemed to be dependent on an OH^- efflux caused by HCO_3^- utilization in photosynthesis (LUCAS 1979). The localized alkalinization is believed to cause precipitation of $CaCO_3$ on the cells (SPEAR *et al.* 1969, LUCAS and SMITH 1973). However, no experimental evidence regarding this has been reported until now. In this paper, we describe a relationship between $CaCO_3$ deposition and alkaline banding on the internodal cells of *Chara braunii*.

Materials and Methods

Plant material

Chara braunii was grown in an aquarium. The aquarium was filled with tap water (20 *l*), and small plastic containers (200 m*l*) containing a total of 500 g of soil were placed at the bottom. 1 g of $Ca(OH)_2$ was mixed with the soil to neutralize the soil and to increase Ca^{2+} in the medium. 10 cm tall *Chara* plants were planted in the soil in the aquarium. The plants grew to about 20 cm at 22°C under 7,000 lux illumination (12 hr light: 12 hr dark) after three weeks, and clear $CaCO_3$ deposits on the internodal cells were observed after one month. Ca^{2+} and Mg^{2+} contents in the medium were determined by an EDTA-chelating titration. The pH of the medium was measured with a combined glass pH electrode.

Observation of $CaCO_3$ deposits on the cells by scanning electron microscopy (SEM)

Internodal cells, averaging 3 cm in length, were separated from intact plants. The cells were fixed at 4°C for 1 hr in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) satuarted with precipitated $CaCO_3$, followed by fixation in 2% osmium tetroxide in the same buffer at 4°C for 1 hr. Then the samples were dehydrated through an ethanol series saturated with precipitated $CaCO_3$, and then dried in a critical point dryer (JCPD-5) after replacement of ethanol with isoamylacetate. They were coated with palladium-gold, and examined with a scanning electron microscope (JSM-F15) at an operating voltage of 15 kV.

Detection of alkaline band on the cell

Small pieces of plants with about five internodal cells were taken from the tips of the intact plants. They were kept under 3,000 lux illumination at 24°C for 2 hr in a 1 mM $NaHCO_3$-containing bathing solution (pH 7.0) consisting of 0.6 mM NaCl and 0.2 mM each of KCl, Na_2SO_4 and $CaCl_2$ and then kept in the dark for 20 min prior to being used in experiments. These pretreated plants were embedded in 0.5% agarose gel (Sigma low gelling-temperature agarose) in a Petri dish. The agarose gel was prepared with the above bathing solution containing 0.2 mM $NaHCO_3$ and 0.1 mM phenol red as a pH indicator. The embedded plants were illuminated at 7,000 lux from two tungsten lamps to detect the alkaline and acid bands formed on the surface of the internodal cells.

Inhibitors

Diamox (acetazolamide), a specific inhibitor of carbonic anhydrase, and DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), a specific inhibitor of photosynthesis, were used to examine their effects on alkaline band formation. Diamox and DCMU were added to the bathing solution containing 1 mM $NaHCO_3$ at 1 mM and at 0.01 mM, respectively. In the case of DCMU, the bathing solution contained 0.05% ethanol to increase the solubility of DCMU. At first, plants were embedded in the agarose gel, as described above, to confirm their ability to form alkaline bands. These plants tested were transferred into a bathing solution containing 1 mM $NaHCO_3$ and 1 mM Diamox or 0.01 mM DCMU, and were incubated at 3,000 lux for 15 hr (for Diamox) or for 3 hr (for DCMU). These plants were embedded in agarose gel containing 0.2 mM $NaHCO_3$ and Diamox or DCMU at the same concentration as above, and were illuminated at 7,000 lux to examine the effect of these inhibitors on the alkalinization on the cells. Then, the embedded plants were carefuly removed from the gel and washed thoroughly with bathing solution for 24 hr to remove the inhibitors incorporated into plants. The washed plants were embedded again in the gel to test resumption of their alkalinizing activity.

Induction of $CaCO_3$ crystals on the cell in gel

Sections of plants which were free from $CaCO_3$ on their cells were embedded in agarose gel containing 0.2 mM $NaHCO_3$, 0.1 mM phenol red and 10 mM $CaCl_2$, and kept at 24°C under illumination at 3,000 lux (12 hr light: 12 h dark) for one month. $CaCO_3$ crystals formed in alkaline bands on the internodal cells were observed under polarized light with a camera (Asahi Pentax SP) through a close-up lens or under normal light with a microscope.

Results

$CaCO_3$ deposition

Chara braunii grown in the aquarium deposited several $CaCO_3$ bands on its internodal cells after one month (Fig. 1). $CaCO_3$ deposition was not observed on the first, youngest internodal cell, but the second and third cells from the tip became encrusted with $CaCO_3$ deposits. Calcification was completed at the fourth internodal cell with about two bands of $CaCO_3$

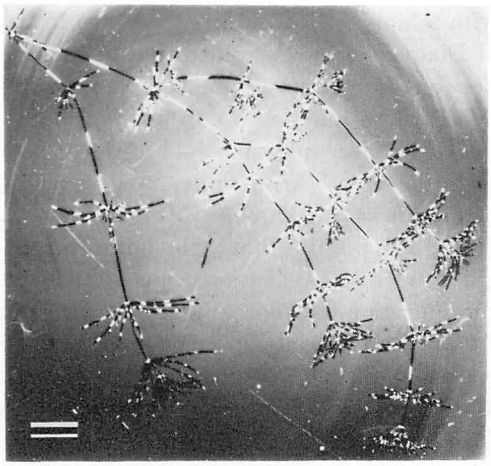

Fig. 1. Heavily calcified *Chara brawnii* grown in Ca^{2+}-enriched pond water. Note white bands of $CaCO_3$ deposited on the internodal cells of main axis and wheels. Photograph was taken under polarized light. Scale=1 cm.

Table 1. Contents of Ca^{2+} and Mg^{2+}, and pH change of culture medium of *Chara braunii*.

Aqarium No.	Ca^{2+} (mM)	Mg^{2+} (mM)	Initial pH	Final pH*
I	0.45	0.21	7.2	8.7
II	0.53	0.25	8.1	9.3
III	0.39	0.15	7.8	9.0
IV	0.59	0.18	7.2	9.2

*Values after one month.

deposits (Okazaki and Furuya 1985). As shown in Table 1, Ca^{2+} and Mg^{2+} concentrations in the culture medium were about 0.5 mM and 0.2 mM, respectively. Calcification was never found when the medium was not enriched with Ca^{2+} by adding $Ca(OH)_2$ in the soil. A remarkable increase in the pH of the culture medium was always observed with the growth of plants. A change from pH 7 to pH 9 was observed after one month, for example, and $CaCO_3$ deposition on the cells was initiated near pH 9. This indicates that an increase in the CO_3^{2-} concentration in the medium took place with alkalinization in the medium, resulting in $CaCO_3$ deposition. Fig. 2 A, B and C show scanning electron micrographs of $CaCO_3$ crystals deposited in a band on the fourth internodal cells. Well-

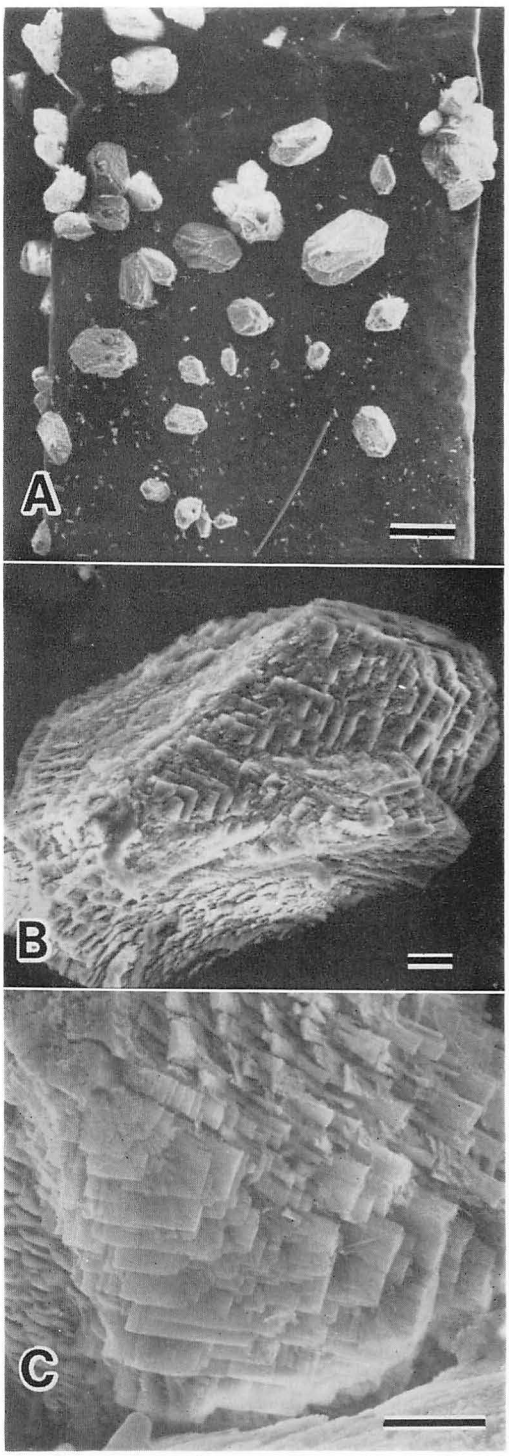

Fig. 2. Scanning electron micrographs of calcite crystals deposited in band on the fourth internodal cell from tip. Note that most crystals are hexagonal and foliated. C shows a magnified view of a crystal in B.
Scale=100 μm (A), 10 μm (B), 5 μm (C).

developed crystals were hexagonal, with a length of about 100 μm (Fig. 2B). They had a foliated structure as shown in Fig. 2 B, C and were identified as calcite by X-ray diffraction of powdered material (data not shown here). Mg^{2+} has been known as a strong inducer of aragonite *in vitro* (KITANO and HOOD 1962). However, in the present case, the degree of Mg^{2+} concentration (about 0.2 mM) was not sufficient to induce aragonite.

Alkaline band formation and effects of Diamox and DCMU on it

A *Chara* plant with a tip was embedded in 0.5% agarose gel prepared with a bathing solution (pH 7.0) containing 0.2 mM $NaHCO_3$ and 0.1 mM phenol red as a pH indicator, and was illuminated at 7,000 lux. Alkaline bands were detected by the change of phenol red color from yellow (below pH 7.0) to reddish (above pH 7.5). The reddish bands appeared on the internodal cells of the main axis and lateral branches after 10 min exposure to the light and the base accumulation continued with time. The reddish bands grew clearer after 30 min (left line in Fig. 3) or 60 min (Fig. 3A). Acid bands, which were indicated by a colour change of phenol red to yellow, alternated with alkaline bands, but acid bands were not seen in Fig. 3, because they were less clear than alkaline bands. The alkaline bands rapidly disappeared in the dark. When the same material was treated with Diamox (at 1 mM) or DCMU (at 0.01 mM), alkaline bands were scarecely detected on the cells even after 120 min in the light (Fig. 3B). This shows the strong inhibitory effect of both compounds on base formation. The inhibition was more complete with DCMU than with Diamox. However, when both inhibitors were washed out from the plants, alkaline bands appeared again as strongly as on the plants prior to exposure to inhibitors, showing a reversible inhibitory effect of both inhibitors (Fig. 3C, compared with 3A). In this experiment, *Chara* plants were pre-incubated with a high concentration (1 mM for 15 hr) of Diamox (acetazolamide), but alkalinization on the cells was not completely inhibited. This may be due to a high concentration of inorganic carbon (1 mM HCO_3^-) and the low permiability of biological membranes to Diamox (MORONEY *et al.* 1985). It is possible that carbonic anhydrase-independent CO_2 fixation occurs in the high concentration of inorganic carbon at pH 7.0 and/or intracellular carbonic anhydrase is not completely inhibited by Diamox, still allowing a low activity of photosynthesis (MORONEY *et al.* 1985, PRICE *et al.* 1985). These results suggest an intimate relationship between base acumulation and the utilization of HCO_3^- in photosynthesis.

Correlation between alkaline bands and $CaCO_3$ bands on the cells

In Fig. 4, the location of alkaline bands (4A) were compared with that of $CaCO_3$ bands (4B). $CaCO_3$ deposits were clearly shown under polarized light. As shown in Fig. 4 A, B, $CaCO_3$ bands on the internodal cells of the main axis and lateral branchs always accompanied alkaline bands although the opposite was not true (arrowhead in Fig. 4A). These photographs suggest an important role of alkaline bands in $CaCO_3$ deposition.

$CaCO_3$ deposition in alkaline bands in vitro

A plant initially free of any $CaCO_3$ deposit was embedded in agarose gel containing 0.2 mM $NaHCO_3$ and 10 mM $CaCl_2$ in bathing solution, as described in Materials and Methods (Fig. 5A). In this experiment, Ca^{2+} was enriched about fifty fold of that of usual bathing solution. The embedded plant was incubated at 3,000 lux at 24°C (12 hr light: 12 hr dark). After about 14 days, several minute crystals appeared in the alkaline band on the internodal cell (arrowheads in Fig. 5B). The number and size of crystals deposited on the cell increased after 19 days (arrowheads in Fig. 5C). Fig. 5D shows light micrographs of calcitic crystals, about 100–200 μm in

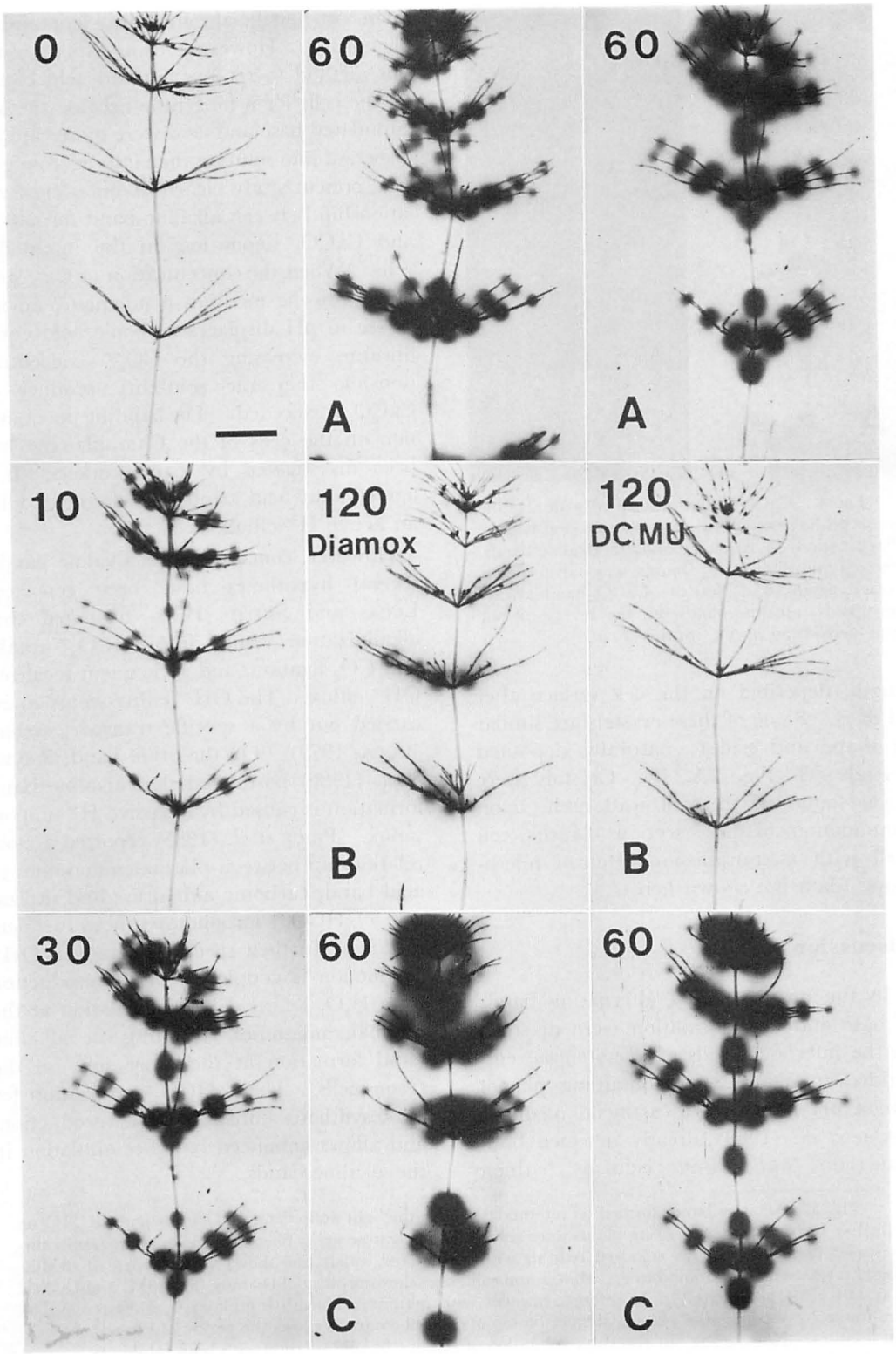

Fig. 3

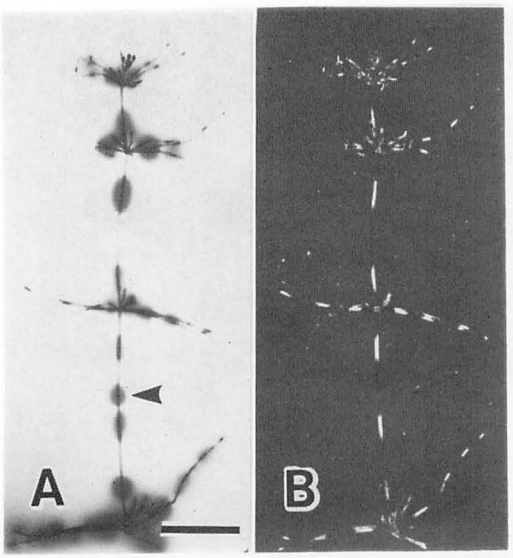

Fig. 4. $CaCO_3$ bands associated with alkaline bands on internodal cells of main axis and wheels. Black regions in A corresponds to alkaline bands. Photograph of $CaCO_3$ bands was taken under polarized light (B). Note that $CaCO_3$ bands always accompany alkaline bands but the reverse is not true (arrowhead in A). Scale=1 cm.

length, deposited on the cell surface after 30 days. Some of these crystals are similar in shape and size to naturally deposited crystals (cf. Fig. 2A, B). Crystals were never found in the cell wall itself, upon examination of thin sections of the cell wall with a transmission electron microscope (data not shown here).

Discussion

In the present study, alternating bands of base and acid formation were detected on the internodal cells of *Chara braunii* embedded in agarose gel containing phenol red, a pH indicator, in artificial medium. SPEAR *et al.* (1969) already detected both bands on *Nitella clevata* cells by bathing them in artificial medium containing phenol red. However, it was difficult using this method to retain base and acid bands on the cells for a long time because the accumulated base and acid were more rapidly dispersed into solution than into agarose gel. Our present study clearly shows a close relationship between alkaline band formation and $CaCO_3$ deposition on the internodal cells. When the concentration of Ca^{2+} and HCO_3^- in the medium is adequate, an increase in pH displaces carbonic acid equilibrium, increasing the CO_3^{2-} concentration, so that the solubility product of $CaCO_3$ is exceeded. The banding phenomenon on the cells of the Charaphyceae has been investigated by many workers. It is agreed that acid bands are dominated by an active H^+ efflux.

However, concerning the alkaline bands, several hypotheses have been reported. LUCAS and SMITH (1973) suggested that alkalinization results from HCO_3^--uptake and CO_2 fixation, and subsequent localized OH^- efflux. The OH^- efflux seems to be carried out by a specific transport system (LUCAS 1979). On the other hand, RAVEN *et al.* (1986) postulated that alkaline band formation is caused by a passive H^+ uniport influx. PRICE *et al.* (1985) reported a close relationship between plasmalemmasomes in acid band, carbonic anhydrase and utilization of HCO_3^- for photosynthesis in *Chara corallina*. In their speculative model, OH^- production is coupled with H^+ production from H_2O, i.e. acid band formation at the plasmalemmasomes, resulting in alkaline band formation at the other sites on the *Chara* cells. Then, HCO_3^- utilization for photosynthesis enhances OH^- production, and allows enhanced base accumulation in the alkaline bands.

Fig. 3. Alkaline bands formed on internodal cells in the light and effects of Diamox and DCMU on alkaline band formation. *Chara* plants were embedded in agarose gel. Black regions in photographs correspond to alkaline bands coloured reddish with phenol red. Left line shows a time course of alkaline band formation without inhibitor. Middle and right lines showing effect of Diamox (at 1 mM) and DCMU (at 0.01 mM), respectively. A, before treatment with inhibitor; B, with inhibitor; C, after removal of inhibitor by washing the plants. Figure on top of each photograph shows the period of exposure to light in minutes. Note strong but reversible inhibition of alkaline band formation with both inhibitors. Scale=1 cm.

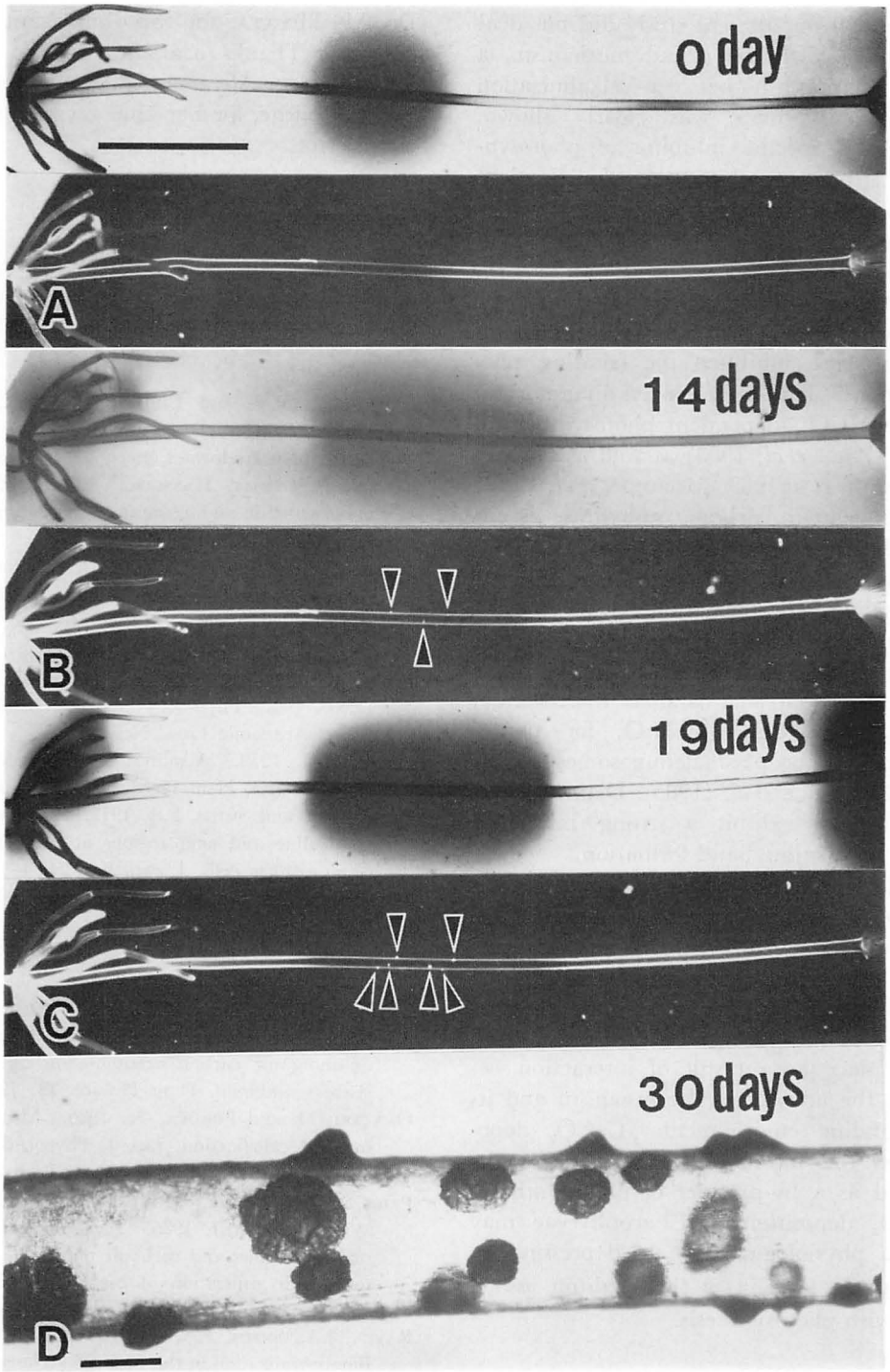

Fig. 5. $CaCO_3$ crystals induced in an alkaline band on internodal cell.
Young internodal cells free of $CaCO_3$ deposits were embedded in agarose gel mounted on a glass slide. A black region in A, B and C corresponds to alkaline band coloured reddish with phenol red. In A, B and C, photographs were taken under normal and polarized light to show crystals deposited (arrowheads). Light micrograph D shows a magnified view of deposited crystals. Scale=0.5 cm (A, B, C), 200 μm (D).

Although our present study did not deal specifically with the causal mechanism, a close relationship between alkalinization and photosynthesis was clearly shown. DCMU, a specific inhibitor of photosynthesis, strongly inhibited the alkalinization. In a freshwater green alga, *Gloeotaenium loitlesbergarianum*, calcium carbonate deposition is inhibited with 10^{-4} M DCMU (DEVIPRASAD and CHOWDARY 1981). Diamox, a specific inhibitor of carbonic anhydrase, also inhibited the banding phenomenon. This enzyme plays an important role in HCO_3^--dependent photosynthesis in *Chara* (PRICE *et al.* 1985) as well as *Chlorella* (MIYACHI *et al.*1983, MORONEY *et al.* 1985). If intracellular carbonic anhydrase is involved ni the reaction, $HCO_3^- \rightarrow CO_2 + OH^-$, in Characeans, an inhibitory effect of Diamox on alkaline band formation can be explained, supporting the hypothesis of Lucas and Smith as described above. On the other hand, if this enzyme catalizes the reaction, $HCO_3^- + H^+ \; CO_2 \rightarrow + H_2O$, for photosynthesis in the plasmalemmasomes as suggested by PRICE *et al.* (1985), Diamox is also expected to exhibit a strong inhibiting effect on alkaline band formation.

Calcification in algae, in general, is grouped into two types (SIMKISS 1986). One is "biologically-induced calcification" and the other is "organic matrix-mediated calcification". *Chara* calcification is considered to be biologically-induced, which takes place as a result of interaction between the activity of the organism and its surrounding environment. $CaCO_3$ deposits on the internodal cells appear to be formed as a by-product of photosynthesis. $CaCO_3$ deposition in Charophyceae may play a physiological role in depressing an increase in the pH of the medium associated with photosynthesis.

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岡崎恵視・時田三恵子：光合成に共役したアルカリバンド形成に基づくシャジクモの石灰化

淡水産の石灰藻であるシャジクモを，Ca^{2+} を強化した池水に入れて水槽中で育てると，水はアルカリ化し，節間細胞の表面に，$CaCO_3$ から成る明瞭なバンドが1～2本形成される。この $CaCO_3$ のバンド中には，六角形をした方解石の結晶が多数沈着しており，各結晶は箔状の結晶が幾つも重なった形状を呈する。

シャジクモを，HCO_3^- とフェノールレッド（pH 指示薬）を加えた人工培地から調製した寒天に埋め込み，光を照射すると，節間細胞表面に赤色のアルカリバンドが1～2本形成される。このアルカリバンド形成は，炭酸脱水酵素の阻害剤ダイヤモックスと光合成阻害剤 DCMU によって，強くかつ可逆的に阻害される。節間細胞表面の $CaCO_3$ のバンドは常にアルカリバンドと結合しているが，アルカリバンドの中には，$CaCO_3$ バンドと一致しないものも観察される。寒天中に十分な HCO_3^- と Ca^{2+} が存在すると，アルカリバンド中に $CaCO_3$ 結晶が誘導されてくる。これらの事実は，光合成時に，$HCO_3^- \rightarrow CO_2 + OH^-$ の反応に従って CO_2 が固定され，その際に排出される OH^- によって形成される細胞表面のアルカリバンドがシャジクモの石灰化に密接に関与していることを強く示唆する（184　東京都小金井市貫井北町4—1—1　東京学芸大学　生物学教室）

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The characteristics of photosynthesis and carbon metabolism in *Heterosigma akashiwo* (Raphidophyceae)

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TAKAHASHI, K. and IKAWA, T. 1988. The characteristics of photosynthesis and carbon metabolism in *Heterosigma akashiwo* (Raphidophyceae). Jpn. J. Phycol. **36**: 202–211.

The characteristics of photosynthetic CO_2 fixation were studied in the marine raphidophycean flagellate *Heterosigma akashiwo*. The rate of photosynthetic CO_2 fixation was saturated about 150 $W \cdot m^{-2}$ and was not inhibited by higher light intensities at least up to 500 $W \cdot m^{-2}$. Maximum rate of photosynthetic CO_2 fixation was about 300 μmol CO_2 mg Chl.a^{-1}. hr^{-1}. The rate was saturated at about 1 mM $NaHCO_3$ and half-saturation for $NaHCO_3$ was about 0.1 mM. Time course of ^{14}C-incorporation into photosynthetic products showed that 3-phosphoglycerate was the initial product, and 80% methanol-soluble β-1,3-glucans were the main reserve products of photosynthetic CO_2 fixation. Pattern of dark $^{14}CO_2$ fixation after preillumination also suggests that photosynthetic CO_2 fixation in this alga may be carried out by the reductive pentose phosphate cycle (C_3 cycle).

The effect of oxygen on the rate of photosynthetic $^{14}CO_2$ fixation was also studied. The highest rate was obtained under 2% O_2. The rate under 100% O_2 was 30% lower than that under 2% O_2. Under 100% O_2 relatively low levels of intermediates of photorespiratory pathway such as glycolate, serine and glycine were accumulated.

These results indicate that *H. akashiwo* has high photosynthetic activity even under the conditions of the high light, low CO_2, and high O_2 concentrations.

Key Index Words: Dark CO_2 fixation—Heterosigma akashiwo (*Raphidophyceae*)—*Light-enhanced dark CO_2 fixation*—Olisthodiscus luteus—*Photosynthesis*—*Photosynthetic CO_2 fixation*—*Storage product.*

The marine raphidophycean flagellate *Heterosigma akashiwo* (Hada) Hada is one of the most abundunt phytoplankton species in the temperate coastal waters of Japan. Previously, this alga was usually referred to as *Olisthodiscus luteus*, but it was recently pointed out that this species should be treated under the name of *H. akashiwo* (HARA *et al.* 1985). In recent years, the number of investigations of the ecology and physiology of this alga has increased considerably since the recognition of its importance as a principal organism in "red tide" blooms (FUKAZAWA *et al.* 1980, HATANO *et al.* 1983, TAKAHASHI and FUKAZAWA 1982, TOMAS 1979, 1980, WADA *et al.* 1985, WATANABE *et al.* 1982).

However, relatively little is known about the photosynthetic process in this alga. TOMAS (1980) has reported the effects of light intensity and temperature on the rate of photosynthesis and the cellular concentrations of nitrogen and carbon in an axenic clone of *O. luteus* following incubation in both indoor and out-door growth chambers. The major photosynthate of *O. luteus* was reported by BIDWELL (1957) to be mannitol, and HELLEBUST (1965) also found it to be the major carbon compound excreted from this alga. However, no investigation has been carried out on the

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carbon pathway during photosynthesis, nor the effect of oxygen on photosynthetic carbon metabolism in the raphidophycean algae including *H. akashiwo*.

The purpose of the present study is to characterize photosynthesis and photosynthetic carbon metabolism and to elucidate carbon fixation in the dark in *H. akashiwo*.

Materials and Methods

Algal culture

Heterosigma akashiwo (Hada) Hada was obtained from M. TAKAHASHI, Department of Botany, University of Tokyo. The alga was originally isolated by S. YAMOCHI, Osaka Prefecture Fisheries Experimental Station, from Tanigawa Fishing Port, Osaka Bay in 1979. Cells were grown axenically in 2-liter Erlenmeyer flasks containing 1 liter of PES medium (PROVASOLI, 1968), together with Jamarine S artificial seawater (Jamarine Laboratory, Osaka, Japan) at 18‰ salinity and enriched with 200 mg $NaNO_3$ and 40 mg Na_2HPO_4 per liter of medium. The medium was adjusted to pH 8.0 with KOH. Illumination was provided by cool-white fluorescent tubes at an intensity of about $12\ W \cdot m^{-2}$ at flask level under continuous bubbling with ordinary air.

Cells in a late exponential phase of growth (6–7 days old) were harvested by gentle filtration through Millipore filter SM (5 μm pore size), washed three times with reaction medium containing 25 mM HEPES and enriched PES medium (pH 8.0), and resuspended in the medium at a concentration of 5–10 μg chlorophyll *a* per m*l*. A small amount of silicon (Toshiba Silicon) was added to the reaction medium to prevent foaming.

Photosynthetic $^{14}CO_2$ fixation

Photosynthetic $^{14}CO_2$ fixation was carried out using 1 m*l* or 6 m*l* of algal suspension placed in spitz-type test tube (15×145 mm or 30×164 mm) at 23°C, and bubbled with CO_2-free air from a long hypodermic needle at a flow rate of $120\ ml \cdot min^{-1}$ throughout preillumination and subsequent photosynthetic $^{14}CO_2$ fixation. The tube was illuminated from one side with a halogen lamp. After 10-min preillumination, photosynthetic $^{14}CO_2$ fixation was started by injecting 240 μCi ($56.1\ mCi \cdot mmol^{-1}$) $NaH^{14}CO_3$ per m*l* of algal suspension, and stopped by treating with methanol as described below. After a scheduled photosynthetic period, suspending algal cells were collected quickly with suction through a glass-filter disc (Whatman GF/A, 25 mm diameter) and the cells were dipped into 80% hot methanol together with the disc. Illumination was continued throughout these processes. The algal suspension was heated in a water bath at 65°C for 5 min. After removal of glass-fiber disc, the suspension was acidified by the addition of acetic acid. A part of the algal suspension was then analysed for ^{14}C fixation products.

Analysis of $^{14}CO_2$-fixation products

The algal cells suspended in methanol were filtered through a Millipore filter (HA type, 0.45 μm pore size, 25 mm diameter). The cells on the membrane filter were extracted several times with a small amount of 80% hot methanol. Extracts were combined (80% methanol-soluble fraction) and a portion of the mixture was removed to determine the radioactivity. The radioactivity of the residue on the membrane filter (80% methanol-insoluble fraction) was determined with a liquid scintillation spectrometer. The rest of methanol extract was dried *in vacuo* at 35°C and dissolved in a small amount of 80% methanol to be chromatograph two-dimensionally on Whatman No. 3MM filter paper. The individual compounds were identified as described by SUZUKI and IKAWA (1985). Free sugars used as standards were also co-chromatographed on Toyo filter paper No. 50 with solvent systems, *n*-butanol-acetic acid-water (5:4:2 v/v), *n*-butanol-pyridine-water (6:4:3 v/v) (FRENCH and WILD, 1953), and ethyl acetate-pyridine-water (6:4:3) (WHISTLER

and HICKSON, 1954).

The three spots of ^{14}C-compounds left in the lower Rf regions after two-dimensional chromatography were eluted with water for treatment with β-1,3-glucanase, which was prepared from *Trichoderma viride* by the method of HORITSU *et al.* (1973). The resulting product giving one radioactive spot on re-chromatography was co-chromatographed with authentic glucose.

Determination of chlorophyll a

Chlorophyll *a* was measured spectrophotometrically in methanol extracts by the procedure of IWAMURA *et al.* (1970).

Results

Effects of light intensity and $NaHCO_3$ concentration on photosynthetic CO_2 fixation

Fig. 1 shows the rate of photosynthesis under ambient air condition as a function of light intensity. The rate of photosynthetic CO_2 fixation was saturated at about 150 $W \cdot m^{-2}$ and was not inhibited by higher light intensities at least up to 500 $W \cdot m^{-2}$. Maximum rate of photosynthetic CO_2 fixation was about 300 μmol CO_2 mg Chl.$a^{-1} \cdot hr^{-1}$.

Fig. 2 shows the effect of $NaHCO_3$ concentrations on the rate of photosynthetic $^{14}CO_2$ fixation at pH 8.0. The rate was saturated at about 1.0 mM and half-saturation for $NaHCO_3$ was 116 μM. These data suggested that characteristics of photosynthetic CO_2 fixation in this alga adapted to a higher light intensisty and a lower concentration of inorganic carbons.

Time course of photosynthetic CO_2 fixation

The total amount of photosynthetic CO_2 fixation increased linearly for 10 min at 0.7 mM $NaHCO_3$ and 250 $W \cdot m^{-2}$ (Fig. 3). The percent of ^{14}C incorporated into the 80% methanol-soluble fraction attained about 84% while those into the insoluble fraction was only 16% after 10-min $^{14}CO_2$ fixation. In addition, about a half of ^{14}C of the latter was localized in the β-1,3-glucans during this period (data not shown). Time course of ^{14}C incorporation into individual compounds are shown in Fig. 4. More than 80% of ^{14}C in the methanol-soluble fraction after 15-sec photosynthesis was incorporated into 3-phosphoglycerate (PGA) and a small portion of other sugar phosphates, but it decreased quickly thereafter. In contrast, the radioactivities of

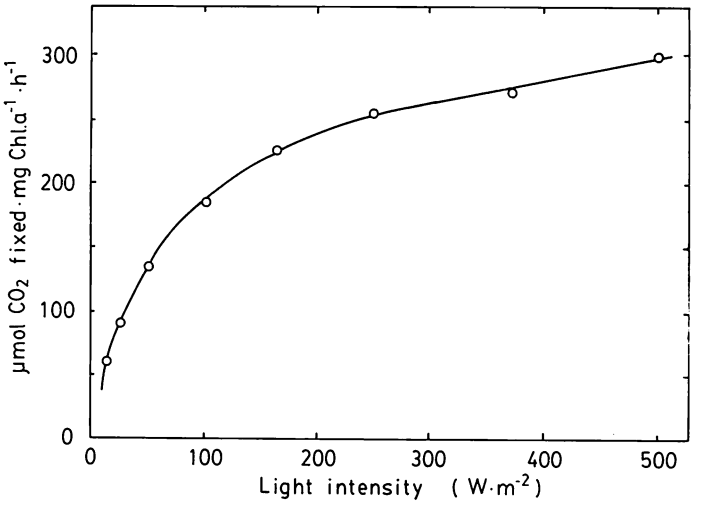

Fig. 1. Effect of light intensity on the rate of photosynthetic $^{14}CO_2$ fixation in *Heterosigma akashiwo* cells under ambient air condition. The rate was calculated from the amount of ^{14}C fixed for 5 min after 10-min preillumination. Incubation temperature and $NaHCO_3$ concentration were 23°C and 0.7 mM, respectively. Other experimental conditions are described in the text.

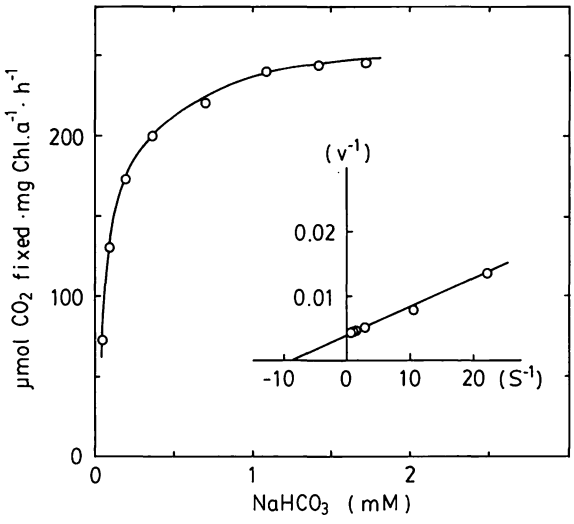

Fig. 2. Effect of $NaHCO_3$ concentration on the rate of photosynthetic $^{14}CO_2$ fixation in *Heterosigma akashiwo* cells under ambient air condition. Chlorophyll *a* content and the light intensity were 5.2 $\mu g \cdot ml^{-1}$ and 250 $W \cdot m^{-2}$. Other experimental conditions are described in the text.

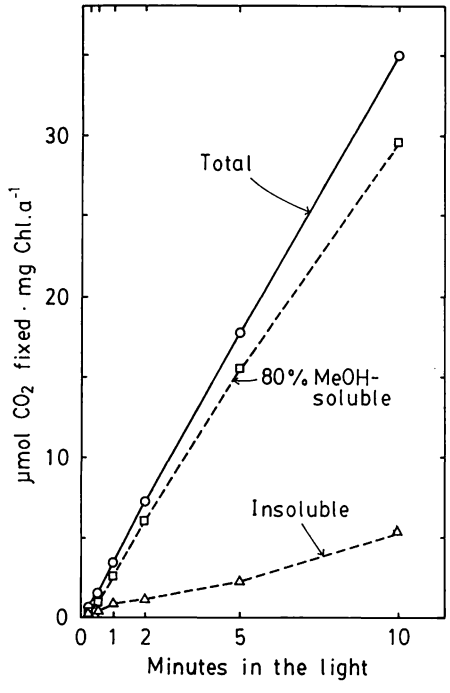

Fig. 3. Time course of ^{14}C-incorporation into 80% methanol-soluble and -insoluble fraction during photosynthetic $^{14}CO_2$ fixation in *Heterosigma akashiwo* cells under ambient air condition. Light intensity and $NaHCO_3$ concentration were 250 $W \cdot m^{-2}$ and 0.7 mM, respectively. Other experimental conditions are described in the text. ○, total activity; □, 80% methanol-soluble fraction; △, 80% methanol-insoluble fraction.

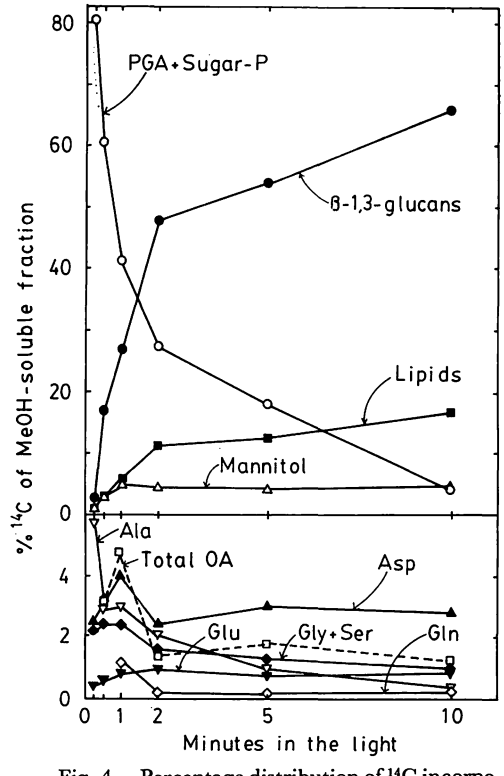

Fig. 4. Percentage distribution of ^{14}C incorporated into individual products *versus* time of photosynthetic $^{14}CO_2$ fixation in *Heterosigma akashiwo* cells. Data are from the experiment described in Fig. 3. Symbols: Ala, alanine; Asp, aspartate; Gln, glutamine, Glu, glutamate; Gly, glycine; Ser, serine; Total OA, total organic acids such as malate, succinate and glycolate.

amino acids as well as β-1,3-glucan are remarkably increased for the first few minutes. The results clearly indicate that PGA was the first product of CO_2 fixation, and that radioactivity was transferred to β-1,3-glucans and amino acids. It may well be that, therefore, the pohtosynthetic CO_2 fixation in *H. akashiwo* is mainly carried out through the reductive pentose phosphate cycle. It should be pointed out here that the ^{14}C-incorporation into β-1,3-glucans of 80% methanol-soluble fraction rapidly increased with time to occupy about 60% of the total activity after 10-min photosynthesis, whereas a very small amount of the activity, i.e. about 4% of the total, were incorporated into mannitol.

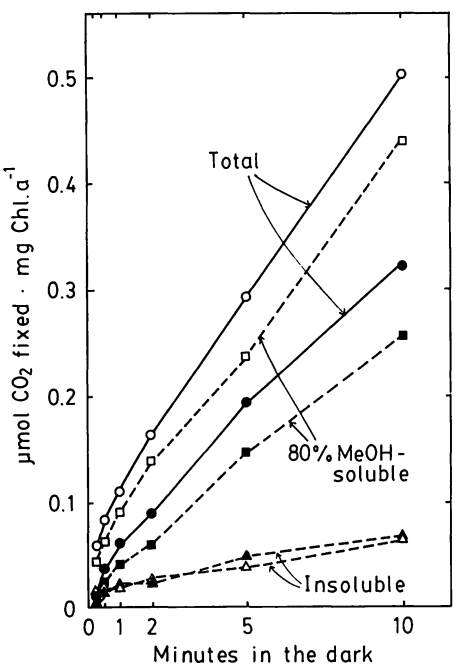

Fig. 5. Time course of ^{14}C-incorporation into 80% methanol-soluble and -insoluble fractions during dark $^{14}CO_2$ fixation by preilluminated and non-preilluminated cells of *Heteosigma akashiwo*. Open symbols: dark $^{14}CO_2$ fixation after 10-min preillumination (250 $W \cdot m^{-2}$) under CO_2-free air condition; closed symbols: dark $^{14}CO_2$ fixation without preillumination. $NaH^{14}CO_3$ solution (0.7 mM) was added in the dark immediately after turning off the light or after 20 min of continuous darkness.
○, ●, total activity; □, ■, 80% methanol-soluble fraction; △, ▲, 80% methanol-insoluble fraction.

Time courses of dark $^{14}CO_2$ fixation by preilluminated and non-preilluminated cells

The incorporation of ^{14}C in the non-preilluminated cells proceeded almost linearly with time and the rate of dark CO_2 fixation was only 1% of that of photosynthesis (Fig. 5). Incorporation of ^{14}C during 10-min dark CO_2 fixation was 40% larger in amount in the preilluminated cells than that in non-preilluminated ones (Fig. 5).

Time courses of ^{14}C incorporation in the individual products during dark $^{14}CO_2$ fixation with and without preillumination are shown in Figs 6 and 7. Aspartate and glutamate were the major products of dark $^{14}CO_2$ fixation with or without preillumination. On the other hand, most of ^{14}C was incorporated into PGA immediately after the addition of $^{14}CO_2$ to the preilluminated cells, but it decreased rapidly during the rest of the time periods. The percentages of ^{14}C incorporations into aspartate and glutamate increased initially with time in either

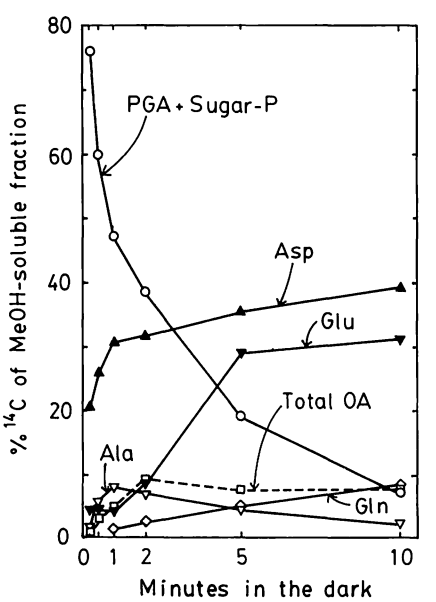

Fig. 6. Percentage distribution of ^{14}C incorporated into individual products *versus* time of dark $^{14}CO_2$-fixation after 10-min preillumination. Data are from the experiment described in Fig. 5. ○, PGA+sugar phosphates; ▽, alanine; ▲, aspartate; ▼, glutamate; ◇, glutamine; □, total organic acids.

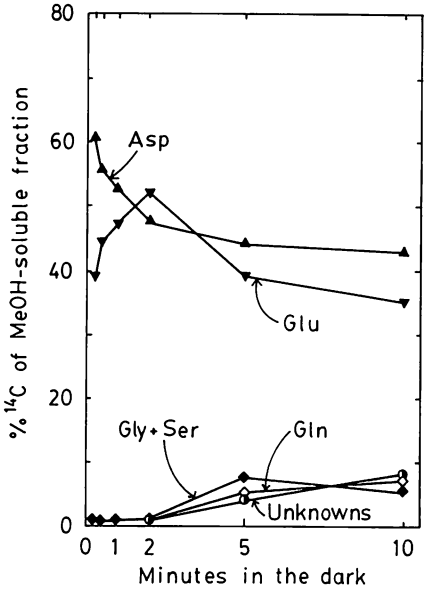

Fig. 7. Percentage distribution of ^{14}C incorporated in individual products *versus* time of dark $^{14}CO_2$-fixation without preillumination. Data are from the experiment described in Fig. 5. ◆, glycine+serine; ◑, unknown compounds, others see legend for Fig. 6.

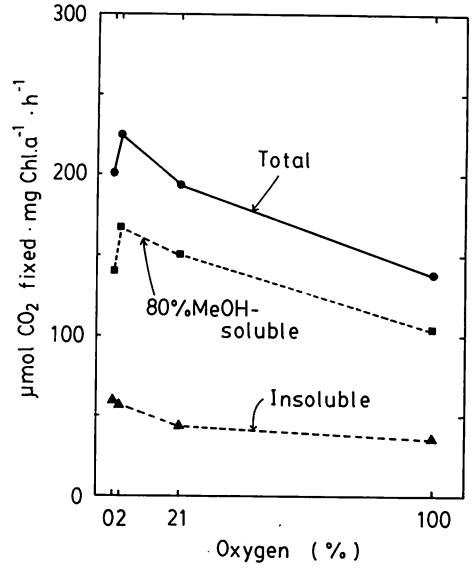

Fig. 8. Effect of oxygen concentration on the distribution of ^{14}C in the 80% methanol-soluble and -insoluble fractions in *Heterosigma akashiwo* cells. The rate was calculated from the amount of ^{14}C fixed for 5 min at 23°C. Light intensity and $NaHCO_3$ concentration were 250 W·m^{-2} and 0.7 mM, respectively. ●, total activity; ■, 80% methanol-soluble fraction; ▲, 80% methanol-insoluble fraction.

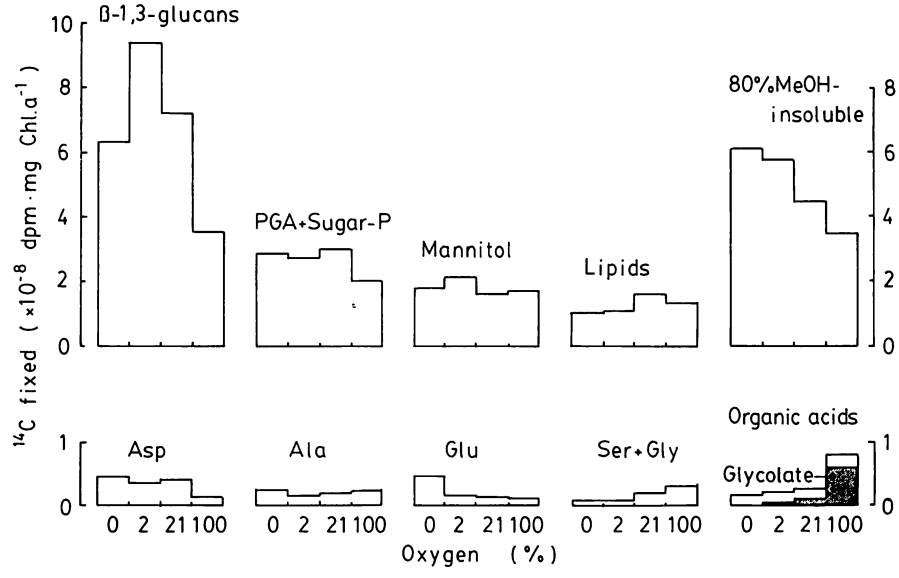

Fig. 9. Effect of oxygen concentration on the distribution of ^{14}C incorporated in individual products during 5-min photosynthetic $^{14}CO_2$ fixation. Data are from the experiment described in Fig. 8. Symbols: Ala, alanine; Asp, aspartate; Glu, glutamate; PGA+Sugar-P, PGA+sugar phosphates; Ser+Gly, serine+glycine.

preillumination or non-preillumination, but it decreased gradually thereafter, while those into other several amino acids, though they were small in amount, tend to increase slightly (Fig. 7).

Effect of oxygen on the photosynthetic CO_2 fixation

The effect of O_2 concentration on the rate of photosynthetic $^{14}CO_2$ fixation was determined at a high light intensity (250 $W \cdot m^{-2}$). As shown in Fig. 8, the highest rate was obtained under 2% O_2. Increase in the O_2 concentration above 2% caused decrease in the rate of photoysnthesis. The rate under 100% O_2 was 30% lower that under 2% O_2. Under anaerobic condition, the rate of photosynthesis was inhibited to about 10% of that under 2% O_2. Since a considerably large amount of ^{14}C was fixed under this concentration of O_2 in the 80% methanol-soluble fraction, some amount of O_2 seemed absolutely to favor ^{14}C-incorporation into this fraction.

Fig. 9 shows the effect of O_2 concentration on the distribution of ^{14}C in the products of 5-min photosynthetic $^{14}CO_2$ fixation. The amount of ^{14}C in 80% methanol-soluble β-1,3-glucans, was predominantly influenced by the O_2 concentration. Although the amounts of ^{14}C in glycolate, glycine and serine increased with increasing O_2 concentration, they were very small under O_2 concentrations up to 21%, and that in glycolate was only 4% of the total ^{14}C fixed even under 100% O_2. These results suggest that photorespiration occurs during photosynthesis at O_2 concentrations higher than 21% at saturating $NaHCO_3$ concentration (0.7 mM), but its inhibitory contribution to the photosynthesis is not very high.

Discussion

Detailed studies on the metabolic pathways of CO_2 fixation have not been made in raphidophycean algae including *Heterosigma akashiwo*, although numerous contributions have dealt with the ecological and physiological features of this alga and *Olisthodiscus*, a species having been identified later as *Heterosigma*. The data obtained in the present experiments suggest that *H. akashiwo* probably fixed CO_2 via the convensional C_3 pathway because PGA was the main primary product formed photosynthetically, and the label of PGA was subsequently transferred to other compounds as in the manner typical of C_3 plants, while C_4 acids comprised only minor part of the labeled compounds (Fig. 4). The main storage products of this photosynthetic process are 80% methanol-soluble β-1,3-glucans of yet unidentified size.

BIDWELL (1957) reported that *Olisthodiscus* sp. accumulated about 35% of total ^{14}C into mannitol and about 10% into an alcohol-insoluble glucan after 12 hr of photosynthesis in the presence of $H^{14}CO_3^-$, and he concluded that mannitol is the main product of photosynthesis in the alga. On the other hand, HELLEBUST (1965) has shown that *Olisthodiscus* sp. cells excreted about 10% of ^{14}C photoassimilates as mannitol in the log phase of growth during 48 hr of alternate 12-hr light and dark periods and it increased to more than 50% during the stationary growth phase.

In the present experiments, however, the percentage of ^{14}C in mannitol attained a maximum stationary level (4%) after 2 min of photosynthesis (Fig. 4). And the amount of ^{14}C-mannitol excreted was only 1.5% of the total ^{14}C fixed in the cells after 5 min of photosynthesis (data not shown). The percentage of radioactivity incorporated into mannitol differed depending on the culture condition. More than 20% of ^{14}C was incorporated into mannitol when the cells were cultured without aeration. Thus the accumulation of mannitol would seem to depend upon physiological and environmental parameters. These results suggest that mannitol may function in part as an osmotic regulation substance in *H. akashiwo* as reported in prasinophycean algae (ASHINO-FUSE and IKAWA 1981, HELLEBUST 1976, KIRST 1975).

On the other hand, most of the ^{14}C was found in the 80% methanol-soluble β-1,3-glucans, which contained over 56% of the total ^{14}C fixed in the cells after 5 min of photosynthesis (Fig. 4), while the amount of ^{14}C fixed in the 80% methanol-insoluble β-1,3-glucan and lipids, which were considered to be storage products in diatoms and brown algae (CRAIGIE 1974, HANDA 1969, HOLDSWORTH and COLBECK 1976, KREMER and BERKS 1978, YAMAGUCHI *et al.* 1968), was relatively small in amount in comparison with that fixed in the 80% methanol-soluble β-1,3-glucans. Furthermore radioactivity in the 80% methanol-soluble glucans in *H. akashiwo* was markedly decreased during the chase period in the dark (data not shown). It is postulated from these facts that the 80% methanol-soluble β-1,3-glucans are the major storage product of photosynthesis in *H. akashiwo*.

Enhancement of dark CO_2 fixation after preillumination in the absence of CO_2 has been observed both in higher plants and in algae (MIYACHI 1979). Analysis of $^{14}CO_2$ fixation products revealed that the main initial product was PGA in C_3 plants whereas it was malate and aspartate in C_4 plants, and the percentage of radioactivity incorparated in the initial $^{14}CO_2$ fixation products continued to decrease rapidly during the rest of time periods (MIYACHI 1979). Light-enhanced dark CO_2 fixation was also observed in *H. akashiwo* (Fig. 5), although the extent of the enhancement in this alga was smaller than those in *Chlorella* and *Anacystis* (HOGETSU and MIYACHI 1970, MIYACHI 1979). Distribution of radioactivity incorporated in the initial ^{14}C fixation product during light-enhanced dark $^{14}CO_2$ fixation (Fig. 6) was considerably different from those during dark $^{14}CO_2$ fixation without preillumination (Fig. 7). About 76% of the total ^{14}C incorporated was found in PGA after 15 sec of light-enhanced dark $^{14}CO_2$ fixation, but the radioactivity decreased rapidly during the rest of the time periods. The pattern of ^{14}C fixation products during light-enhanced dark $^{14}CO_2$ fixation is consistent with those in C_3 plants (MIYACHI 1979). These result also suggest that *H. akashiwo* is a C_3 plant.

Photosynthesis in terrestrial C_3 plants is inhibited considerably by oxygen even under ambient air conditions (21% O_2, 0.03% CO_2). The inhibition is mainly associated with photorespiration derived from oxygenase activity of RuBP carboxylase/oxygenase (BECK 1979).

Oxygen inhibition of photosynthesis has been also observed in many species of various algal divisions (WARBURG 1920, GAFFRON 1940, TAMIYA and HUZISIGE 1949, BEARDALL and MORRIS 1975, COLEMAN and COLMAN 1980, KREMER 1980, SHELP and CANVIN 1980, BIRMINGHAM *et al.* 1982). Other algae, on the other hand, seem to exhibit a different photosynthetic response to O_2. Little or no effect of O_2 on photosynthesis was reported in several algae (LLOYD *et al.* 1977, COLEMAN and COLMAN 1980, BEER and ISRAEL 1986). Furthermore, oxygen enhancement of photosynthetic $^{14}CO_2$ fixation has been observed in the blue-green alga *Anacystis nidulans* (MIYACHI and OKABE 1976), and in the cryptophycean alga *Chroomonas* sp. (SUZUKI and IKAWA 1984a, b and 1985).

In *H. akashiwo* cells, the pattern of photosynthetic $^{14}CO_2$ fixation closely resemble those of C_3 plants (Fig. 4). However, the effect of O_2 on photosynthesis in *H. akashiwo* cells seems to differ from that in terrestrial C_3 plants. Photosynthetic $^{14}CO_2$ fixation was inhibited by anaerobiosis as well as high concentrations of O_2, and the highest rate of CO_2 fixation was obtained under 2% O_2 (Fig. 7). The percent radioactivity incorporated into the intermediates of the photorespiratory pathway, such as glycolate, glycine and serine, was almost negligible at concentrations up to 21%, and very small even under 100% O_2 (Fig. 8). These results were consistent with those in *Chroomonas* (SUZUKI and IKAWA 1985).

The lack of inhibition of photosynthesis at high light intensity (Fig. 1), low sensitivity to O_2 (Fig. 7), and relatively high af-

finity to inorganic carbon (Fig. 2) may be important features to cause a bloom in natural waters.

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高橋京子・猪川倫好：ラフィド藻 *Heterosigma akashiwo* における光合成炭素代謝特性

海産ラフィド藻 *H. akashiwo* の光合成炭酸固定活性は，最大約 300μ mol CO_2·mg Chl.a^{-1}·hr^{-1} で他の近縁の藻類に比べ非常に高い活性を持つことが示された。またこの活性は 500 W·m^{-2} 以上の強光下でも阻害されず，無機炭素に対し比較的高い親和性を持つこと（$Km_{app(HCO_3^-)}$=0.1 mM）が明らかにされた。光合成 $^{14}CO_2$ 固定産物の経時的変化の解析から，光合成炭酸固定は炭素還元回路（C_3 回路）によって行われ，主要な貯蔵産物として80%メタノール可溶性のβ-1,3-グルカンを生成することが示された。また光合成炭酸固定に対する酸素の影響を調べたところ，本藻は C_3 植物であるにもかかわらず，高い酸素耐性を有することが明らかになった。これらの特性は，本藻が昼間，海表面付近で活発な光合成を行うことによりもたらされると推定される低炭酸，高酸素環境下でも，十分高い光合成活性を保つ上で，非常に有利であると考えられる。（305 つくば市天王台1-1-1 筑波大学生物科学系）

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Effect of nitrogen starvation on photosynthetic carbon metabolism in *Heterosigma akashiwo* (Raphidophyceae)

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TAKAHASHI, K. and IKAWA, T. 1988. Effect of nitrogen starvation on photosynthetic carbon metabolism in *Heterosigma akashiwo* (Raphidophyceae). Jpn. J. Phycol. **36**: 212–220.

The distribution patterns of ^{14}C during photosynthetic $^{14}CO_2$ fixation were studied in nitrogen-enriched and -limited cells of the marine raphidophycean flagellate *Heterosigma akashiwo*. In addition, pulse-chase experiments were conducted under light and dark conditions. The initial products of photosynthetic CO_2 fixation was 3-phosphoglycerate in cells of both groups. In nitrogen-enriched cells about 45% of the total fixed ^{14}C was incorporated into 80% methanol-soluble β-1,3-glucans, which gradually increased during the chase in the light but decreased rapidly in the dark. In nitrogen-starved cells, on the other hand, 40% of the fixed ^{14}C was incorporated into the soluble β-1,3-glucans, which decreased rapidly during the chase in the light as well as in the dark. The ^{14}C in mannitol attained a maximum stationary level (below 7%) in cells from both groups after 2 minutes of photosynthesis. These results suggest that the main storage product of photosynthetic CO_2 fixation in both nutrient conditions may not be mannitol, but the 80% methanolsoluble β-1,3-glucans.

The nitrogen starvation leads to the activation of catabolic metabolism or dark respiration and to the depression of photosynthetic CO_2 fixation.

Key Index Words: Carbon metabolism—Heterosigma akashiwo (*Raphidophyceae*)—*β-1,3-Glucan*—*Mannitol*—*Nitrogen starvation*—Olisthodiscus luteus—*Photosynthesis*—*Photosynthetic CO_2 fixation*—*Storage product.*

The marine raphidophycean flagellate *Heterosigma akashiwo* (Hada) Hada (formerly called *Olisthodiscus luteus*, HARA *et al.*, 1985) is the organism which causes extensive "red tide" bloom during the summer in the temperate coastal waters of Japan. There are a number of studies on the ecology and physiology of this alga (FUKAZAWA *et al.*, 1980; HATANO *et al.*, 1983; TAKAHASHI and FUKAZAWA, 1982; TOMAS, 1979, 1980; WADA *et al.*, 1985; WATANABE *et al.*, 1982). These studies have shown that this alga exhibited diurnal vertical migration similar to other red tide dinoflagellates, such as *Gonyaulax* (EPPLEY *et al.*, 1968) and *Gymnodinium* (CULLEN and HORRIGAN, 1981) swimming down-ward before the start of the dark period and upward before the end of the dark period. TAKAHASHI and FUKAZAWA (1982) and YAMOCHI and ABE (1984) suggested that this migration is favorable for their growth, as it allows them to absorb necessary nutrients such as nitrogen, Mn and vitamin B_{12} at the nutrients-rich bottom layer during the night and carry out photosynthesis effectively near the surface during the daytime, consuming the nutrients absorbed. Furthermore, HATANO *et al.* (1983) reported that nitrogen-starved cells of *H. akashiwo* showed no vertical migration, but that after the addition of a nitrogen source such as nitrate and ammonia, they recovered their migratory ability. Little is known, however, about the changes in photosyn-

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thetic carbon metabolism that accompany nitrogen starvation in this alga.

In the previous paper (TAKAHASHI and IKAWA, 1988) we have shown the characteristics of photosynthesis and carbon metabolism in nitrogen-enriched culture cells of this alga. In the present paper we studied the effect of nitrogen starvation on photosynthetic carbon metabolism and the activity of dark respiration in *H. akashiwo*.

Materials and Methods

Algal culture

Heterosigma akashiwo cells were grown axenically in N-enriched or N-limited culture media at 18°C with a 12-hr light and 12-hr dark cycle. For N-enriched culture conditions, PES medium (PROVASOLI 1968) was used, together with Jamarine S artificial seawater at 18‰ salinity and enriched with 2.35 mM $NaNO_3$ and 0.28 mM Na_2HPO_4. For the N-limited culture conditions, the alga was grown in modified PES medium which contained only 1% of the full-strength concentration of $NaNO_3$ (25 μM). The pH of the medium was adjusted to 8.0 with KOH. Illumination was provided by cool-white fluorescent tubes at an intensity of about 12 $W \cdot m^{-2}$ at flask level. Cultures were bubbled continuously with filtered air without supplementary CO_2.

Cells in the late exponential phase of growth (6–7 days old) were harvested as previously described (TAKAHASHI and IKAWA 1988).

Photosynthetic $^{14}CO_2$ fixation

Photosynthetic $^{14}CO_2$ fixation was carried out using 6 m*l* of algal suspension placed in a spitz type test tube at 23°C, and bubbled with CO_2-free air from a long hypodermic needle at a flow rate of 120 $ml \cdot min^{-1}$ throughout the period of preillumination and subsequent photosynthetic $^{14}CO_2$ fixation. The tube was illuminated from one side at 200 $W \cdot m^{-2}$ with a halogen lamp. After 10-min preillumination, $^{14}CO_2$ fixation was started by injecting $NaH^{14}CO_3$ and stopped with methanol as previously described (TAKAHASHI and IKAWA 1988).

Pulse-chase labelling experiments

For pulse-chase labelling experiments, 6 m*l* of algal suspension was placed in a test tube. After a 5-min photosynthesis period in medium containing $NaH^{14}CO_3$ (0.7 mM initial concentration) according to the method described above, $NaH^{12}CO_3$ (10 mM final concentration) was added to the reaction tube, and the reaction was successively carried out in the light or in the dark. At intervals, 0.5-m*l* aliquots of the reaction mixture were removed with a micropipette and the reaction was stopped with methanol. The amount of ^{14}C was determined using a liquid scintillation spectrometer. Other details were described previously (TAKAHASHI and IKAWA 1988).

Determination of chlorophyll a

Chlorophyll *a* was measured spectrophotometrically in methanol extracts as described by IWAMURA *et al.* (1970).

Measurement of cellular oxygen consumption

The rate of cellular oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Yellow Spring Instrument Co.) fitted to a 2-m*l* acryl cell thermostated at 25°C. Assuming the oxygen concentration of air-saturated water to be 0.26 μmol $O_2 \cdot ml^{-1}$ at 25°C, calibration was performed using dithionite and air-saturated water (DELLIEU and WALKER 1972).

Results

Time courses of photosynthetic $^{14}CO_2$ fixation

The total amount of photosynthetic $^{14}CO_2$ fixation products increased linearly for 5 min in cells from both N-enriched and N-depleted cultures (Fig. 1). In N-starved cells the rate of photosynthetic $^{14}CO_2$ fixation was about 20% lower than that in N-enriched cells. However, the level of ^{14}C in the 80% methanol-insoluble fraction was

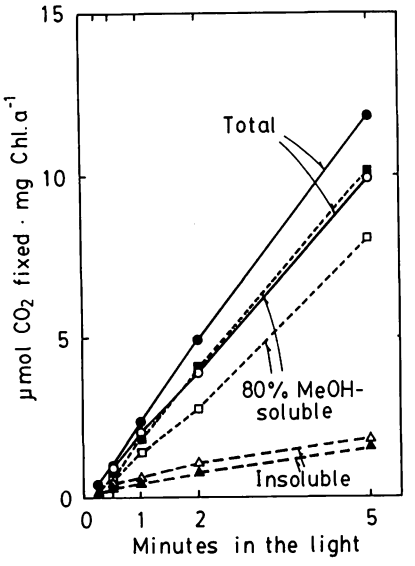

Fig. 1. Time courses of ^{14}C incorporation into 80% methanol-soluble and -insoluble fractions during photosynthetic $^{14}CO_2$ fixation in N-enriched and N-starved cells of *Heterosigma akashiwo*. Solid lines, total activity; dotted lines, 80% methanol-soluble fraction; broken lines, 80% methanol-insoluble fraction. Closed symbols, N-enriched cells; open symbols, N-starved cells. Incubation conditions were the same as described in the text.

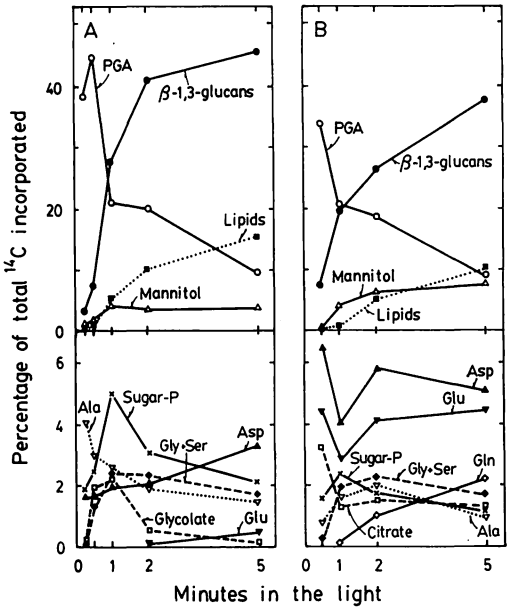

Fig. 2. Percentage distribution of ^{14}C in individual products of photosynthetic $^{14}CO_2$ fixation *versus* time in N-enriched (A) and N-starved (B) cells of *H. akashiwo*. Data are from the experiment described in Fig. 1. Symbols: Ala, alanine; Asp, aspartate; Gln, glutamine; Glu, glutamate; Gly+Ser, glycine plus serine; Sugar-P, sugar phosphates.

little higher in N-starved cells than in N-enriched cells. About 50% of ^{14}C in the insoluble fraction was detected in glucose after hydrolysis with β-1,3-glucanase (data not shown). The result indicates that half of the ^{14}C in this fraction was incorporated into a β-1,3-glucan. Time courses of percentage distribution of ^{14}C in compounds during photosynthetic $^{14}CO_2$ fixation are shown in Figs. 2A and B. Most of ^{14}C fixed during the first 30 sec was found in 3-phosphoglycerate (PGA), and it decreased rapidly during the rest of the time period. The labelling patterns of intermediates indicate that photosynthetic CO_2 fixation in *H. akashiwo* cells is mainly carried out through the reductive pentose phosphate cycle in both nutrient conditions.

In N-enriched cells, the percentage of ^{14}C incorporated into 80% methanol-soluble β-1,3-glucans increased to reach about 50% of total activity, while that into lipids gradually increased to reach 15% after 5 min of photosynthesis (Fig. 2A). In N-starved cells, on the other hand, the percentages of ^{14}C incorporated into these fractions were lower than those in N-enriched cells, and these levels during the initial 2 min of photosynthesis were half those in N-enriched cells (Fig. 2B).

The percentage distribution of radioactivity in mannitol attained a maximum stationary level in both groups of cells after 2 min of photosynthesis (4% and 7% in N-enriched and N-starved cells, respectively). This figure also shows that the amounts of ^{14}C incorporated into amino acids, in particular aspartate, glutamate and glutamine, were much higher in N-starved cells than in N-enriched cells. ^{14}C-Glycolate was detected in N-enriched cells, although the amount was relatively small under the experimental conditions we used (bubbled with CO_2-free air).

Pulse-chase experiments

After a 5-min photosynthetic $^{14}CO_2$ fixa-

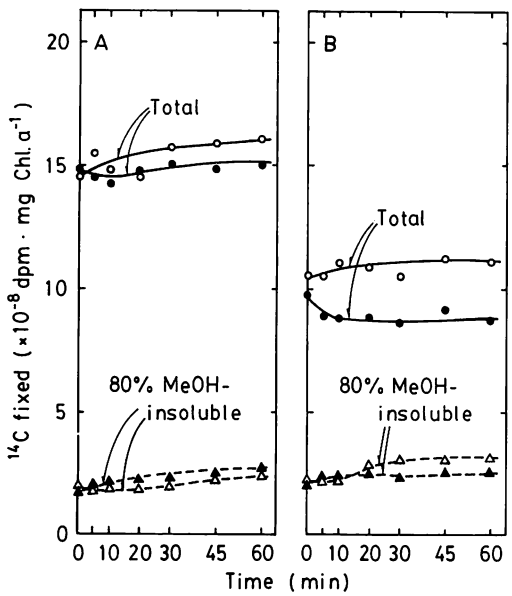

Fig. 3. Changes in the distribution of ^{14}C in total and 80% methanol-insoluble fractions in N-enriched (A) and N-starved (B) cells during the chase periods in the light and in the dark after a 5-min $^{14}CO_2$ pulse in the light. Experimental conditions are described in the text. Open symbols, chase in the light; closed symbols, chase in the dark.

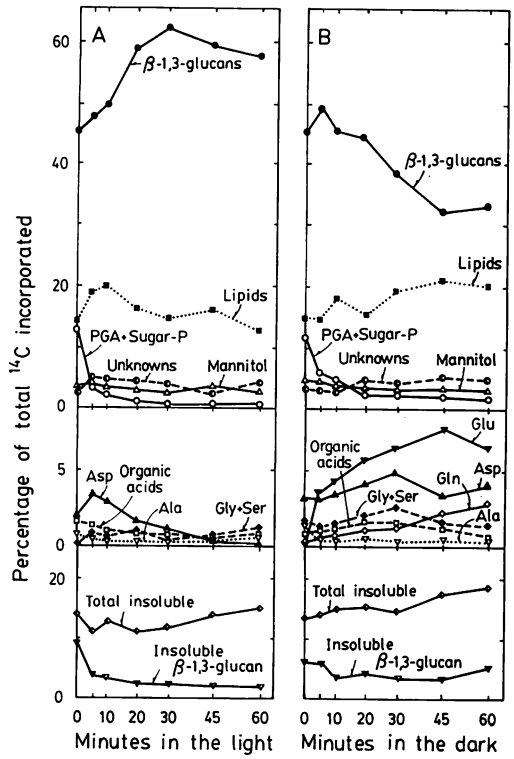

Fig. 4. Changes in the distribution of radioactivity among ^{14}C-labeled compounds in the light (A) and in the dark (B) in N-enriched *H. akashiwo* cells during pulse-chase experiments after a 5-min $^{14}CO_2$ pulse in the light. Data are from the experiment described in Fig. 3. Symbols: Total insoluble, total radioactivity of 80% methanol-insoluble fraction; others see legend for Fig. 2.

tion (pulse), non-labelled bicarbonate was added to the reaction mixture to reduce the fixation of $^{14}CO_2$, and labelled carbon was chased in the light or in the dark. Figures 3A and B show that the total amounts of ^{14}C fixed in the cells became relatively constant after 5 min of chasing under all reaction conditions, while the amount of ^{14}C incorporated into the 80% methanol-insoluble fraction gradually increased during the chase period.

Time courses of percentage distributions of the radioactivity incorporated into individual products during chasing in the light and in the dark in N-enriched cells are shown in Figs. 4A and B. The radioactivity in PGA and sugar phosphates quickly decreased during the chase. The pronounced negative slope of the curve for PGA and sugar phosphates indicated clearly that PGA was the first product of CO_2 fixation, and that the radioactivity appeared later in β-1,3-glucans and the level of β-1,3-glucans gradually decreased after 30 min in the light. The amount of label in lipids increased initially and then fell to a steady level (Fig. 4A). The level of 80% methanol-insoluble fraction shows the reverse changes to the 80% methanol-soluble β-1,3-glucans. Under these conditions the methanol-insoluble fraction contained a small and relatively constant proportion (ca 2%) of β-1,3-glucans except for early 20 min (Fig. 4A). The remaining ^{14}C-methanol-insoluble fraction was not yet analyzed. On the other hand, when a photosynthetic pulse was followed by cold incubation in the dark, the percentage of β-1,3-glucans decreased gradually with chase time, whereas those of lipids, the 80% methanol-insoluble fraction and amino acids (particularly glutamate, glutamine

and aspartate) increased (Fig. 4B). The level of mannitol remained constant in both the light and the dark (Figs. 4A and B).

In contrast, when the pulse-label was chased at the same light intensity in N-starved cells, the distribution of radioactivity in individual compounds differed from those in N-enriched cells (Figs. 5A and B). After the pulse-label, the percentage of radioactivity in 80% methanol-soluble β-1,3-glucans decreased rapidly even under light conditions, although the amount of ^{14}C incorporated into this compound had increased linearly during the 5-min photosynthetic pulse labelling period. Accompanying this drop were increases in the radioactivities in the 80% methanol-insoluble fraction, lipids, amino acids (particularly glutamine and alanine) and unidentified compounds (probably amino acids). Under these conditions, one-third of the ^{14}C in the 80% methanol-insoluble fraction was detected in β-1,3-glucan. Also, the label in organic acids such as citrate, malate and succinate rose sharply and then fell in the first 10 min of the chase (Fig. 5A). Similar results were obtained when the pulse-label was chased under the dark conditions, but more pronounced changes in the distribution of ^{14}C compounds were observed under the experimental conditions (Fig. 5B). The level in mannitol decreased gradually with chase time, unlike the case in N-enriched cells. These results suggest that both photosynthesis and dark respiration in *H. akashiwo* cells are considerably affected by changes in the concentrations of nitrate available to the cells.

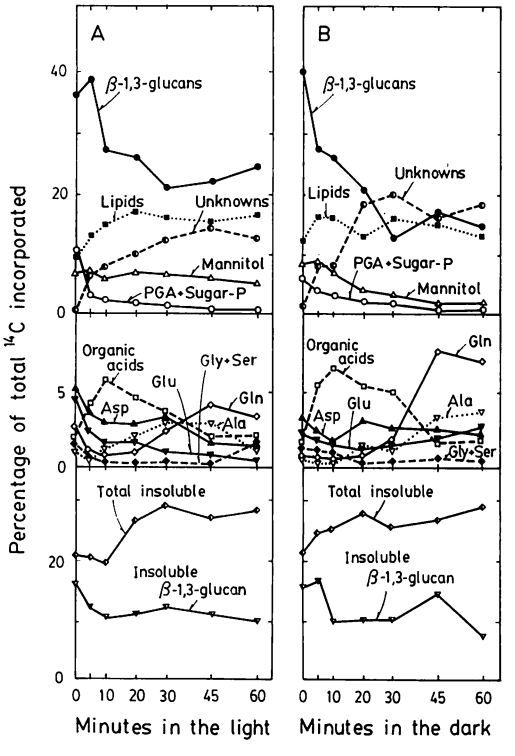

Fig. 5. Changes in the distribution of radioactivity among ^{14}C-labeled compounds in the light (A) and in the dark (B) in N-starved *H. akashiwo* cells during pulse-chase experiments after a 5-min $^{14}CO_2$ pulse in the light. Symbols are the same as those of Fig. 4.

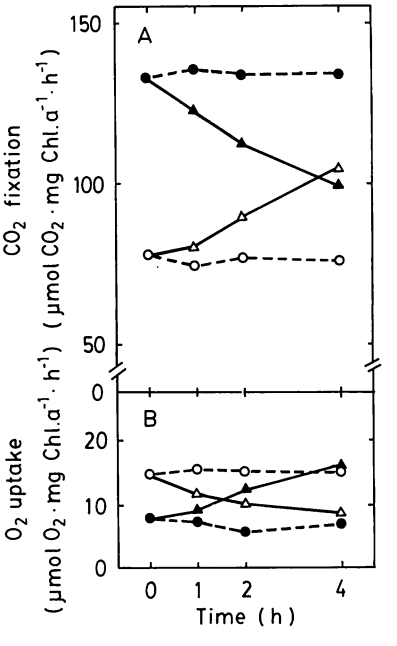

Fig. 6. Effects of nitrate on the rate of photosynthetic CO_2 fixation (A) and dark respiration (B) in *H akashiwo* cells. N-enriched or N-starved cells were collected by filtration, washed with sterile N-free culture medium and resuspended in incubation medium. N-enriched cells were incubated in either N-enriched (2 mM $NaNO_3$) (●) or N-depleted (100 μM $NaNO_3$) (▲) culture medium, and N-starved cells were incubated in either N-depleted (20 μM) (○) or N-enriched (2 mM $NaNO_3$) (△) culture medium for 4 hr in the light.

Effect of nitrate concentration on the rates of photosynthesis and dark respiration

The changes in the rates of photosynthesis and dark respiration occurring when the concentrations of nitrate in the culture medium were changed are shown in Fig. 6. When the N-enriched cells were transferred to the N-depleted culture medium, the rate of respiration of the cells was increased two-fold, and the rate in N-starved cells decreased to the level that in N-enriched cells within 4 hr after the transfer to N-enriched conditions. On the other hand, a reverse response was observed between the change in the rate of photosynthesis and that of the concentration of nitrate in the culture medium.

Discussion

Photosynthetic CO_2 fixation

In the previous paper (TAKAHASHI and IKAWA 1988), we reported that *Heterosigma akashiwo* probably fixes CO_2 via the conventional C_3 carbon-reduction pathway and the main storage product of photosynthesis is the 80% methanol-soluble β-1,3-glucans. Furthermore, we suggest that the mannitol is second to β-1,3-glucans in importance as the reserve substance, in disagreement to BIDWELL (1957) who had reported the former to be the main product of photosynthesis.

These ideas are supported by the pulse-chase experiment in the present study. The percentage of ^{14}C in mannitol attained a maximum stationary level (4%) in N-enriched cells after 2 min of photosynthesis (Fig. 2) and did not change during a 60-min chase period either in light or in dark conditions (Figs. 4A and B). On the other hand, more than 45% of the fixed ^{14}C was recovered in the 80% methanol-soluble β-1,3-glucans after 5 min of photosynthesis (Fig. 2A), and this percentage exceeded 60% during the chase period in the light in N-enriched cells (Fig. 4A). The radioactivity markedly decreased during the chase period in the dark (Fig. 4B). In chrysophycean and phaeophycean algae 80% methanol insoluble β-1,3-glucan and lipids are considered to be reserve substances (CRAIGIE 1974, HANDA 1969, HOLDSWORTH and COLBECK 1976, KREMER and BERKS 1978, YAMAGUCHI *et al.* 1968). However, percentage distribution of ^{14}C in these substances were relatively smaller than those in the 80% methanol-soluble β-1,3-glucans in *H. akashiwo* (Figs 4A and B). These facts suggest that the 80% methanol-soluble β-1,3-glucans are the major storage product of photosynthesis in *H. akashiwo*.

Effect of nitrogen starvation on the photosynthetic carbon metabolism and dark respiration

When *H. akashiwo* cells were cultured in N-depleted medium, the rate of photosynthetic CO_2 fixation was reduced by 80% (Fig. 1). Accompanying this, ribulose 1,5-bisphosphate carboxylase activity and chlorophyll contents in extracts of N-starved cells decreased to 80% and 67%, respectively (data not shown). Thus the decrease of photosynthetic capacity may occur as a consequence of a decrease in the contents of chlorophylls and ribulose 1,5-bisphosphate carboxylase, as shown in *Ankistrodesmus braunii* (HIPKIN and SYRETT 1977), and *Oscillatoria rubescens* (FEUILLADE *et al.* 1982). During short-term photosynthesis in the marine diatom *Phaeodactylum tricornutum*, GLOVER *et al.* (1975) showed that more than 50% of fixed ^{14}C was incorporated into amino acids such as glycine, serine, alanine, asparagine and glutamine, and N-starvation reduced the proportion incorporated into asparagine and glutamine to almost undetectable levels, although it had little effect on the proportion of ^{14}C of the total amino acids. N-starvation also decreased the relative synthesis of sugar phosphate and increased the proportion of ^{14}C assimilated into intermediates of the tricarboxylic acid cycle. On the other hand, FEUILLADE *et al.* (1982) reported that N-starvation caused a significant qualitative change in the distribution of short-term photosynthetic $^{14}CO_2$ fixation of the cya-

nophyte *O. rubescens*. In unstarved *O. rubescens* cells all of the ^{14}C fixed in a 2-sec period of photosynthesis was found in 3-phosphoglycerate, whereas in N-starved cells most of the fixed ^{14}C was found in aspartate (up to 41%) and malate as is the case of an aspartate-C_4 plant.

However, N-starved *H. akashiwo* cells showed a similar labelling pattern in short-term photosynthesis to N-enriched ones, although in N-starved cells the amounts of radioactivity incorporated into amino acids and organic acids were enhanced with the accompanied decrease in β-1,3-glucans (Fig. 2A and B). The most significant difference was observed in the distribution of radioactivity in 80% methanol-soluble β-1,3-glucans in the N-starved cells when the pulse-label was chased under light conditions (Fig. 5A). The percentage of radioactivity in the glucans decreased rapidly and those in organic acids such as citrate, malate and succinate, and in amino acids increased during the chase period (Fig. 5A). The distribution pattern of ^{14}C in N-starved cells during the light-chased period looks similar to that in dark-chased N-enriched cells (Fig. 4B) and N-starved cells (Fig. 5B). These results suggest that nitrogen deficiency leads to the activation of catabolic metabolism or dark respiration in *H. akashiwo* cells. This assumption was supported by the results shown in Fig. 6B. During the four hours of nitrogen starvation, the rate of dark respiration in N-enriched cells showed a two-fold increase, while the rate of photosynthetic CO_2 fixation reduced to 75% of that obtained at zero time.

The results of this study, although reflecting the consequences of a special set of conditions (nitrate enrichment and starvation), may provide clues to the normal physiological regulation of the carbon metabolism in *H. akashiwo* undergoing diurnal vertical migration in the laboratory, swimming upward before the end of the dark period and downward in the middle of the day when nitrate was present in the culture medium, but accumulated at the bottom of the flask throughout the day when nitrate was depleted.

In preliminary experiments that we have conducted, the activity of nitrate reductase in this alga showed diurnal variation, increasing before the end of the dark period and decreasing in the middle of the day. N-starvation in the cells of *H. akashiwo* may thus be a fairly common occurrence in nature when the external supply of nitrate is not adequate at the surface of a natural body of water and when the activity of nitrate reductase in the cells is reduced to a low level, even when the external nitrate supply is adequate.

These results suggest that some product(s) of nitrogen assimilation may play an important role in the regulation of vertical migration in *H. akashiwo* cells, although other mechanisms, such as geotaxis and phototaxis, may be operating at the same time.

Acknowledgements

Thanks are due to Dr. M. TAKAHASHI, the University of Tokyo for supplying the strain of *Heterosigma akashiwo*, and to Dr. Y. HARA of this Institute for valuable suggestions.

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高橋京子・猪川倫好：ラフィド藻 *Heterosigma akashiwo* の光合成炭素代謝に及ぼす窒素欠乏の影響

海産ラフィド藻 *H. akashiwo* は，窒素源を除いて培養すると，硝酸塩を十分に与えて培養した細胞に比べ光合成炭酸固定速度が約20%低下する。$^{14}CO_2$ 固定産物の分析結果から，この低下は主として80%メタノール可溶性 β-1,3-グルカンへの取り込みの低下によることが明らかになった。さらに，5分間光合成 $^{14}CO_2$ 固定させた後 $NaH^{12}CO_3$ を加え，明及び暗条件下で ^{14}C 化合物の変動を追跡した結果，硝酸塩を十分に与えて培養した細胞では，明条件で追跡中 β-1,3-グルカンへの ^{14}C の取り込みが増加し，暗条件下では β-1,3-グルカンの ^{14}C が減少し，脂質，アミノ酸，80%メタノール不溶性画分などへ ^{14}C が移動した。しかし，窒素欠乏細胞では，明条件

下でも β-1,3-グルカンの ^{14}C は急激に減少するなど，暗条件下と同様に異化的代謝が活発に進行していることが示唆された。このことは，窒素欠乏状態の進行に伴い，呼吸活性が上昇することからも明らかにされた。以上の結果から，*H. akashiwo* において窒素欠乏状態は，光合成炭素代謝の制御に重要な役割をもつことが明らかになった。(305 つくば市天王台1-1-1 筑波大学生物科学系)

新刊紹介

西澤一俊・村杉幸子：(1988) **海藻の本**—食の源をさぐる—研成社 215頁 1,300円

海藻にはアルギン酸，寒天，カラギーナン，フコイダン，ラミナランなど，他の植物群に見られない特殊な多糖類が多数，しかも多量に存在する。最近，海藻は肥満を防ぐ，血糖値の上昇を抑制する，動脈硬化を防ぐ，腸がんなどを予防するなどの効果があるすぐれた健康食品であるというニュースをよく見，そしてよく聞くようになった。私達は藻類に興味をもち，藻類の研究を行っているものであるが，上記の多糖類がどのような生化学的性質をもつのか，海藻が健康によいのはどのような理由によるのかなどについて改まって質問されたとき，充分に答えられるものはそう多くないように思われる。専門書をひもどけば解答は得られるはずであるが，それを容易に出来るものもそう多くないようである。本学会元会長の西澤一俊博士は，共同執筆者の村杉氏と上記の質問に容易に，しかもわかりやすく答えることの出来る本を作って下さった。

本書は次の10章からなる。1．日本人と海藻，2．おもな海藻多糖類，3．おもな食用海藻と有効成分，4．食用海藻の風味と消化性，5．各国の海藻食品，6．海藻とバイオテクノロジー，7．海藻の養殖，8．有用海藻とその分布，9．バイオマスと海藻，10．海藻の生物学.

2．3．4 章が本書の最も特徴的な部分であり，西澤先生ならではの優れた記述が随所に見られ，教えられる所が多い。2章では代表的な多糖類であるアルギン酸，寒天，カラギーナン，フコイダン，及びラミナラン硫酸について，性質，用途，生物活性などが解説され，3章では代表的な食用海藻，ノリ，コンブ，ワカメ，ヒジキについて，生産状況，製品と加工方法，おもな栄養素及び特殊成分の薬理作用などが記述される。4章は日本人が好む海藻特有の味，香り，においの成分が中心である。5章以降は，栄養価の上で見直されつつある海藻資源の一層の利用と開発の方法，その基礎知識と最近の研究動向及び成果などの紹介にあてられる。

厚生省が行った国民栄養調査によると，食事の洋風化により，近年日本人の食物繊維摂取量は大幅に低下しているという。そしてこれに反比例するかのように，わが国の糖尿病患者数や直腸がん・結腸がんなどによる死亡率は増加の傾向にあるという。多くの海藻類はあらゆる食品中で最も食物繊維に富んでいる。本書は藻類を研究するものにとってはぜひ読んでおきたい本であり，また健康食品づくりに取組むために，一般家庭の方達にも広く奨めたい本である。(筑波大学生物科学系 千原光雄)

Jpn. J. Phycol. (Sôrui) **36**: 221–229, September 20, 1988

Sub-ice microalgal strands in the Antarctic coastal fast ice area near Syowa Station

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Sub-ice microalgal strands, collected in the fast ice area near Syowa Station, Antarctica, are reported and described floristically. In mid-July, no strands were seen on the bottom of the ice. Strands 10–15 cm in length were observed hanging from the sea ice in early November which grew up to 50–60 cm in early December. The strands were mainly pennate diatoms, especially those that form long colonies, including *Amphiprora kufferathii*, *Berkeleya rutilans*, *Nitzschia lecointei*, *Nitzschia stellata*, *Nitzschia turgiduloides*, and several species of *Nitzschia* in a section *Fragiraliopsis* with a small abundance of a solitary cell species of *Navicula glaciei*. Cluster analysis performed on samples collected from a 10 m long sweep with a net under the ice suggests that the seasonal succession of the organisms composing strands from November to December was not significant.

Key Index Words: Antarctic—coastal fast ice—ice algae—ice diving—microalgal strands—sub-ice assemblage—Syowa Station.

Many types of ice algal assemblages have been reported from various sea ice areas (HORNER 1985). Among them, reports on a sub-ice assemblage, attached to the underside of the ice, forming strand colonies and extending into the water column, seem to be limited to those by CROSS (1982) from the Canadian Arctic, by SULLIVAN *et al.* (1982) from McMurdo Sound, by SASAKI and WATANABE (1984) from Lützow-Holm Bay and by MCCONVILLE *et al.* (1985) from near Davis Station. The loss of this type of assemblage from core samples is likely to be the reason why it has been reported so rarely.

To investigate the sub-ice assemblage in the coastal fast ice area near Syowa Station (69°00′S, 39°35′E), underwater observations and collections were made, and the results of a floristic study of the sub-ice microalgal assemblage in November and December 1983 are described in this paper.

Materials and Methods

Under-ice diving was carried out for observation and collection of the sub-ice assemblage on July 14, November 5 and December 9 and 12 1983 at the same site, where the water was about 17 m deep, in Kita-no-seto Strait near Syowa Station (Fig. 1). The equipment employed in the SCUBA diving was described earlier (WATANABE *et al.* 1982, 1986). Strands of ice algal assemblage (microalgal strands) hanging from the undersurface of undisturbed sea ice were collected on November 5 and December 9, using 50 ml plastic disposable syringes and a hand net with five openings arranged vertically (5 cm high × 20 cm wide mouth with 100 μm mesh net each). With the syringe, microalgal strands (samples Syr. 1 and 2 collected on November 5 and Syr. 3–5 on December 9) were sampled at several locations. The hand net was towed horizontally for about 10 m beneath the sea

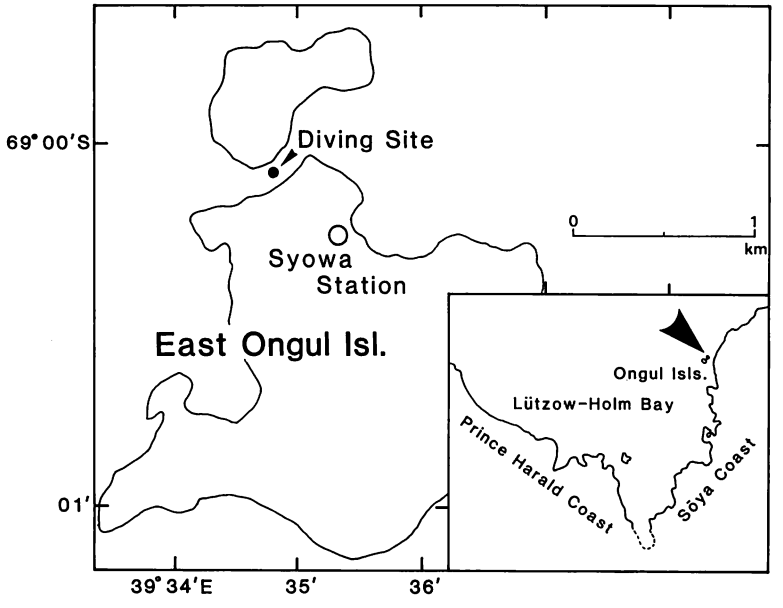


Fig. 1. Map showing under ice observation and collection site in the fast ice area near Syowa Station, 1983.

ice with the uppermost beam touching the bottom of the sea ice. Three hand net samples, H.N. 1, 2 and 3 in November and H.N. 4, 5 and 6 in December were collected from the microalgal strands in the layers of 0–5, 10–15 and 20–25 cm from the bottom of the sea ice, respectively. Samples were transported to a laboratory at Syowa Station and fixed with a formalin and acetic acid (1:1) mixture (HASLE 1978).

Species were identified using a JEOL T-100 scanning electron microscope (SEM) after being cleaned with distilled water or concentrated HCl and $KMnO_4$ mixture (SIMONSEN 1974), air dried and ion sputtered with gold. Two subsamples were taken from each sample to determine the relative abundance of selected species. 1200–2300 intact cells were counted in water mounted subsamples at a magnification of 400X or 200X, using a Nikon Diaphoto-TMD phase contrast inverted microscope. Species composition on the basis of relative abundance was compared between samples and Percent Similarity Index (PSI) by WHITTAKER (1952) was calculated as follows,

$$PSI(sample_{a,b}) = \sum_i \text{minimum } \% [species_i(sample_a, sample_b)],$$

where $i = (1,2,\ldots n$ species) in a comparison of similarity between samples a and b. Percent Similarity Index was applied for cluster analysis and a dendrogram was drawn representing the single-linkage method.

Results and Discussion

No discoloration by ice algae was observed at the bottom surface of the sea ice around the diving hole on July 14 1983. On a second dive, made on November 5, microalgal strands were found hanging from the bottom of the sea ice which was then about 120 cm thick (Fig. 2). The length of the strands was mostly less than 15 cm. This agrees with the seasonal variation of standing crop in the fast ice area at Stn. I about 100 m away from the diving hole where the spring increase of chlorophyll *a* in the bottom layer of ice began in mid-August (WATANABE and SATOH 1987). As the strands were short, the amount of mi-

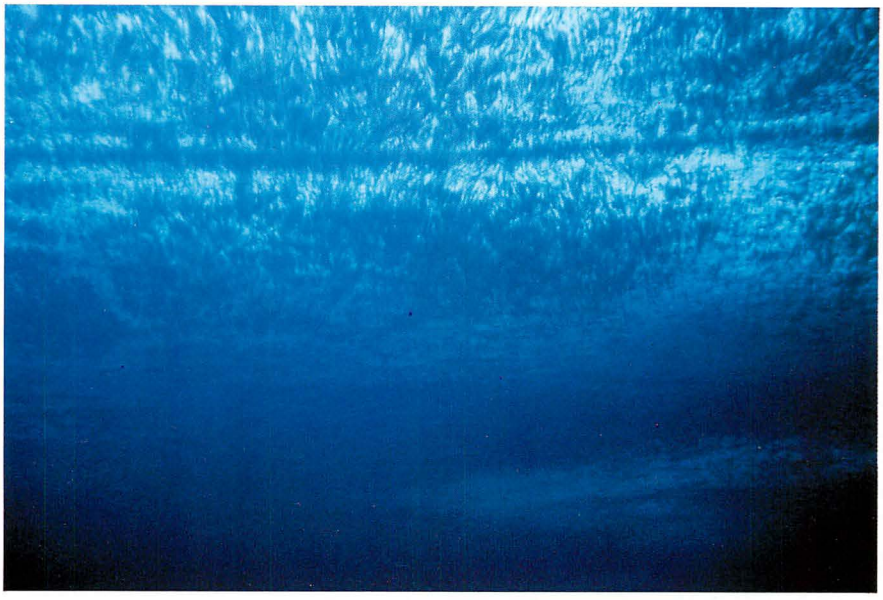
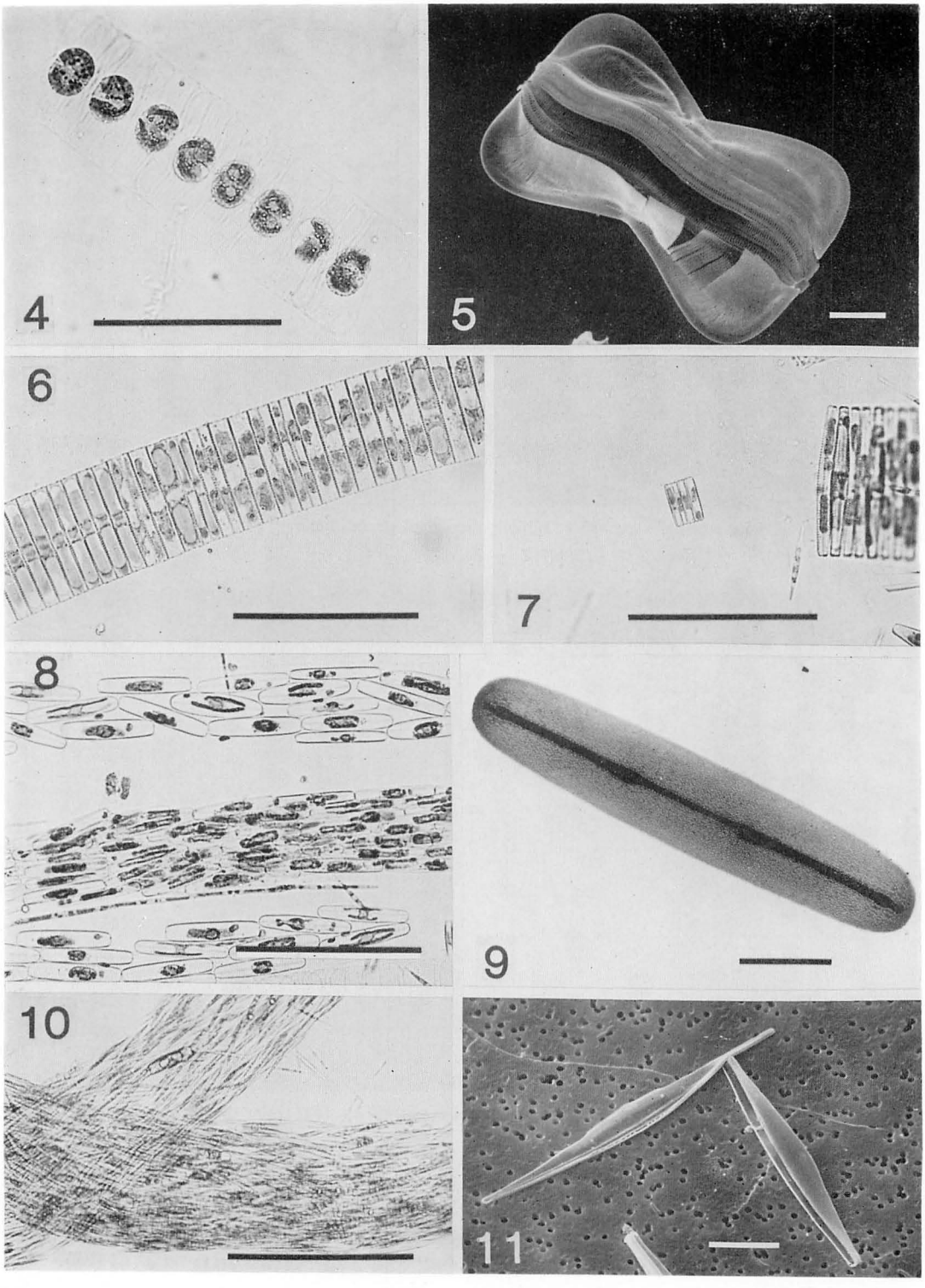


Fig. 2. Under-water photograph of sub-ice microalgal strands hanging from the fast ice in Kita-no-seto Strait near Syowa Station on November 5, 1983.

Fig. 3. Sub-ice microalgal strands extending about 50–60 cm into water column on December 12, 1983.

croalgae collected by the hand net from 20–25 cm beneath the ice in November (H.N. 3) was much less than that of H.N.1 (0–5 cm) and H.N.2 (10–15 cm). These microalgal strands appeared slightly different in their colony form and density on the ice from those reported from near Davis (Figs. 2 and 3 in McConville *et al.*, 1985). On November 5, strands were more densely distributed beneath a crack in the sea ice than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elsewhere. This unevenness of algal abundance was apparently due to the higher irradiance penetrating through the crack.

The strands were found to be longer (up to 50–60 cm) on December 9 and 12 (Fig. 3). The amount of microalgae in samples H.N.4, 5 and 6 did not differ greatly. On December 9, both short (Syr.3) and long (Syr.4) microalgal strands and globular colonies ca. 5 mm in diameter (Syr. 5), attached to the bottom of the sea ice, were collected from different locations. The strands were so fragile that they were often detached and broken by the water movement or by air bubbles exhausted from a diver. Therefore, it is presumed that the growth of sub-ice microalgal strands requires calm water. There were patches where no microalgal strands were found. This might reflect ice melting from the bottom surface which correlates with a marked decrease of ice algal standing crop in the fast ice area near Syowa Station in November and December (WATANABE and SATOH, 1987).

Most of the species appearing in the microalgal strand in this study were diatoms. Of the eleven selected taxa on which relative abundance were determined, seven formed colonies. The microalgal strands, hanging into water column, would possibly benefit zooplankton and micronekton which feed on ice algae because they are more easily accessible to grazers than microalgae inside the sea ice.

Amphiprora kufferathii MANGIN and *Nitzschia* spp. in group 1 and group 2 formed a ribbon-shaped colony (Figs. 4–7). Both groups of *Nitzschia* spp. belong to a section *Fragilariopsis* (HASLE 1972) forming similar colony in a water mount, but were distinguished on the basis of the fact that the former's valve costae were coarse enough to be seen in girdle view by light microscopy (LM) at a magnification of 200X, whereas the latter ones could hardly be seen at a magnification of 400X. By SEM examination of cleaned samples, *Nitzschia* spp. in group 1 and group 2 were found to include at least *N. obliquecostata* (VAN HEURCK) HASLE and *N. sublineata* HASLE, and *N. curta* (VAN HEURCK) HASLE and *N. cylindrus* (GRUNOW) HASLE, respectively. *Berkeleya rutilans* (TRENTEPOHL) GRUNOW and *Nitzschia lecointei* VAN HEURCK formed a tubular colony, in which cells were packed (Figs. 8–11). Sometimes, both species were found in the same tube. *B. rutilans* was abundant in the sub-ice assemblage of the coastal fast ice area near Davis (MCCONVILLE *et al.* 1985). HASLE (1964) reported that *N. lecointei* occurred in great numbers in ice samples and that its proper habitat seems to be the under-surface of ice. *Nitzschia stellata* MANGUIN formed a stellate colony (Fig. 12) and *N. turgiduloides* Hasle formed short chains with cell ends overlapping (Fig. 13). *N. turgiduloides* has been reported to be abundant in the samples from the under-surface of pack-ice in the Atlantic Ocean (HASLE, 1965). The only centric diatom species *Porosira pseudodenticulata* (HUSTEDT) JOUSE, found, appeared in chains with valve faces connected to each other (Figs. 14, 15). It was the dominant species (96%) in the faintly colored bottom layer of sea ice 80 m off Langhovde in Lützow-Holm Bay (WATANABE 1982). Three other species, *Navicula glaciei* VAN HEURCK, *Nitzschia closterium* (EHRENBERG) W. SMITH and *Pleurosigma*

Figs. 4, 5. *Amphiprora kufferathii*. 4. Light micrograph (LM) showing the ribbon-shaped colony in a water mount. 5. Scanning electron micrograph (SEM) of a valve.

Fig. 6. A ribbon-shaped colony of *Nitzschia* species in group 1 in a water mount (LM).

Fig. 7. A short fragment of a colony of *Nitzchia* species in group 2 in a water mount in the center of this photograph (LM). Note that the valve costae cannot be seen in girdle view, while those of *Nitzschia* species in group 1 in the right are recognizable.

Figs. 8, 9. *Berkeleya rutilans*. 8. Tubular colony in a water mount (LM). 9. Transmission electron micrograph (TEM) of a valve.

Figs. 10, 11. *Nitzschia lecointei*. 10. Tubular colony fully packed with the cells in a water mount (LM). 11. SEM of two cells. (Scale bar: 100 μm for Figs. 4, 6, 7, 8 and 10; 10 μm for other figures.)

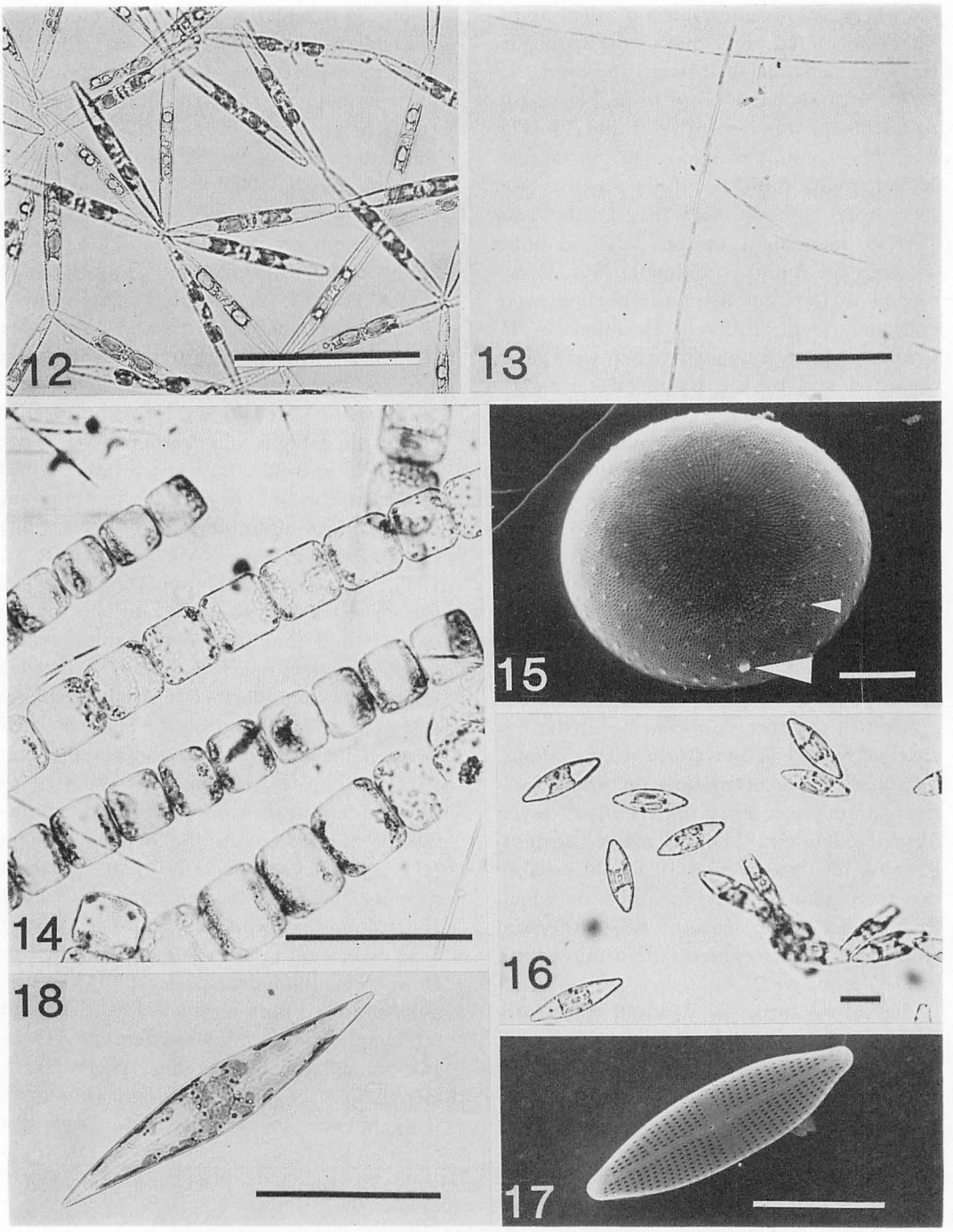

Fig. 12. *Nitzschia stellata*, forming characteristic stellate colonies in a water mount (LM).
Fig. 13. *Nitzschia turgiduloides*, forming a chain-shaped colony in a water mount (LM).
Figs. 14, 15. *Porosira pseudodenticulata*. 14. Chain-shaped colonies in a water mount (LM). 15. Valve view with a labiate process (large arrow) and strutted processes (small arrow) (SEM).
Figs. 16, 17. *Navicula glaciei*. 16. Cells in a water mount (LM). 17. SEM.
Fig. 18. *Pleurosigma directum* in a water mount (LM).
(Scale bar: 100 μm for Figs. 12, 13, 14 and 18; 10 μm for other figures.)

directum GRUNOW appeared as solitary cells (Figs. 16–18). Of these, *N. glaciei* was found to be abundant in the coastal tide-crack overflow region in Signy Is. (WHITAKER, 1977) and in Ongulkalven Is. near Syowa Station (WATANABE, unpublished), and *P. directum* was dominant (73%) in ice-algal assemblage in the off Prince Olav Coast as *Pleurosigma* sp. (WATANABE, 1982).

Species composition of the sub-ice samples are shown in Table 1. All samples were dominated by pennate diatoms (94.1–99.8%). The dominant taxa which appeared in more than 10% of samples were *Nitzschia lecointei, Amphiprora kufferathii, N. stellata, Berkeleya rutilans, Navicula glaciei, Nitzaxhia turgiduloides* and *Nitzschia* species in group 1. The results of a cluster analysis based on PSI (%), of the species composition of sub-ice samples are shown in Fig. 19. The globular sub-ice colony found on December 9 (Syr. 5), which was domi-

Table 1. Relative abundance (%) of selected taxa in sub-ice assemblages collected in Kita-no-seto Srait mear Syowa Station in 1983. Samples were collected with a syringe (Syr. samples) and with a hand net (H.N. samples). See text for more details.

Sampling Date	Nov. 5					Dec. 9					
Species/Samples	Syr. 1	Syr. 2	H.N. 1	H.N. 2	H.N. 3	Syr. 3	Syr. 4	Syr. 5	H.N. 4	H.N. 5	H.N. 6
Amphiprora kufferathii	6.6	11.4	13.4	12.5	13.2	2.5	4.7	45.0	11.6	16.2	20.1
Berkeleya rutilans	6.8	7.1	0.5	0.5	0.3	10.4	10.0	0.9	5.4	0	0
Navicula glaciei	0.7	11.5	17.8	16.3	4.7	1.2	4.9	2.8	6.3	15.0	16.6
Nitzschia closterium	0.5	0.6	0.8	0.4	0.2	2.0	1.3	2.6	2.2	1.4	1.7
N. lecointei	65.8	13.8	26.6	24.2	7.5	16.5	10.6	7.8	49.2	30.9	30.2
N. stellata	3.6	2.0	9.1	13.7	14.4	1.2	1.0	21.6	10.1	12.0	13.4
N. turgiduloides	4.5	10.7	0.6	1.8	1.1	11.0	3.4	0	0.3	0.6	0
N. spp. in group 1	2.3	35.4	10.0	21.3	53.7	35.0	42.9	5.9	7.7	4.0	4.6
N. spp. in group 2	1.1	0.8	0.1	0	1.4	1.1	3.4	0.1	0.7	1.8	0
Pleurosigma directum	1.9	0.3	0.7	0.2	0.3	0.1	0	5.5	0.3	0.9	0.7
Porosira pseudodenticulata	0.3	1.7	0.3	0.2	0.3	5.7	0.8	0	0.2	0.3	0.1
Pennales	99.5	98.3	99.7	99.7	99.7	94.1	98.8	99.1	99.8	99.6	99.4
Centrales	0.3	1.7	0.3	0.3	0.3	5.9	1.2	0.1	0.2	0.3	0.1

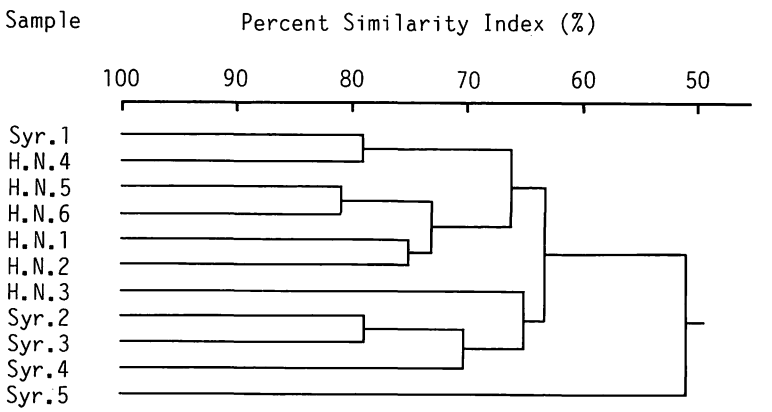

Fig. 19. A dendrogram showing the results of cluster analysis based on Percent Similarity Index (PSI) of the species composition of sub-ice samples. Samples were collected with a syringe (Syr. samples) and with a hand net (H.N. samples). Syr. 1 and 2 and H.N. 1–3 were collected on November 5 and Syr. 3–5 and H.N. 4–6 on December 9.

nated by *A. kufferathii* and *N. stellata*, has least similarity to others. However, samples from long and short strands collected on December 9 (Syr. 4 and Syr. 5, respectively) did not differ greatly (PSI=66.9%). They were dominated by *Nitzschia* species in group 1, *N. lecointei* and *Berkeleya rutilans*. As the hand net was towed for 10 m, samples collected by it represent averaged species composition in the area swept. Among hand net samples, H.N.5 and 6 collected on December 9 and H.N. 1 and 2 collected on November 5 had a high PSI; 79.6 and 74.0%, respectively. *Nitzschia lecointei, Navicula glaciei, Amphiprora kufferathii* and *Nitzschia stellata* dominated. H.N.3 sample had least similarity to other hand net samples. It contained *Nitzschia* species in group 1, *N. stellata* and *A. kufferathii*. These taxa which make long tubular or stellate colonies, may play an important role in forming long microalgal strands at an early stage of their development.

Acknowledgments

The author expresses his gratitude to Prof. T. HOSHIAI (N.I.P.R.) for his valuable advice during this study and critical reading of the manuscript. He is grateful to Dr. H.J. MARCHANT (Australian Antarctic Division) for reviewing the manuscript and helpful suggestions. He also thanks Prof. S. MAE (Univ. Hokkaido), the leader of the 24th Japanese Antarctic Research Expedition and the members of the party, especially Drs. H. KANDA (N.I.P.R.), H. SATOH (Tokyo Univ. Fish.) and E. TAKAHASHI (Univ. Kobe) for their helpful assistance in the ice diving operations.

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渡辺研太郎：南極昭和基地周辺定着氷下に見られたヒモ状微細藻類群体

南極昭和基地近くの定着氷下面に成長する微細藻類の太いヒモ状群体を海中から観察・採集し，種組成を調べた。7月中旬には海氷下面に群体は見られなかったが，11月上旬，10～15 cm の長さの微細藻類から構成される太ヒモ状群体が海氷から水中へ垂れ下がっているのが観察され，12月上旬には50～60 cm の長さに達するものが見られた。太ヒモ状群体を構成していたのは主に羽状目珪藻類，特に長い群体を形成する *Amphiprora kufferathii*, *Berkeleya rutilans*, *Nitzschia lecointei*, *Nitzschia stellata*, *Nitzschia turgiduloides*, section *Fragilariopsis* に属する *Nitzschia* 属の数種，および単独性の *Navicula glaciei* だった。海氷下面を手持ちネットで 10 m 区間採集したサンプルをクラスター分析したところ，11月と12月のサンプル間には顕著な種の遷移は認められなかった。（173 板橋区加賀1-9-10　国立極地研究所）

新　刊　紹　介

TRONO, G.C. & GANZON-FORTES, E.T.: Philippine Seaweeds, 328 pp, paperback, 1988, National Book Store, Inc., Manila, Price unknown.

著者達は Marine Science Inst., Univ. of Philippines 所属の海藻学者であり，開発途上国であるフィリピンでの海藻資源の研究をしている。

第1章は序論。第2章は pp. 6–196 で，総種数141についての線画とカラー写真を伴った記載であるが，写真は通常紙印刷のせいもあって明瞭でないものも少くない。緑藻は77種，褐藻は31種，紅藻は80種を掲載。種の記載は中程度の詳しさ。第3章は pp.197–204 で，海藻の現地での利用面を扱い，第4章は pp. 205–244 で，資源としての海藻を扱い，学名，地方名，利用区分，利用程度，野生と養殖の別から成る表（pp. 206–228），*Gracilaria*, *Eucheuma*, *Caulerpa*, *Porphyra* の海中や池中での養殖技術（pp. 229–236），収穫と市場価値（pp. 236–238），収穫物の処理（pp. 238–244）と寒天，カラゲナン，アルギン酸の製造法が述べられる。第5章（pp. 245–255）は海藻産業を扱い，輸出入，価額，問題点その他が述べられる。最後に，術語解，文献リスト（約200件），付録としての，食用海藻の調理法（12 pp.），海藻漁穫関連法令，標本作製法，*Eucheuma* と *Caulerpa* の海中と池中養殖の詳細（カラー写真多数），種名と件名索引が付いている。

浅海産の大型および普通種に限定されたので，名属又は科内のフィリピン産の全容を知ることはできぬ。現地人以外にとってはむしろ種の記載＋図よりも，現地独特の上記2属の養殖の現状が興味深いと思われる。ともあれ，東南アジアからこのような出版物が現われるようになったことは評価すべきと考える。

著者の訂正メモによれば，Figs 142 と 143 の説明が交換されてミスプリントとなっている。（191 日野市日野6498-4-103　赤塚伊三武）

新刊紹介

WOMERSLEY, H.B.S.: The Marine Benthic Flora of Southern Australia. Part II. 484 pp. Government Printer, Adelaide (1987).

〝南オーストラリア海産底生植物相〟は第1部（海草，緑藻植物，車軸藻植物，1984年），第II部（今回の紹介）及び第III部（紅藻植物，2－3年内に発行予定）のシリーズからなり，LUCAS（1936）の〝南オーストラリアの緑藻類と褐藻類〟及びLUCAS & PERRIN（1947）の〝同，紅藻類〟に代わるものとして発行された南オーストラリアの海産植物誌である。

対象地域は，西オーストラリア州，南オーストラリア州，ビクトリア州及びタスマニア州のオーストラリア南部の全沿岸で，東部の寒冷温帯海域（夏季平均水温 15°～19℃）から中部及び西部の温暖温帯海域（19°～25℃）に及んでいる。

第I部では，海産植物の採集と保存，研究史，生態及び生物地理学について詳細に解説しているので，第II部では省略されている。

褐藻綱を13目に分類しているが，Ralfsiales（イソガワラ目）を認めていない。ナガマツイモ目には8科があり，その中に Scytothamnaceae（新科）を設けて，近縁の Splachnidiaceae から，胞子嚢が皮層中に散在し，また枝に洋梨形の頂端細胞をもたないという特性によって区別している。同目にイソガワラ科（*Ralfsia*, *Pseudolithoderma*, *Hapalospongidion*）を入れている。アミジグサ目は豊富で，15属40種があって，世界のどの温帯海よりも多い。また，枝の先端が陥入して，その頂端細胞が枝の縦方向にあって，その両側へ枝を発出する特性をもつ Scoresbyellaceae（新科）（*Scoresbyella*, 新属）を設けている。ウルシグサ目の中に，船舶によって運ばれたと思われる西部大西洋及び地中海産の *Arthrocladia villosa*（Arthrocladiaceae）が発見されている。褐藻綱の中に，他に7種の外来種があり，ある種は居着いているという。ケヤリモ目はケヤリモ科だけで，世界にある10属のうち8属（2新属：*Austronereia*, *Sporochnema*）が存在する。他の2属はクィーンズランド州にある *Tomaculopsis* と，ニュージランドにある *Perisporochnus* であって，オーストラリア海域は同目の分布の中心となっている。NIZAMUDDIN & WOMERSLEY（1960）によってナガマツモ目に分類されていた *Notheia anomala* が，ヒバマタ目に似ているが，生殖窠内にできる大配偶子は運動性であることからNotheiales を新設して入れている。

南半球にはコンブ目植物は少ないが，Lessoniaceae（*Lessonia*, *Macrocystis*）及びチガイソ科（カジメ属）が知られている。また，タスマニア州，ビクトリア州及び南オーストラリア州の寒冷海に *Durvillaea potatorum*（Durvillaeales）が生育する。ヒバマタ目に，NIZAMUDDIN(1962)が新設したSeirococcaceae(NIZAMUDDINが1987年にラテン記載をする）があり，同沿岸固有の3属（*Phyllospora*, *Seirococcus*, *Scytothalia*）が知られている。ウガノモク科には，世界にある16属のうち9属が存在し，そのうちの *Cystophora* は南アフリカ産の1種（*Cystophora fibrosa*）を除いて23種（2新種を含む）があって，同沿岸の最大の分類群となっている。ホンダワラ科はホンダワラ属のみで，3亜属（*Phyllotrichia*, 6種；*Arthrophycus*, 5種；*Sargassum*, 4種）に分類されている。

同沿岸から世界の褐藻綱の全目（Tilopteridales と Ascoseirales を除く）が知られ，34科104属231種が分類されている。固有種は131種（57%）で，また，オーストラリア外からの61種（26%）が記録されている。

最後に，黄金色藻植物―黄緑藻綱―フシナシミドロ目―フシナシミドロ科にフシナシミドロ属5種が知られている。

目科属にはそれぞれの検索表があり，分類群の特性（藻体の形態，生長様式，生殖法，生活史）が記述されている。多くの種の藻体写真，顕微鏡スケッチ，フィールドの藻体のカラー生態写真が載せられている。引用文献に続いて，9頁にわたる術語の解説もある。

前述の如く，本書（第I－III部）は南オーストラリアの海産底生植物相を集大成したものであり，また，最近の同類の分類学の進歩をしめす大著である。

（京都大学農学部熱帯農学専攻　梅崎　勇）

Jpn. J. Phycol. (Sôrui) **36**: 231–236, September 20, 1988

Additional notes on the life history of *Nemalion vermiculare* SURINGAR (Nemaliales, Rhodophyta)

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MASUDA, M. and HORIUCHI, K. 1988. Additional notes on the life history of *Nemalion vermiculare* SURINGAR (Nemaliales, Rhodophyta). Jpn. J. Phycol. **36**: 231–236.

Further investigations into the life history of the red alga *Nemalion vermiculare* SURINGAR were conducted at Oshoro Bay on the west coast of Hokkaido. The appearance, abundance and size of the upright gametophytes were related to tidal height and the degree of wave exposure. During the autumn and winter months, acrochaetioid filaments were found growing on barnacles on which *N. vermiculare* gametophytes grew in the summer. Sterile fragments of these filaments cultured in laboratory formed tetrasporangia under a short day regime at 20°C. Tetraspores germinated to produce a filamentous prostrate system with upright, multiaxial, terete axes that were similar to young plants of field-collected *N. vermiculare* gametophytes.

Key Index Words: filamentous tetrasporophyte—life history—Nemaliales—Nemalion—N. vermiculare —*Rhodophyta*.

Culture studies have demonstrated that the life history of *Nemalion vermiculare* SURINGAR (Nemaliales, Rhodophyta) comprises the alternation of a multiaxial, upright gametophyte with an acrochaetioid tetrasporophyte (UMEZAKI 1967, MASUDA and UMEZAKI 1977), as has been shown in other species in this genus (FRIES 1967, 1969, CHEN *et al.* 1978). However, the tetrasporophyte has never been observed in nature. In this paper, we report the occurrence of the tetrasporophyte in nature and present culture studies that confirm it as a phase in the life history of *N. vermiculare*. We relate patterns in the growth and reproduction of the gametophyte to its environment.

Materials and Methods

Twelve survey sites were established at Oshoro Bay (43°13'N, 140°51'E) on the west coast of Hokkaido (Fig. 1) and field observations and samplings were made twice a month from May 1986 to October 1986 and monthly from November 1986 to February 1987. These sites were selected to represent the diverse habitats of *Nemalion vermiculare* at Oshoro Bay: (1) A, C, G and L, below the low watermark and fully wave-exposed; (2) J, near the low watermark and moderately wave-exposed; (3) B, E, H and K, above the low watermark and in shallow tide pools made by large, breaking waves; and (4) D, F and I, above the low watermark and sheltered from wave action. Gametophytes, when present, and several substrates such as barnacles (*Semibalanus cariosus* and *Chthamalus challengeri*), mussels (*Mytilus edulis* and *Septifer virgatus*), perennial algae (*Sargassum thunbergii*, *Carpopeltis affinis* and *Chondrus pinnulatus*) and rock fragments were collected.

Culture experiments were conducted with carpospores of *Nemalion vermiculare* and excised filaments of the putative tetrasporophytes. These were cultured according to methods similar to those described earlier

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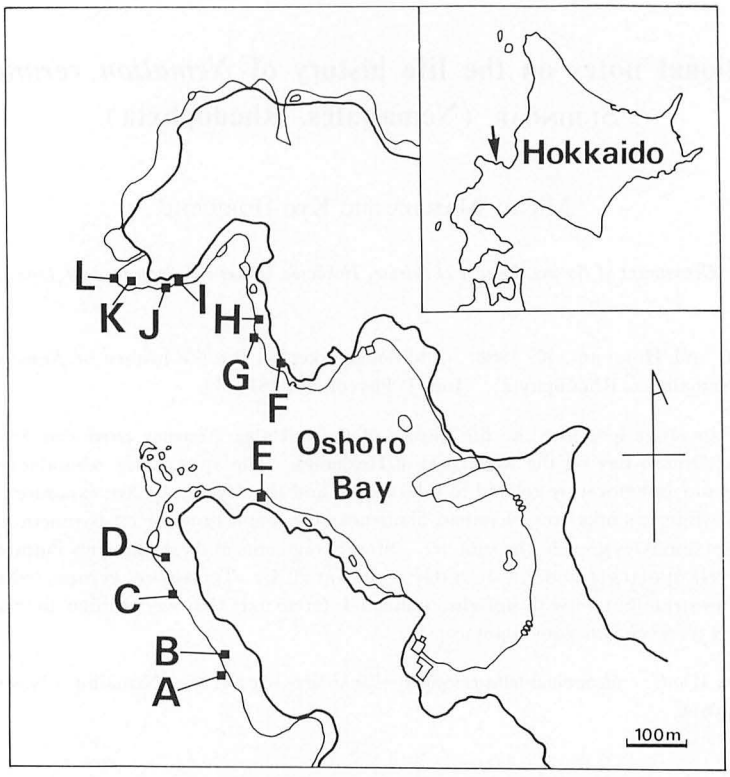


Fig. 1. Map of Oshoro Bay, showing twelve study sites.

(MASUDA and UMEZAKI 1977). The temperatures and photoperiods were regulated as follows: 5°C, 16:8 h LD (light and dark cycle); 5°C, 8:16 h LD; 10°C, 16:8 h LD; 10°C, 8:16 h LD; 15°C, 16:8 h LD; 15°C, 8:16 h LD; 20°C, 16:8 h LD; and 20°C, 8:16 h LD. The following materials were used for culture experiments: gametophytes collected on August 15, 1986 at site C and on August 28, 1986 at site E; and tetrasporophytes collected on October 25, 1986 and on November 25, 1986 at site B. Voucher specimens are deposited in the Herbarium of Faculty of Science, Hokkaido University, Sapporo (SAP 051052–051063).

Results

Phenology of upright gametophytes

Upright gametophytes (Fig. 2), less than 1 mm long, appeared in early May at all sites except the sheltered sites D, F and I, where they appeared about one month later. These young upright thalli were superficially similar to young plants of

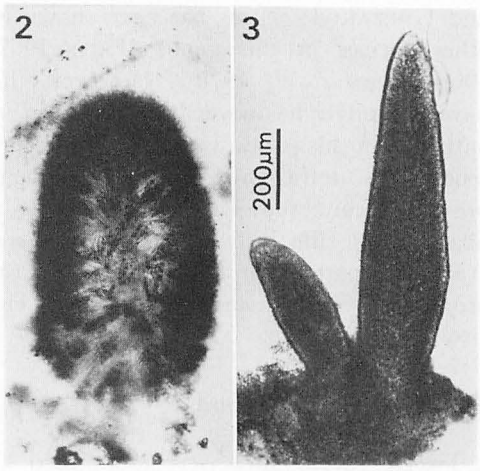
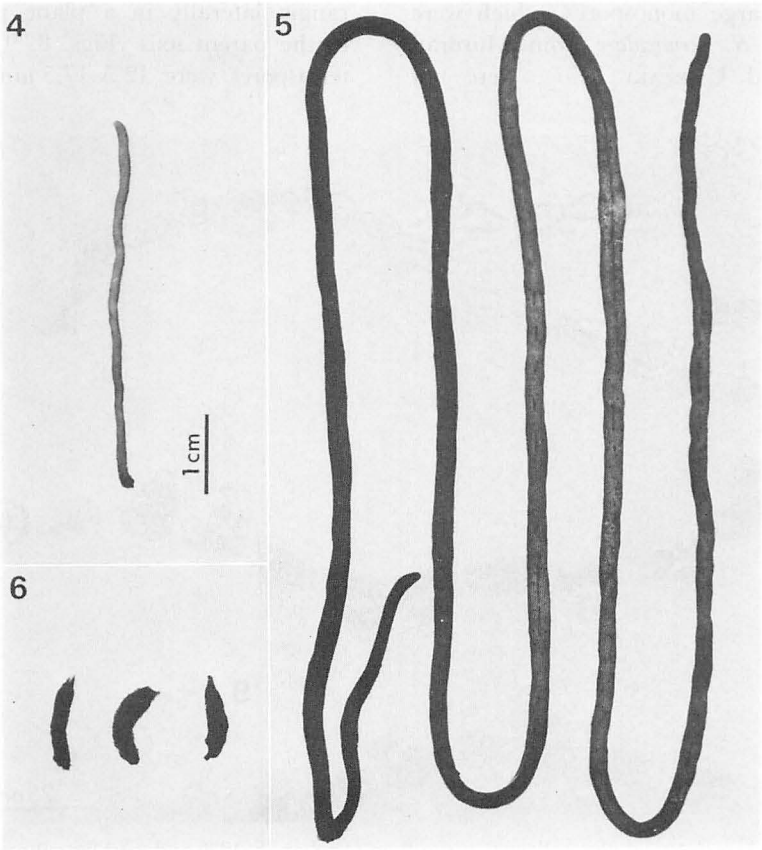


Fig. 2. Young upright thallus of *Nemalion vermiculare* collected at site C on May 9, 1986. Fig. 3. Young plant of *Gloiopeltis furcata* collected at site C on May 9, 1986. Scale in Fig. 3 applies also to Fig. 2.

Gloiopeltis furcata (Fig. 3), but *Nemalion* thalli could be distinguished from the latter by their soft, mucilaginous texture and rounded apices.

Upright thalli grew rapidly and reached maximum lengths during July and August. The number of *Nemalion* plants varied according to survey sites. The *Nemalion* plants grew abundantly at fully wave-exposed sites A, C, G and L, whereas they were less frequent at other sites. The size of upright thalli also varied according to site (Figs. 4, 5). The maximum length of thalli growing at fully wave-exposed sites was 56 cm (Fig. 5) and ten times longer than that of plants growing in shallow tide pools (Fig. 4). Plants characteristic of wave-exposed sites were thick and dark red in color.

Reproductive structures were not found on upright thalli collected in May. In mid-June, plants proved to be monoecious gametophytes, bearing both spermatangia and carpogonia near their apices. In late June, cystocarps developed at the apices while spermatangia and carpogonia continued to be produced basipetally. Assimilatory filaments constituting the cortices of fertile areas disintegrated after carpospore discharge, leaving axial filaments that also eventually eroded. Although plants declined in length as the season progressed, cystocarps continued to develop in proximal portions. In late October through November, plants only 1 cm in length (Fig. 6) formed cystocarps near their holdfasts.



Figs. 4–6. Upright gametophytes of *Nemalion vermiculare*: 4, mature plant growing in a shallow tide pool (site K) and collected on July 29, 1986; 5, mature plant growing at a fully wave-exposed place (site L) and collected on July 29, 1986; 6, old plants growing at a fully wave-exposed place (site C) and collected on October 25, 1986. Scale in Fig. 4 applies also to Figs. 5 and 6.

Culture experiments with carpospores

Isolated carpospores, 12.5–17.5 μm in diameter, were incubated under the full range of culture conditions described in Materials and Methods. They germinated and grew into acrochaetioid plants under all conditions. The plants formed tetrasporangia in a manner similar to that reported for *N. vermiculare* from Muroran (MASUDA and UMEZAKI 1977). Plants grown at 20°C, 8:16 h LD began to form tetrasporangia 12 days after inoculation, those grown at 15°C, 8:16 h LD formed tetrasporangia 14 days after inoculation, those grown at 10°C, 8:16 h LD 35 days after inoculation and those grown at 5°C, 8:16 h LD 70 days after inoculation. Liberated tetraspores were 12.5–17.5 μm in diameter. Large monospores, which were reported for *N. vermiculare* from Muroran (MASUDA and UMEZAKI 1977), were not observed. Plants grown under long day regimes did not produce tetrasporangia at any of the experimental temperatures.

Naturally occurring tetrasporophytes

Substrates (listed in Materials and Methods) were collected at the survey sites and examined under a dissecting microscope in the laboratory. From October 1986 to February 1987, sterile filaments of an acrochaetioid alga resembling the cultured tetrasporophyte of *Nemalion vermiculare* (MASUDA and UMEZAKI 1977) were found in the grooves between the longitudinal ribs of barnacle plates (Fig. 7). Excised filaments were cultured at 20°C, 16: 8 h LD for 2 weeks, and transferred to 20°C, 8:16 h LD to induce tetrasporogenesis. After 2 weeks, filaments began to produce sessile tetrasporangia laterally in a plane perpendicular to the parent axis (Figs. 8, 9). Liberated tetraspores were 12.5–17.5 μm in diameter

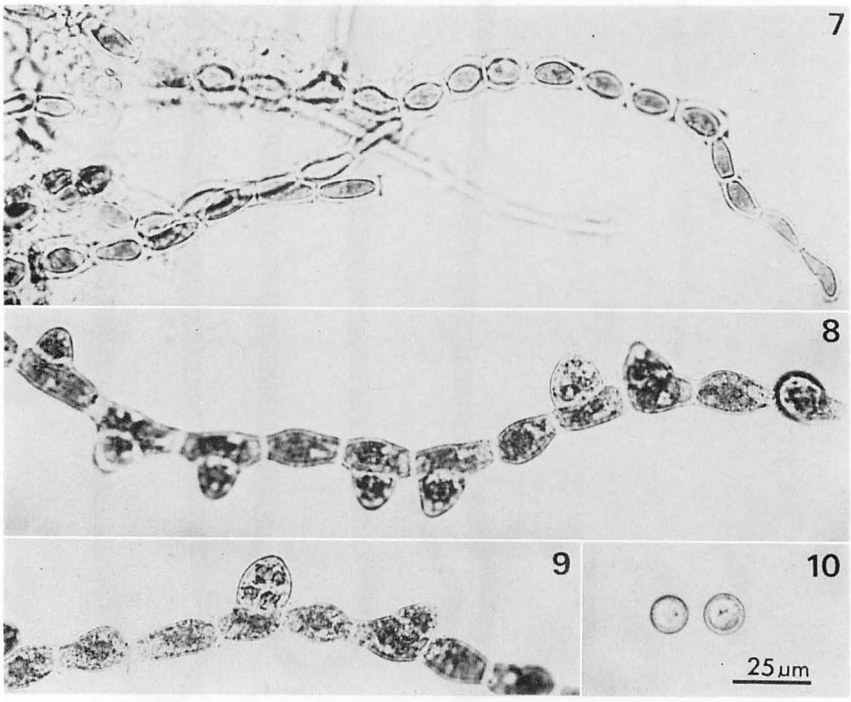
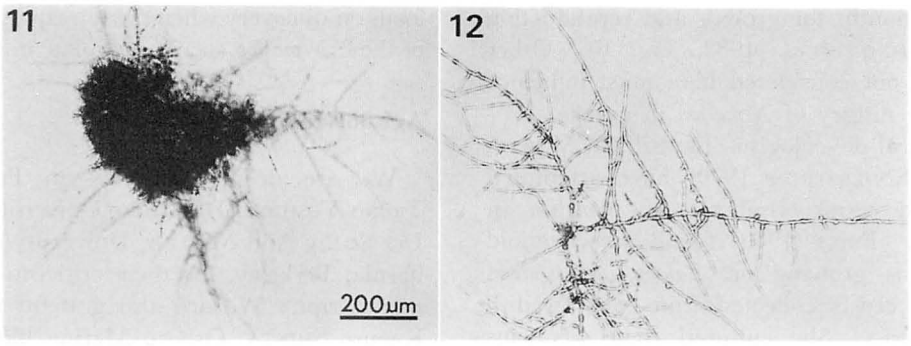


Fig. 7. Acrochaetioid alga collected at site B on October 25, 1986 and used for culture experiments [microphotographed from a specimen stained with 0.5% (w/v) cotton blue in a lactic acid/phenol/glycerol/water (1:1:1:1) solution]. Figs. 8, 9. Cultured fertile tetrasporophyte of *Nemalion vermiculare* derived from the acrochaetioid alga shown in Fig. 7. Fig. 10. Two tetraspores released from the plant shown in Figs. 8 and 9. Scale in Fig. 10 applies also to Figs. 7 to 9.



Figs. 11, 12. Twenty-one-day-old gametophytes of *Nemalion vermiculare* derived from tetraspores from the plant shown in Figs. 8 and 9: 11, grown at 15°C, 16:8 h LD and bearing an upright thallus primordium; 12, grown at 15°C, 8:16 h LD. Scale in Fig. 11 applies also to Fig. 12.

(Fig. 10). Large monospores were not observed.

Laboratory-induced tetraspores from field-collected sporophytes were cultured at 15°C, 16:8 h LD, 15°C, 8:16 h LD, 10°C, 16:8 h LD and 10°C, 8:16 h LD. Primordia of upright thalli appeared at 15°C, 16:8 h LD (Fig. 11) and 10°C, 16:8 h LD 3 weeks after spore inoculation. Each primordium consisted of a compact group of filaments arising from the center of a system of prostrate filaments. Each developed into a multiaxial *Nemalion* thallus (Figs. 13, 14). However, upright thalli did not develop at 15°C, 8:16 h LD (Fig. 12) and 10°C, 8:16 h LD.

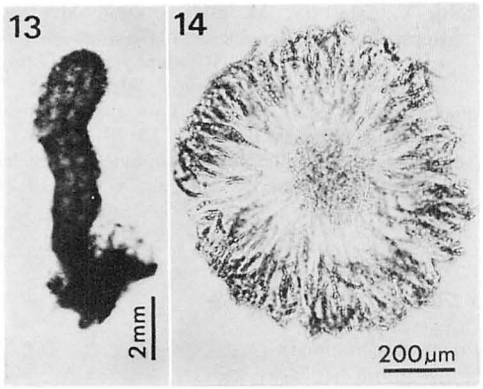


Fig. 13. Six-month-old gametophyte of *Nemalion vermiculare* derived from a tetraspore from the plant shown in Figs. 8 and 9 and grown at 15°C, 16:8 h LD. Fig. 14. Cross section of the upright thallus shown in Fig. 13.

Discussion

Our surveys show that populations of *Nemalion vermiculare* at Oshoro Bay attain their maximum abundance and size at wave-exposed localities from May through August. The summer occurrence of *N. vermiculare* gametophytes is consistent with the results of our culture experiments which indicate that upright thalli develop only under long day conditions. The tidal height has a narrow range along the coast of the Sea of Japan, but the seasonal change is large: the mean sea level is low in winter and high in summer (Japan Meteorological Agency 1985). The monthly mean sea level at Otaru near Oshoro Bay is the lowest in March (9.3 cm) and the highest in August (29.1 cm). It reaches 21.0 cm high in June (Japan Meteorological Agency 1985). This increase may account for the delayed appearance of macroscopic gametophytes in sheltered localities near the high watermark.

Under short day regimes at 5–20°C, cultured tetrasporophytes grow quickly, becoming visible to the naked eye and reproductively mature within 10 weeks after germination. Tetrasporangia are formed more rapidly at the higher temperatures in culture. Field-collected tetrasporophytes are inconspicuous and apparently sterile during the autumn and winter, even though our culture experiments indicate that light and temperature conditions are suitable during

these months for growth and reproduction (cf. OHNO *et al.* 1982, Fig. 6). Other factors not considered here must influence the life history of *Nemalion* in nature.

Several phycologists (FRIES 1969, MARTIN 1969, SÖDERSTRÖM 1970) have attempted to find tetrasporophytes of *Nemalion* in nature. FRIES (1969) found acrochaetioid filaments growing on *Balanus* shells and *Ralfsia* crusts collected from the Swedish west coast. She cultured these filaments in the laboratory and succeeded to produce tetrasporangia with the same appearance as those in her previous cultures of *Nemalion multifidum* (WEBER et MOHR) J. AGARDH (FRIES 1967). MARTIN (1969) followed carposporelings of *N. helminthoides* (VELLEY) BATTERS on the Anglesey coast of Wales during several years. These acrochaetioid carposporelings growing on linpet shells and barnacle plates formed monosporangia instead of tetrasporangia in the field. They survived in the winter and produced upright gametophytes in May. SÖDERSTRÖM (1970) suggested that tetrasporophytes of *N. multifidum* in Scandinavia are induced to form tetrasporangia by rising temperature and increasing daylength. On the basis of laboratory culture experiments CHEN *et al.* (1978) concluded that tetrasporangia can be formed under various conditions of temperature and photoperiod (10–20°C, 12:12–16:8 h LD), but that a prior excursion into winter conditions (5°C, 8:16 h LD) is necessary to induce tetrasporogenesis for the Canadian *N. helminthoides*. According to our present culture experiments and those of MASUDA and UMEZAKI (1977), such an excursion is unnecessary for the Japanese *N. vermiculare*. Further investigations should focus on discovery when the tetrasporophytes of these *Nemalion* species sporulate in nature.

Acknowledgments

We are deeply indebted to Professor Tadao YOSHIDA, Hokkaido University, and Dr. Kathy Ann MILLER, University of California, Berkeley, for their criticism of the manuscript. We are also grateful to Mr. Kazuro SHINTA, Oshoro Marine Biological Station, for his help in collecting materials; and to Dr. Shin KUBOTA, Hokkaido University, for his identification of barnacles and mussels.

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増田道夫・堀内　京*：紅藻ウミゾウメンの生活史についての続報

北海道西岸の忍路湾において，紅藻ウミゾウメン *Nemalion vermiculare* SURINGAR の生活史を調査した。直立配偶体の出現，生育量及び生長は生育環境，すなわち，潮位と波の影響の度合に関連していることが示された。本種の着生していたフジツボに秋から冬にかけて，アクロケチウム様糸状体が生育していた。この糸状体には四分胞子嚢の形成はみられなかった。実験室で培養された糸状体は，20℃の短日条件下で四分胞子嚢を形成した。放出された四分胞子は発芽して，ウミゾウメンの若い直立体に類似した多軸型の藻体に生長した。（060 札幌市北区北10条西8丁目　北海道大学理学部植物学教室　*現住所：049-15 北海道松前郡松前町字白神928　松前町立白神小学校）

Jpn. J. Phycol. (Sôrui) **36**: 237–240, September 20, 1988

Shigeru KUMANO: Sexual reproductive organs of *Bostrychia flagellifera* POST (Ceramiales, Rhodophyta) from Japan

Key Index Words: Bostrychia flagellifera—*procarp*—*spermatangia*—*3-celled carpogonial branch.*
Shigeru Kumano, Department of Biology, Faculty of Science, Kobe University, Rokko-dai, Nada-ku, Kobe, 657 Japan.

POST (1936) described *Bostrychia flagellifera* as a new species from Paramatta River in Sydney, Australia. POST (1961) identified Japanese specimens collected from Sakura-jima Volcano as *B. flagellifera* and reported the structure of thallus, tetrasporangial stichidia and cystocarps, but no observations are available on the procarps and spermatangia.

This paper deals with observations on sexual reproductive organs based on the specimens of *Bostrychia flagellifera*, collected by the late Dr. Hiroshi ITONO of Kagoshima University, from Sonoyama-ike in Sakura-jima Volcano, Kagoshima Prefecture in Japan, on September 5, 1981. The specimens examined were deposited in the Herbarium of Faculty of Science, Kobe University.

Bostrychia flagellifera is monoecious, the spermatangial sorus (Figs. 2, 7 and 8) is found to form at the terminal portion of fertile branches in conjunction with a procarp. The axial cell (Figs. 2 and 5, AC) divides longitudinally to produce four to five pericentral cells, called as primary parent cells (Figs. 2 and 5, PPC).

Each primary parent cell divides to produce secondary parent cells (Figs. 2 and 5, SPC), each of which divide successively to produce tertiary parent cells (Figs. 2 and 5, TPC). The tertiary parent cells (Figs. 2 TPC, PC) produce 1–2 spermatangia (Figs. 2 and 5, SP).

FALKENBERG (1901) observed that the axial cell of *Bostrychia tenella* is surrounded by pericentral cells and one layer of cortical cells, each of which directly produces a spermatangium. SMITH and NORRIS (1988) reported the linear arrangement of a single primary parent cell (Fig. 3, PPC) connection to chains of secondary parent cells (Fig. 3, SPC) for *B. binderi*. Spermatangia (Fig. 3, SP) of this species were produced from both primary and secondary parent cells. Each primary parent cell (Fig. 4, PPC) of *B. montagnei* was pit-connected to at least three spermatangium producing, secondary parent cells (Fig. 4, SPC) in a di- or trichotomous arrangement.

In the present study, it is observed that each primary parent cell (Fig. 5, PPC) of *B. flagellifera* produces successively secondary (Fig. 5, SPC) and tertiary parent cells (Fig. 5, TPC, Figs. 2 and 8, PC), which have some analogy with cortical cells. The tertiary, and sometimes secondary parent cells (Figs. 2 and 8, PC) were observed to produce 1–2 spermatangia (Figs. 2 and 8, SP).

The axial cells of the terminal portion of the thallus produce 3–4 pericentral cells and usually one procarp is formed on each sector. A sterile pericentral cell divides transversely into two tier cells in the same manner as the vegetative portion of the thallus. Procarp consists of a supporting cell (Figs. 1 and 2, S) and 3-celled carpogonial branch with one sterile cell (Fig. 1, SR) or none (Figs. 1, lower one, and 2). Since the four-celled carpogonial branch (Fig. 1, lower one) is observed only once on Japanese specimens collected from Sakura-jima Volcano, the 3-celled carpogonial branch is more common than 4-celled carpogonial branch.

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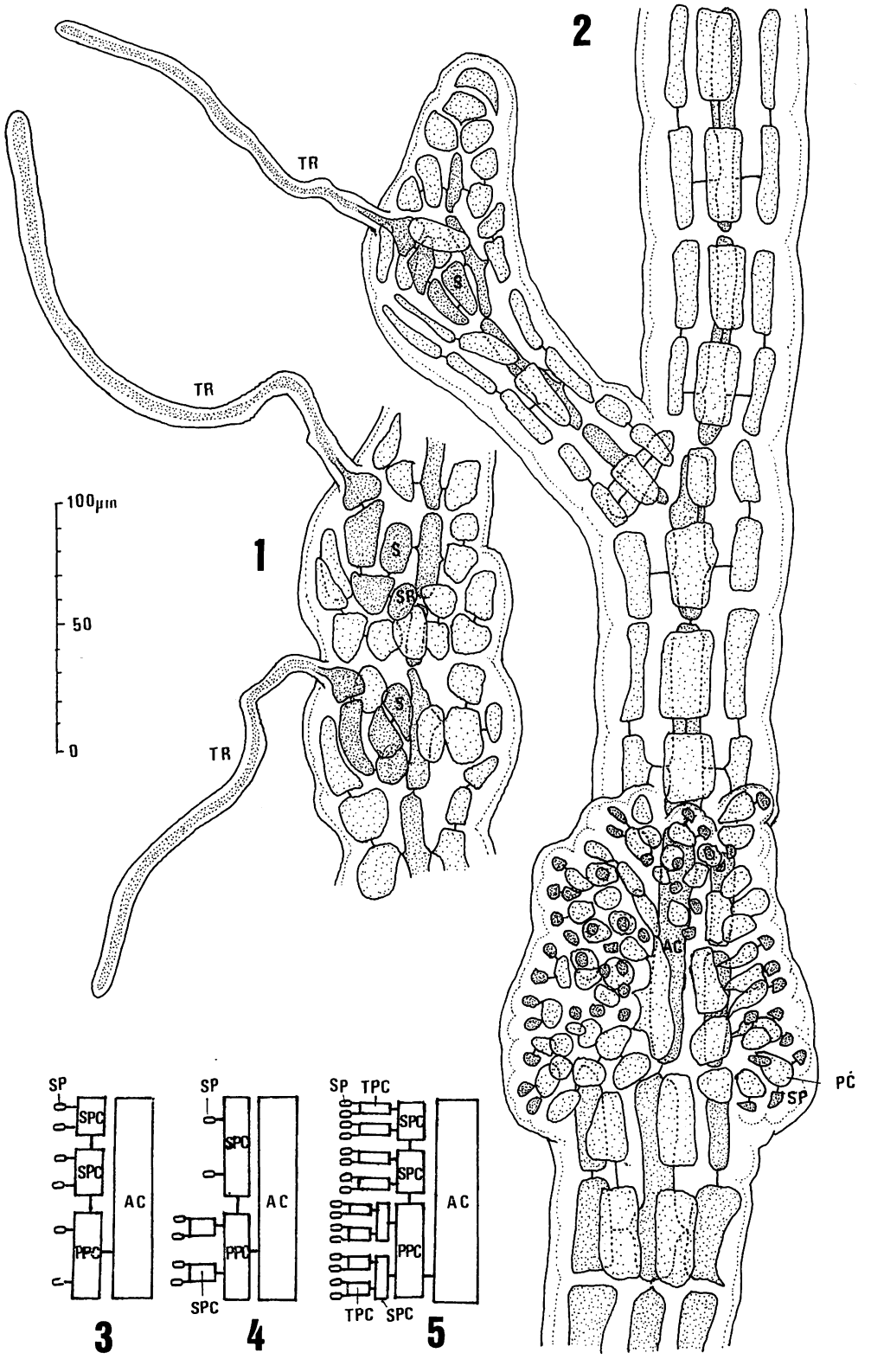
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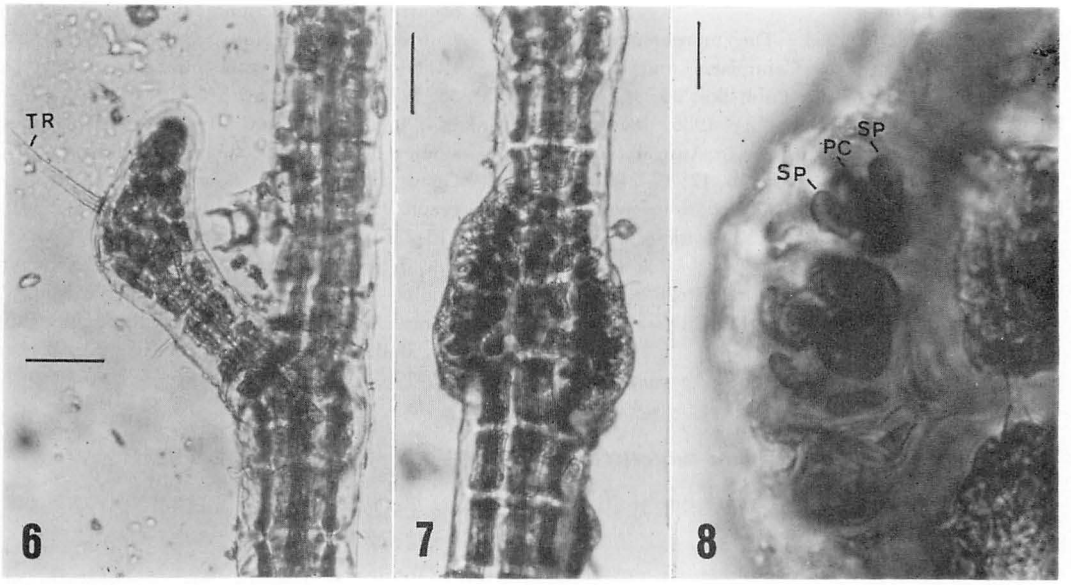

Figs. 6–8. *Bostrychia flagellifera* Post 6. A procarp with a mature trychogyne projecting obliquely toward the tip of the thallus; 7. A spermatangial sorus; 8. A part of a spermatangial sorus. (AC, axial cell; PC, parent cell; PPC, primary parent cell; S, supporting cell; SP, spermatangium; SPC, secondary parent cell; SR, sterile cell; TPC, tertiary parent cell; TR, trichogyne. Scale bars; 50 μm for Figs. 6 and 7; 10 μm for Fig. 8)

The carpogonial branch is directed toward the apex of the thallus and the carpogonium occupies a position lateral to the supporting cell. The mature trichogyne (Figs. 1, 2 and 6, TR) is up to 200 μm long and projects obliquely toward the tip of the thallus.

Most taxon of the Ceramiales have been observed invariably to produce 4-celled carpogonial branches. Many authors such as Hommersand (1963), Cordeiro-Marino (1979), Tanaka and Chihara (1984a, b), King and Puttock (1986) have reported the standard Ceramiales-type of 4-celled carpogonial branches in *B. arbuscula* Harvey, *B. kelanensis* Grunow in Post, *B. pinnata* Tanaka et Chihara, and *B. radicans* (Montagne) Montagne. Four-celled carpogonial branch of Japanese specimens of *B. flagellifera* Post collected from Sonoyamaike was observed once, and the post-fertilization and carposporophytes have not been observed.

The author wishes to express his sincere thanks to the late Dr. Hiroshi Itono of Kagoshima University for collecting specimens.

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Figs. 1–2. *Bostrychia flagellifera* Post 1. An upper procarp consisting of a supporting cell and 3-celled carpogonial branch with a sterile cell, and a lower procarp consisting of a supporting cell and 4-celled carpogonial branch without a sterile cell; 2. A spermatangial sorus formed at the terminal portion in conjunction with a procarp consisting of 3-celled carpogonial branch without a sterile cell.

Figs. 3–5. Comparison of male reproductive structures for three species. 3. Male reproductive structure of *Bostrychia binderi* Harvey (Smith and Norris 1988, modified); 4. Male reproductive structure of *B. montagnei* Harvey (Smith and Norris 1988, modified); 5. Male reproductive structure of *B. flagellifera* Post (present study).

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熊野　茂：*Bostrychia flagellifera* POST（イギス目・紅藻植物）の有性生殖器官

故糸野　洋博士が鹿児島県桜島の園山池から採集した *Bostrychia flagellifera* POST の標本を観察した。本種は雌雄同株である。皮層細胞と似た方法で，周心細胞から造り出された精子嚢母細胞は 1-2 ケの精子嚢を造る。嚢果は発見出来なかったが，プロカルプは，1 ケの支持細胞，0-1 ケの sterile cell, 3 細胞性（例外的に 4 細胞性）の造果枝からなる。（657 神戸市灘区六甲台町1-1 神戸大学理学部生物学教室）

藻類 Jpn. J. Phycol. **36**: 241–245, September 20, 1988

台湾産ヒメバショウ（紅藻，コノハノリ科）の原標本について

三　上　日　出　夫

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MIKAMI, H. 1988. On the original specimens of *Neoholmesia neurymenioides* (OKAMURA) WYNNE (Delesseriaceae, Rhodophyta) from Taiwan. Jpn. J. Phycol. **36** : 241–245.

Morphological studies were carried out on the original specimens of *Neoholmesia neurymenioides* (OKAMURA) WYNNE and the following characters were confirmed : 1) the thallus is polystromatic except the growing margins, 2) the thallus grows by means of a single transversely-dividing apical cell, 3) intercalary cell divisions are absent in the primary cell row, 4) initial cells of second-order cell rows reach the thallus margin, 5) tertiary initials do not always reach the thallus margin, 6) spermatangial sori are scattered over the thallus surface, 7) spermatangial mother cells are formed by repeated divisions of an initial cell, 8) the initial cells are formed pericentrally from a vegetative cell, 9) tetrasporangial sori are scattered over the thallus surface, 10) tetrasporangial primordia are cut off from surface cells, and 11) tetrasporangia are 20–32 μm in diameter and cruciately or tetrahedrally divided.

Key Index Words: Delesseriaceae—Morphology—Neoholmesia neurymenioides—*Rhodophyta—Taxonomy.*

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ヒメバショウは1925年に台湾の三貂角で採集され，OKAMURA（1929）により *Botryocarpa neurymenioides* として新種記載が行なわれた。しかし間もなく彼は本種を *Holmesia* 属に移し *H. neurymenioides*（OKAMURA）OKAMURA（1932）と改めた上で日本海藻誌（岡村 1936）に登載した。その後本種が採集された記録は全く見当らない。ところが，かねて本種の扱いに疑念を示していた WYNNE（1970, 1985）は本種の原記載を検討した末，ヒメバショウに対して *Neoholmesia neurymenioides*（OKAMURA）WYNNE（1985）の新併合名を発表した。

幸いなことに，ごく最近になって北大理学部腊葉庫中（SAP, herb. OKAMURA）に本種のタイプ標本を含む原標本が見いだされ，早速観察を行なったところ今までに確認できていなかった幾つかの知見が得られたので次に報告する。

外形：原標本5葉のうち，Fig. 1 は本種のタイプ標本である。添付されているラベル（第35号）上には，大正14年4月29日採集，産地は三貂角，type specimen と記されている。原記載に示されているように，本種は紅藻スジムカデ（*Grateloupia ramosissima*）の体上に数個ずつ着生し，外形はバンド状，披針状又は舌状で，体の下方に向い次第に細くなる。単条又は不規則に分岐し，長さ 2–6 cm，幅は 5–10 mm である。体は始め平滑であるが，後に体の両面より小葉を点状に生ずる。縁辺は全縁又は小葉をふさ状につける。

生長点と内部構造：Fig. 2 は主葉の生長点，Fig. 3 は縁辺小葉の，そして Fig. 4 は主葉上に副出する点状小葉の生長点を示す。即ち，明らかに 1）横に関節する頂端細胞（a）を持ち，2）第1位細胞列内に介生分裂は認められず，そして 3）第3位細胞列の始原細胞（i_3）はそのすべてが体の縁辺に達するとは限っていない。次に Fig. 5 は体の縁辺部，Fig. 6 は新小葉の，そして Fig. 7 は主葉の基部それぞれにおける断面を示す。体は生長点及びその付近の縁辺部を除き多層構造を示しており，体内部に向って柔細胞は次第に大型となる。

精子嚢：本種の精子嚢斑は体の両面上に散在して生ずる（Fig. 8）。Fig. 9 はその断面を示す。本種の精子嚢母細胞は始原細胞の反復分裂によって形成され，始原細胞は体細胞から pericentrally に形成される。

四分胞子嚢：本種の四分胞子嚢斑は体の両面上に直接散在して生ずる。Fig. 10 は四分胞子嚢原基（p）の表面観，Fig. 11 はその時期の断面を示す。即ち，本種の四分胞子嚢原基（p）は皮層内部からではなくて，（KYLIN 1924）のいう surface cell から切り出される。

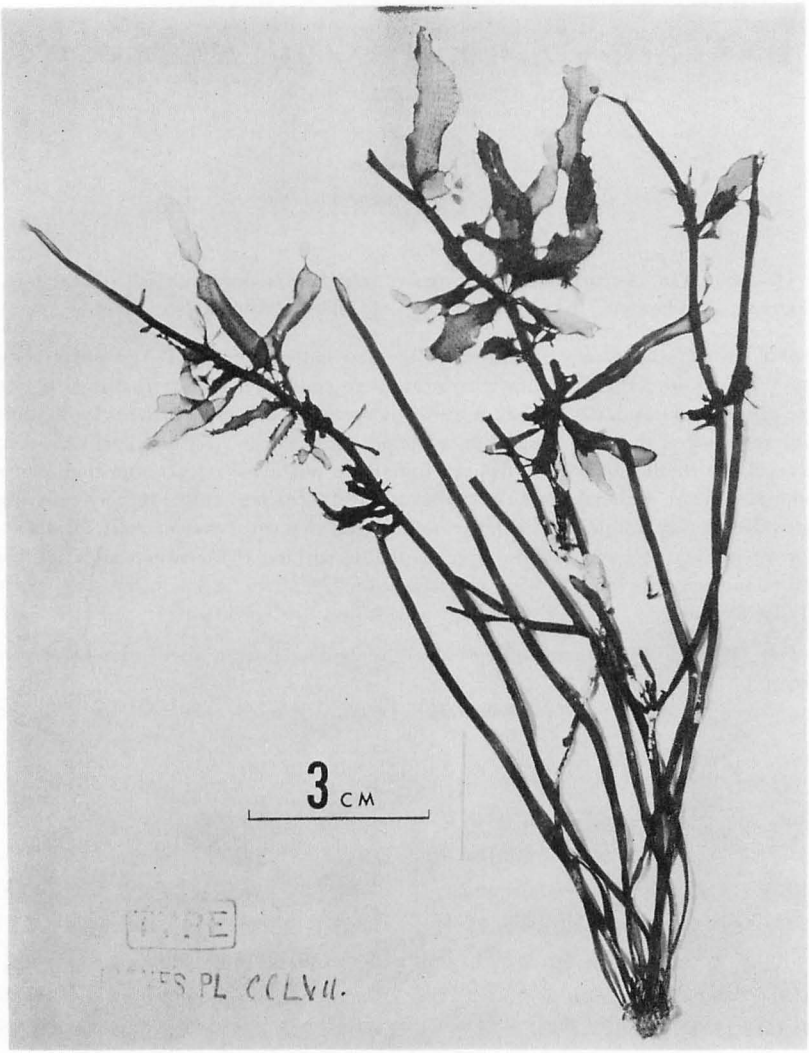
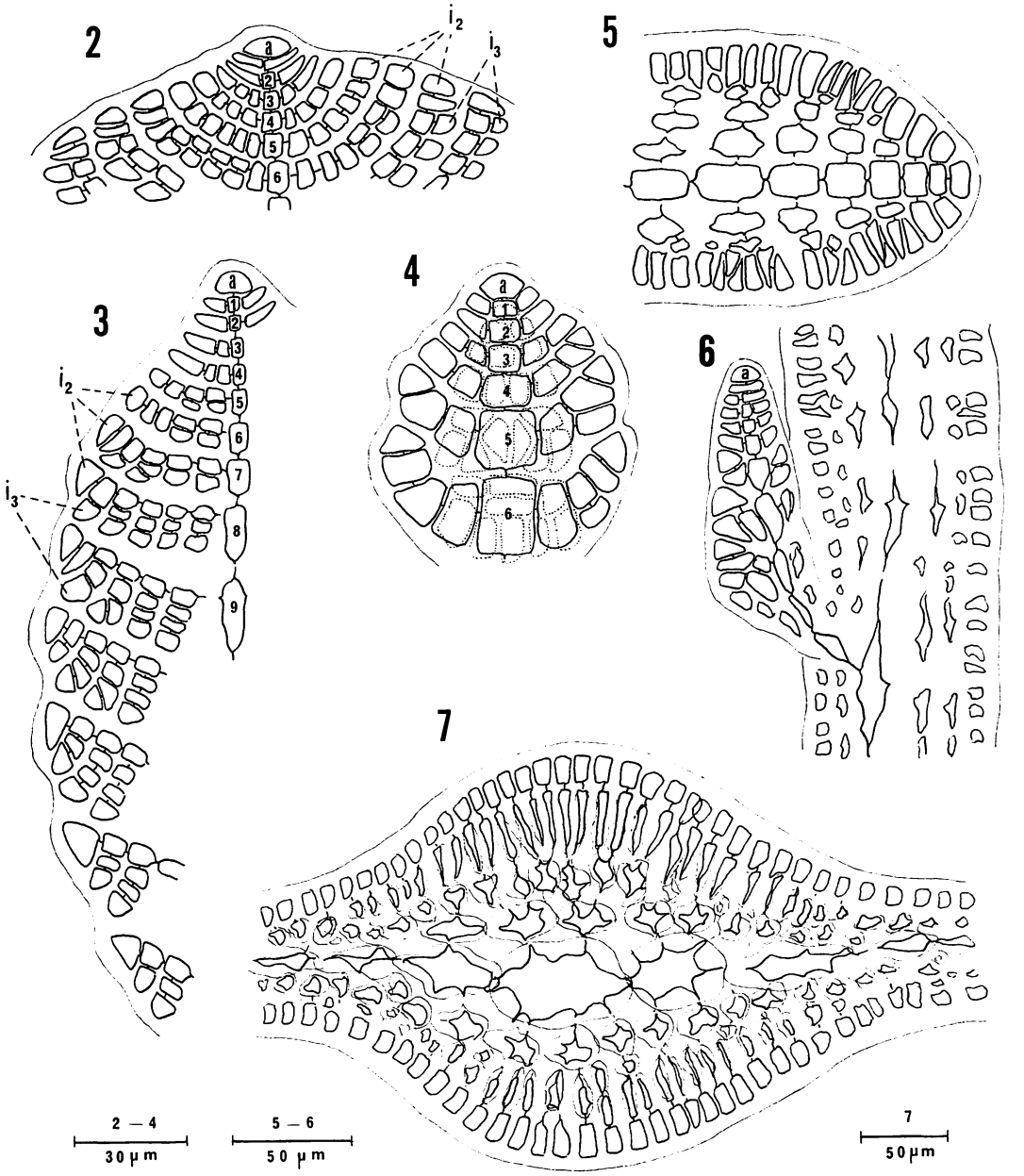


Fig. 1. *Neoholmesia neurymenioides* (OKAMURA) WYNNE. Holotype specimen collected from Taiwan (Santenkaku) on April 29, 1925, (SAP, herb. OKAMURA).

Fig. 12 はほぼ成熟期に達したと見られる四分胞子嚢群の表面観，そして Fig. 13 はその時期の断面を示す。四分胞子嚢は乾燥状態下で径 20–32 μm，多くは十字様に分割するが，時に三角錐状分割も認められる。

考　　察

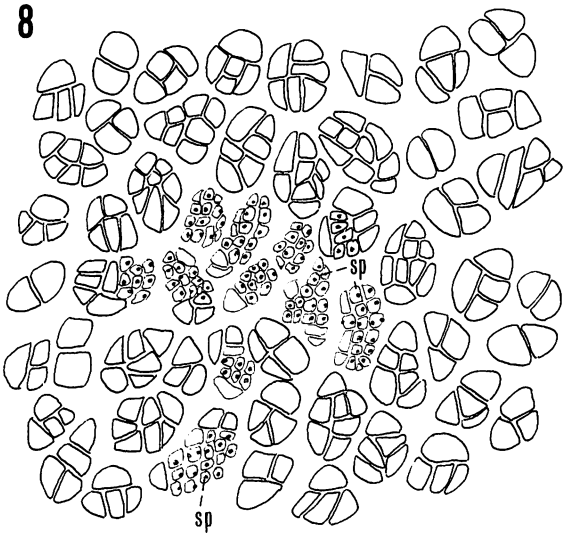
ヒメバショウは初め *Botryocarpa* 属の新メンバーとされたが（OKAMURA 1929），間もなくその生長点構造がコノハノリ亜科中の *Membranoptera* 型（KYLIN 1924）に当たるとされて *Holmesia* 属に移された（OKAMURA 1932）。しかしその際，その根拠となるような正確な図版等は少しも示されなかった。まず，ヒメバショウの生長様式確認は他の仲間に比べて確かに容易ではない。それは本種が多層構造を持つため，生長点にごく接近してすみやかに皮層細胞形成が始まり多層化に向かうためである。それゆえに第 1 位，第 2 位及び第 3 位細胞列の各細胞は皮層細胞層の下におおい隠されてしまうからである。今回，幸いにも比較的体質の薄い雄性体が初めて見い出され，それを用いることにより本種の生長様式は，まさに *Membranoptera* 型であることを確かめることができた。即ち，1）横に関節する 1 個の頂端細胞を持つこと，2）第 1 位細胞列内に介生分裂が存在しないこと，及び 3）第 3 位細胞列の始

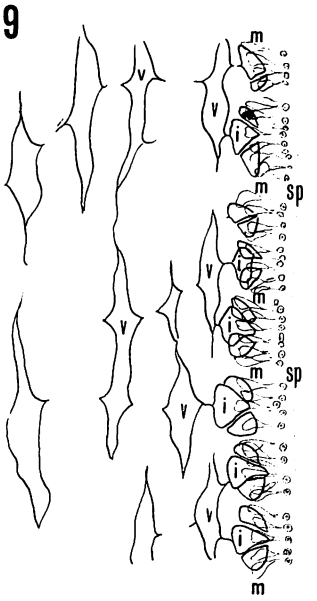


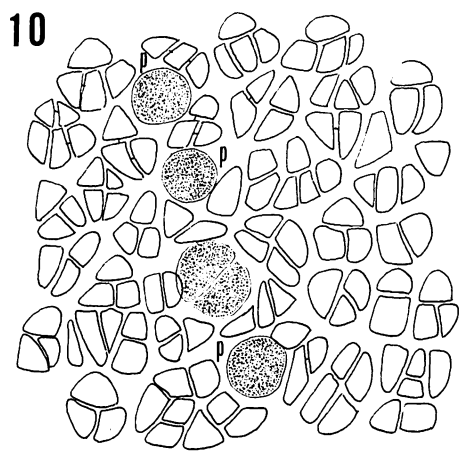
Figs. 2–7. *Neoholmesia neurymenioides*. 2. Apex of blade showing apical segmentation (cortical cells not shown). 3. Apex of marginal proliferation (cortical cells not shown). 4. Small leaflet on surface of primary blade. 5. Cross section of marginal region of blade. 6. Origin of leaflet. 7. Cross section of basal portion. a, apical cell; numerals 1–9, segments of apical cell; i_2, i_3, initials of 2nd and 3rd order rows.

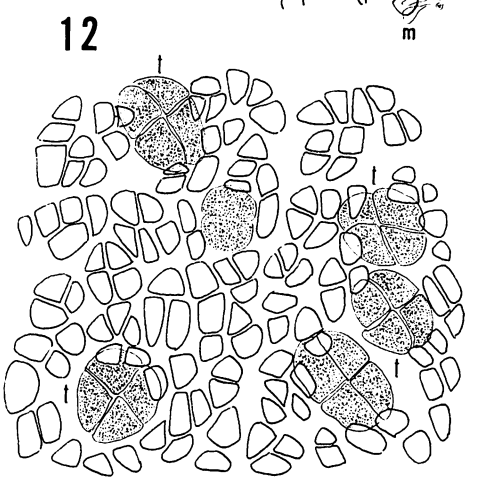
原細胞のすべてが必ずしも体縁辺に到達するとは限らないことである。次に本種の四分胞子嚢は体の表面上に直接散在して生ずることが既に Okamura (1929) により示されていたが，このたびの観察を通してそれが確かめられた上に，本種の四分胞子嚢原基(p)は内部の皮層細胞からではなくて，surface cell から生じ Kylin (1924) のいう *Delesseria* 型起源であることもあわせて解明された。

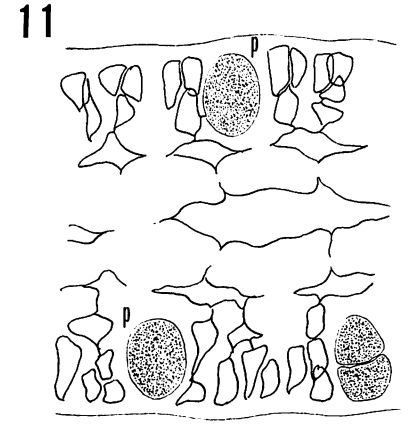
ところで *Holmesia* 属 (J. Agardh 1890) における四分胞子嚢は体表上に直接生ずることはなく，体表上

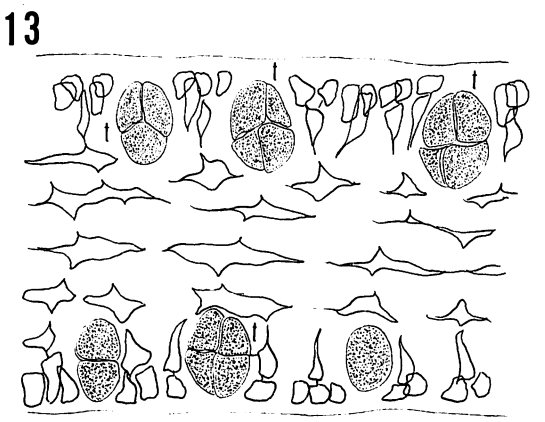












8 — 13
30 μm

に房状に群生する特別小葉上に形成される特性をもっている（Wagner 1954）。従って本種ヒメバショウを依然として *Holmesia* 属にとどめおくことは妥当ではなく，近縁の *Neoholmesia*（Mikami 1972）に移すべきであるとした Wynne（1985）の見解に一応同意することができる。更に今回の観察で初めて本種の雄性体が見い出され，その精子嚢母細胞の発生様式は他の多くのコノハノリ仲間の場合と同様 Tazawa（1975）の示した D_3 型であることも確かめられた。そこで残る不安の点は本種の雌性体が今もって発見されていないことである。従って将来，本種の雌性体が採集されて，そのプロカルプ構造と嚢果形成に至る過程が究明されたとき，始めて本種ヒメバショウの正しい所属についての断定を下すことができるものと考えられる。

終りに貴重な原標本の閲覧と共に終始懇切な助言と配慮をいただいた北大理学部植物分類学講座の吉田忠生教授に深謝申し上げる。

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Figs. 8–13. *Neoholmesia neurymenioides*. 8. Surface view of spermatangial sorus. 9. Cross section of spermatangial sorus. 10. Surface view of tetrasporangial primordia. 11. Cross section showing tetrasporangial primordia cut off from surface cells. 12. Surface view of mature tetrasporangial sorus. 13. Cross section of tetrasporangial sorus. i, initial cell of spermatangial mother cell; m, spermatangial mother cell; p, tetrasporangial primordia; sp, spermatangium; t, tetrasporangium; v, vegetative cell.

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Review

The *Chlamydomonas* cell walls and their degrading enzymes

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The unicellular algae like *Chlamydomonas* and their related colonial members in the Volvocales are surrounded by extracellular matrices or cell walls which are composed of glycoproteins with little, if any, polyglucans characteristic of higher plant and other algal walls. Their architecture, chemistry, synthesis, secretion, *in vitro* assembly, and mutation are becoming increasingly clear particularly in the cell wall of *C. reinhardtii*, which will be briefly reviewed. Moreover, in *C. reinhardtii* a lytic enzyme (gamete wall-autolysin), which is responsible for digestion of the gamete cell wall during mating, and a hatching enzyme (sporangium wall-autolysin), which acts on the sporangium cell wall to liberate zoospores, have been purified and characterized. Recent work on the specificity, cleavage sites, intracellular storage form, location, and excretion signal of the two degrading enzymes will be discussed and their nature will be compared. Finally, discussion will be extended to some systematic approaches which employ the cell walls and their degrading enzymes as important phylogenic keys in *Chlamydomonas* and in the Volvocales.

Key Index Words: Cell wall—chemotaxonomic key—Chlamydomonas—*glycoprotein—hatching enzyme—lytic enzyme—protease—Volvocales.*

Chlamydomonas is a biflagellated, green unicell which has been used as a valuable experimental alga for almost 100 years. The genus which contains nearly 500 species is divided into 9 sub-groups (ETTL 1976, 1983), and is placed in the family Chlamydomonadaceae and the order Volvocales (BOLD and WYNNE 1985). Individual species of *Chlamydomonas* is either homothallic or heterothallic. In the homothallic strains like *C. monoica* (VANWINKLE-SWIFT and AUBERT 1983), mating can occur within clones, presumably by interconversion of the mating-type genes as observed in yeasts (HERSKOWITZ and OSHIMA 1981). Such mating-type switches, however, are absent in the heterothallic species such as *C. reinhardtii*, *C. eugametos* and *C. moewusii*, where two sexually compatible clones having either mating-type plus (mt^+) or minus (mt^-) nuclear genes exist, and no sexual reproduction occurs within clones. Copulation may occur between motile gametes which are morphologically alike (isogamy; *e.g. C. reinhardtii*, *C. eugametos*, *C. moewusii*) or morphologically different (anisogamy or heterogamy; *e.g. C. zimbabwiensis*) or between gametes differentiated as egg and sperm (oogamy; *e.g. C. suboogama*, *C. pseudogigantea*) (TSCHERMAK-WOESS 1959, 1962).

Fig. 1 shows the asexual and sexual cell cycles of an isogamous heterothallic alga, *Chlamydomonas reinhardtii*. The mt^+ and mt^- cells reproduce asexually by repeating a cycle of growth, mitosis and cytokinesis (Fig. 1A, B). When all cell divisions are complete, a new cell wall is formed around each daughter cell, and 4–16 daughter cells

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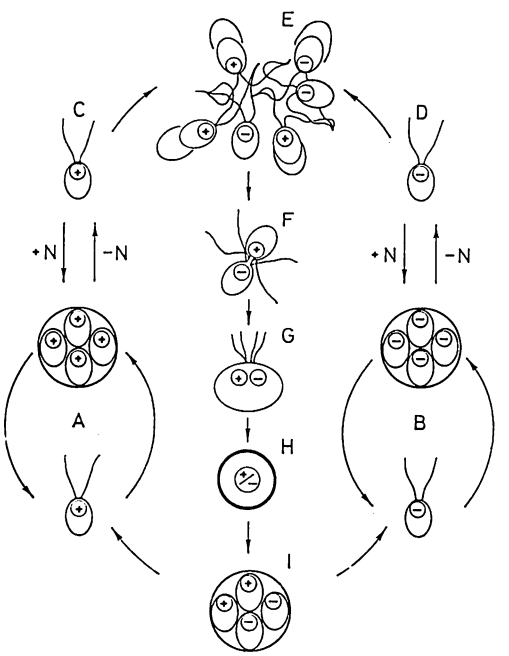

Fig. 1. The life cycle of an isogamous, heterothallic alga, *Chlamydomonas reinhardtii*. A and B, asexual reproduction of mt^+ and mt^- vegetative cells; C and D, mt^+ and mt^- gametes induced by nitrogen starvation; E, flagellar agglutination and cell wall loss after mixing the two mating-type gametes; F, pairing and protoplasmic fusion; G, quadriflagellated young zygote; H, mature zygote with thick zygotic cell wall; I, meiotic cell division.

(zoospores) are released by breaking down the mother cell wall (sporangium cell wall). The asexual cell cycle can be synchronized by the light-dark regime (Bernstein 1960; Kates and Jones 1964).

Switching to the sexual cell cycle in *C. reinhardtii* occurs when the asexually growing vegetative cells are subjected to nitrogen starvation (Sager and Granick 1954). Gametic differentiation of either mating-type can occur in the absence of cells of the opposite mating-type and without sexual cell division (Fig. 1C, D) (Schmeisser *et al.* 1973; Matsuda *et al.* 1978). At the level of the light microscope, vegetative cells and gametes are indistinguishable. At the ultrastructural and biochemical levels, however, gametogenesis in *C. reinhardtii* involves: (1) the construction of a mt^+ or mt^- mating structure for protoplasmic fusion (Friedmann *et al.* 1968; Cavalier-Smith 1975; Goodenough and Weiss 1975; Martin and Goodenough 1975; Triemer and Brown 1975b; Goodenough *et al.* 1982), (2) the synthesis of mating-type specific agglutinin molecules, their transportation to the flagellar surface for agglutination (Wiese 1965; Goodenough *et al.* 1985; Snell 1985) and their accumulation in the cell body as a reservoir of molecules (Saito *et al.* 1985; Snell 1985), and (3) the shift in the storage form of cell wall lytic enzyme for digestion of the gametic wall (Matsuda *et al.* 1987b; see later for details).

General features of the sexual cell cycle which occur between the mt^+ and mt^- gametes in heterothallic species are similar with minor exceptions and consist of the following steps (Fig. 1): the initial clumping of gametes by flagellar adhesion (Fig. 1E), pairing of gametes by firm flagellar tip-to-tip adhesion, protoplasmic fusion (Fig. 1F, G), flagellar deadhesion and retraction, zygotic wall formation (Fig. 1H), zygote maturation, meiosis, cytokinesis (Fig. 1I), and ultimately the liberation of zygospores (4 or more) by breaking down the thick zygotic cell wall (Snell 1985; van den Ende 1985). In *C. reinhardtii*, flagellar agglutination triggers the excretion of a cell wall lytic enzyme so that paired gametes are naked before fusion (Cavalier-Smith 1975; Goodenough and Weiss 1975; Triemer and Brown 1975b; Matsuda *et al.* 1978; Goodenough *et al.* 1982; Weiss 1983). In *C. eugametos* and *C. moewusii*, the gamete wall is partially broken down at the apical end, and through the resulting hole, a plasma tube elongates towards that of its partner with which it eventually fuses. The pair joined by a narrow protoplasmic bridge, which is referred to as a "vis-a-vis" pair, swims about for several hours, and then the rest of the gamete wall is released to allow complete cell fusion (Brown *et al.* 1968; Triemer and Brown 1975a; Homan *et al.* 1980; Musgrave *et al.* 1983).

Several surveys on asexual and sexual reproduction in *Chlamydomonas* have recently been made by many authors (Wiese 1984;

ADAIR 1985; SNELL 1985; VAN DEN ENDE 1985; MATSUDA and SAITO 1986; TRAINOR and CAIN 1986; BLOODGOOD 1987). In this article, I will therefore concentrate on information on the *Chlamydomonas* cell walls and their degrading enzymes and discuss how the synthesis and degradation of cell walls are regulated temporally and spatially in the life cycle. The rest of this review will discuss some new approaches where the cell walls and degrading enzymes are used to systematize a variety of organisms in *Chlamydomonas* and in the Volvocales.

I. Cell Wall

The following information on the *Chlamydomonas* cell wall has been obtained mainly by using the isogamous, heterothallic species, *C. reinhardtii*.

1. *Vegetative cell wall*

The vegetative cell wall of *Chlamydomonas* covers the cell surface except for that of the flagella. Therefore, the wall is not continuous at the opening through which the flagella protrude: the two tunnels in the cell wall are lined by flagellar collars, cylinders of wall material (ROBERTS *et al.* 1975; SNELL 1983). The vegetative cell wall is composed of glycoproteins with none of the polysaccharide polymers characteristic of higher plant walls (ROBERTS *et al.* 1985b). The protein portion accounts for about 30% of the wall by mass and is rich in hydroxyproline to which oligosaccharides are attached (MILLER *et al.* 1972; ROBERTS *et al.* 1972). The carbohydrate portion which accounts for about 47%, contains galactose, arabinose and mannose as the most abundant sugars (MILLER *et al.* 1972; ROBERTS 1974; CATT *et al.* 1976), and is modified by sulphation (ROBERTS *et al.* 1980). Flagellar collars are different from the rest of the wall in both structure and chemical composition (ROBERTS *et al.* 1975).

Ultrastructurally, the intact cell wall was originally described as having seven layers, numbered W1–W7 (Fig. 2A) (ROBERTS *et al.* 1972). However, recent images (Fig. 2B) obtained by the quick-freeze deep-etch technique (GOODENOUGH and HEUSER 1985) indicate that W3 and W5 are actually spaces. The innermost (W1) and outermost (W7) layers constitute a "warp", while the central triplet layers, which are composed of an inner amorphous layer (W2), a medial granular layer (W4) and an outer crystalline layer (W6), form a "weft". The outer crystalline layer is further divided into inner (W6A) and outer (W6B) sublayers (Fig. 2B). The W4 and W6 layers can be solubilized by chaotropic salts (*e.g.* sodium perchlorate, lithium chloride), whereas the inner layers, W1 and W2 cannot (DAVIES 1972; HILLS 1973; ROBERTS 1974; HILLS *et al.* 1975; GOODENOUGH and HEUSER 1985). The salt-solubilized fraction is

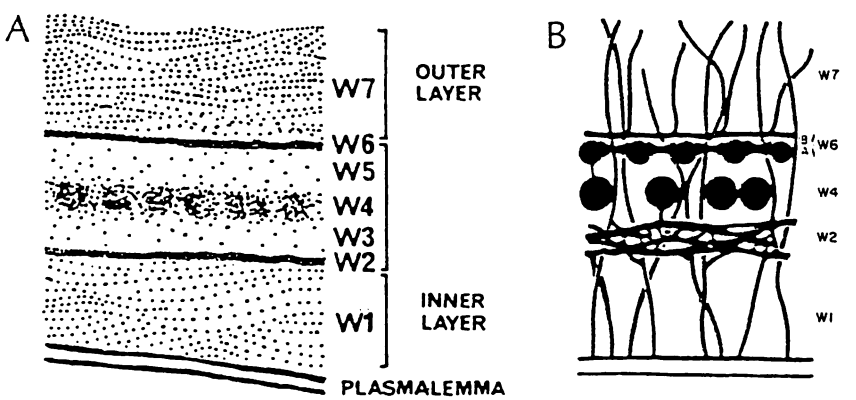

Fig. 2. Multilayered structure of the *C. reinhardtii* cell wall. A, diagram from ROBERTS *et al.* (1972); B, diagram from GOODENOUGH and HEUSER (1985).

further separated by gel filtration on Sepharose 2B into two fractions, 2BI which derives from W4, and 2BII which derives from W6 (CATT *et al.* 1976, 1978; ROBERTS 1981; GOODENOUGH *et al.* 1986). The salt-insoluble fraction can be seen under phase contrast microscopy as an extremely thin wall-shaped structure (HILLS *et al.* 1975). The salt soluble and insoluble fractions have very similar amino acid and sugar compositions (HILLS *et al.* 1975), but are composed of distinctly different species of polypeptides and glycopolypeptides in SDS-PAGE (IMAM *et al.* 1985; MATSUDA *et al.* 1985). The salt-soluble fraction contains four major fibrous glycopolypeptides (>350, 270, 150, 130 kDa) that make up 2BII subfraction (CATT *et al.* 1976; ROBERTS *et al.* 1985a; GOODENOUGH *et al.* 1986) and several minor polypeptides, whereas the insoluble fraction is composed of several polypeptides among which a 100 kDa polypeptide is prominent (GOODENOUGH and HEUSER 1985; IMAM *et al.* 1985; MATSUDA *et al.* 1985). In *C. eugametos* the cell wall contains two major glycoproteins and several minor glycoproteins (MUSGRAVE *et al.* 1983; ADAIR *et al.* 1987).

When the salt-soluble and insoluble fractions are mixed and dialyzed against water, a complete cell wall is reconstituted, as judged by light and electron microscopy (HILLS 1973; HILLS *et al.* 1975). The 2BII subfraction itself, when dialysed against water, can self-assemble into a crystalline lattice structure while 2BI cannot (HILLS 1973; HILLS *et al.* 1975; CATT *et al.* 1978; GOODENOUGH *et al.* 1986). Oligosaccharide residues, especially the terminal mannose residues of 2BII glycoproteins, are important for the self-assembly process (CATT *et al.* 1978; O'NEILL and ROBERTS 1981). If 2BI is added to 2BII and dialysed, the former is incorporated into the lattice structure, resulting in the formation of sandwiches of W6–W4–W6 layers, but they are still fragments (GOODENOUGH *et al.* 1986). Thus, the presence of the salt-insoluble fraction (W1 and W2) is essential for the reconstruction of a complete cell wall having W1–W2–W4–W6 layers (HILLS 1973; HILLS *et al.* 1975; ADAIR *et al.* 1987). The inner wall layer (W2) is considered to serve as a template onto which glycoproteins of the outer crystalline layers specifically bind (HILLS *et al.* 1975; ADAIR *et al.* 1987). The cell wall-less mutant strains, *cw*-2 and *cw*-18, are deficient in some aspects of the inner wall layer, and continue to shed the crystalline wall components into the medium (HILLS *et al.* 1975; LANG and CHRISPEELS 1976; MONK *et al.* 1983; ADAIR *et al.* 1987).

The inner wall layer is the first component to be laid down around the plasma membranes of naked daughter cells after cell divisions in the asexual cell cycle (VOIGT 1986; GRIEF and SHAW 1987). Glycoproteins of the outer layers are synthesized in the endoplasmic reticulum, glycosylated in the Golgi stack, transported to the cell surface directly or *via* the contractile vacuole, and then self-assembled onto the inner wall layer (GRIEF and SHAW 1987). When vegetative cells are cultured synchronously, turnover of the inner wall layer occurs during the cell growth period (VOIGT 1985a). It has been assumed that extension of the cell wall occurs by cleaving some cross links of the inner wall layer and incorporating additional components (VOIGT 1985a).

2. *Gametic cell wall and mother cell wall*

Differentiation of non-synchronized vegetative cells into gametes occurs under nitrogen starvation with no apparent cell division (SCHMEISSER *et al.* 1973; MATSUDA *et al.* 1978, 1987b). Moreover, vegetative cells separate their cell walls (which then become the sporangium cell walls) from the protoplasts during the initial stage of cell divisions (HARPER and JOHN 1986). Therefore, both gamete cell wall and sporangium wall are actually the vegetative cell wall itself. In fact, the cell walls and their salt-soluble extracts from vegetative cells, gametes and sporangia have very similar glycoprotein compositions on SDS-PAGE

(DAVIES 1972; MUSGRAVE *et al.* 1983; VOIGT 1985b). However, a conformational change in the vegetative cell wall seems to occur during the mitotic cell division period. MUSGRAVE *et al.* (1983) have reported that an antiserum raised against one of the major cell wall glycopolypeptides of *C. eugametos* does not react with the intact wall of vegetative cells but bind to the sporangium cell walls, suggesting that the antigenic sites become exposed in the mother cell wall due to the conformational change. Moreover, SCHLÖSSER (1976) found that a hatching enzyme, which is responsible for digestion of the mother cell wall to release daughter cells (see below), does not act on the vegetative or gametic cell walls.

3. *Zygote wall*

The zygote wall differs from the vegetative wall in both chemical composition and architecture. It consists of 60–70% sugar and 5–10% protein rich in hydroxyproline (CATT 1979; GRIEF *et al.* 1987). Glucose, present only in trace amounts in vegetative cell walls, is the principal sugar in zygote walls and accounts for about half of the sugar residues (CATT 1979). The presence of a homopolymer, β-1,3 glucan is indicated by GRIEF *et al.* (1987), and this polyglucan appears to surround the fibrous hydroxyproline-rich layer. Chaotropic agents which can solubilize the outer layers of the vegetative cell wall do not solubilize the zygote wall (CATT 1979). Structurally, zygospores are surrounded by primary and secondary walls. The primary wall is formed soon after gametic fusion. Zygote specific glycoproteins are synthesized, and become associated with fibers of the early cell walls in young zygotes, but are also secreted into the culture medium (MINAMI and GOODENOUGH 1978). The secondary wall develops inside the primary wall during the maturation period, which requires several days. It is thick and composed of two layers, the outer of which is highly convoluted and appears orange under light microscope (BROWN *et al.* 1968; CAVALIER-SMITH 1976).

II. Cell Wall Degrading Enzymes

1. *Lytic enzyme (gamete wall-autolysin)*

Chemical nature

In *C. reinhardtii* and many other species of *Chlamydomonas* (*C. iyengarii*, *C. indica*, *C. chlamydogama*, *C. gymnogama*, *C. media*, *C. zimbabwiensis*), the protoplast escapes from its enclosing gametic cell wall (Fig. 1E) during mating as a necessary prelude to cell fusion (MITRA 1949; BOLD 1949; DEASON 1967; CLAES 1971; MILLER *et al.* 1974; CAVALIER-SMITH 1975; HEIMKE and STARR 1979). Gametes of *C. reinhardtii* slip out of their cell walls by breaking down the apical region and concurrently excrete into the culture medium a cell wall lytic factor (CLAES 1971), referred to as "gamete wall-autolysin" (SCHLÖSSER 1976) or "cell wall lytic enzyme" (TAMAKI *et al.* 1981). The lytic enzyme in the medium continues to disintegrate the stripped walls and also attacks other walled gametes from the outside.

Lytic enzyme can dissolve the cell walls at all stages of the life cycle (Fig. 1; vegetative cell, gamete and sporangium) with the exception of the zygote wall (CLAES 1971; SCHLÖSSER 1976). Therefore, bioassays for lytic activity measure either the formation of protoplasts from walled cells (CLAES 1971; KASKA and GIBOR 1982; SNELL 1982) or the liberation of daughter cells from sporangia (SCHLÖSSER 1976; TAMAKI *et al.* 1981), the latter assay being 20–40 times as sensitive as the former assay (MATSUDA *et al.* 1984). The enzymatic nature of gamete wall-autolysin was first indicated by SCHLÖSSER (1976) who showed that it is heat labile, non-dialysable, precipitated from the mating medium with ammonium sulfate and inactivated with $HgCl_2$, EDTA and papain. TAMAKI *et al.* (1981) developed procedures for a sensitive and quantitative assay of lytic activity using glutaraldehyde-fixed sporangia as substrate.

They then developed a method for the purification of lytic enzyme from the mating medium under stabilized conditions. Subsequently, MATSUDA *et al.* (1984, 1985, 1987b) succeeded in purifying and characterizing the enzyme (Table 1). The purified enzyme is a single glycoprotein with a molecular mass of 62–65 kDa. Activity is inhibited by metal ion chelators (EDTA, CDTA, EGTA, α,α'-dipyridyl and 1,10-phenanthroline), SH-blocking agents (*p*-chloromercuribenzoic acid, $HgCl_2$, iodoacetate, diethyl pyrocarbonate and copper acetate), certain amino acids, α_2-macroglobulin (a protease inhibitor) and phosphoramidon (a metalloprotease inhibitor). However, lytic enzyme is insensitive to the inhibitors of serine, thiol, and carboxyl proteases (phenylmethylsulfonyl fluoride (PMSF), ε-aminocaproic acid, pepstatin A, antipain, chymostatin, leupeptin and E-64). These inhibitor specificities, together with the finding that the purified enzyme contains zinc, led MATSUDA *et al.* (1985) to conclude that cell wall lytic enzyme (gamete wall-autolysin) is a metalloprotease.

Lytic enzyme digests casein as analyzed by SDS-PAGE (MATSUDA *et al.* 1985) and reversed phase chromatography (MATSUDA *et al.* unpublished data). In order to determine the polypeptide linkages cleaved by lytic enzyme, we are currently analysing points of cleavage in enzyme-treated peptides with known amino acid sequence. Our results show that lytic enzyme does not cleave the oxidized B chain of insulin, β-endorphin or α-mating factor, but does split the peptide bonds of Phe^4-Leu^5 in dynorphin and Pro^{11}-Tyr^{12} in neurotensin (MATSUDA *et al.* unpublished data).

Wall digestion

Recent studies from three laboratories (GOODENOUGH and HEUSER 1985; IMAM *et al.* 1985; MATSUDA *et al.* 1985) have independently concluded that lytic enzyme acts on only the salt-insoluble fraction, that is, the inner wall layer (W2) of the *C. reinhardtii* cell wall. IMAM *et al.* (1985) and MATSUDA *et al.* (1985) observed by phase contrast microscopy that the "ghost" like structure of the salt-insoluble wall becomes completely invisible after lytic enzyme treatment. MATSUDA *et al.* (1985) showed by SDS-PAGE analysis that lytic enzyme does not act on any of the major or minor polypeptides of the salt-soluble fraction, but does degrade some polypeptides of the salt-insoluble fraction. GOODENOUGH and HEUSER (1985) observed by electron micro-

Table 1. Properties of cell wall degrading enzymes in *Chlamydomonas reinhardtii*.

Property	Lytic enzyme	Hatching enzyme
Molecule	glycoprotein	glycoprotein
Molecular mass		
gel filtration	65 kDa	114 kDa
SDS-PAGE	62 kDa	125 kDa
pH optimum	7.5	9.0
Temperature optimum	35°C	35°C
Isoelectric point	6.5	>10.0
Wall digestion	mother cell wall vegetative cell wall gametic cell wall	mother cell wall
Inhibitors of		
serine protease	insensitive	sensitive
metalloprotease	sensitive	sensitive
thiol protease	sensitive	sensitive
acid protease	insensitive	insensitive

scopy that as the W2 layer is degraded by lytic enzyme, fibrous units, shaped like "fishbones", are observed in the medium. Since the prominent 100 kDa polypeptide in the salt-insoluble fraction appears not to be degraded by the enzyme (IMAM *et al.* 1985; MATSUDA *et al.* 1985), it may constitute the "fishbones", while lytic enzyme may attack some core polypeptide(s) which interconnects the "fishbones". If the core or framework is degraded by lytic enzyme, the whole assemblage of the cell wall may break down.

Enzyme topography

Is lytic enzyme synthesized *de novo* during gametic induction or is it already stored in the vegetative cell? The pioneering work on the lytic factor by CLAES (1971) has shown that lytic activity found in the mating medium can also be detected in vegetative cells and gametes which have been broken by sonication. The same author also reported (CLAES 1977) that cell homogenates obtained using a French press yielded no activity unless they were subjected to sonication. Recently, we (MATSUDA *et al.* 1987b) confirmed CLAES's preliminary but suggestive findings through biochemical studies and presented some important topographic aspects of lytic enzyme in cells. Both vegetative cells and gametes contain lytic enzymes, but their storage forms are quite different (Fig. 3). In vegetative cells, lytic enzyme is stored in an inactive and insoluble form (V-form). The activation and solubilization of V-form enzyme occur either when vegetative cell homogenates from the French press are subjected to sonication or when the cells are freeze-thawed prior to homogenization. In contrast, the lytic enzyme is always found in gametic cell homogenates in an active and soluble form (G-form). When the V-form enzyme is activated and then purified, it is a glycoprotein with an apparent molecular mass of 67 kDa by gel filtration and 62 kDa by SDS-PAGE, and is sensitive to metal ion chelators and SH-blocking agents. These

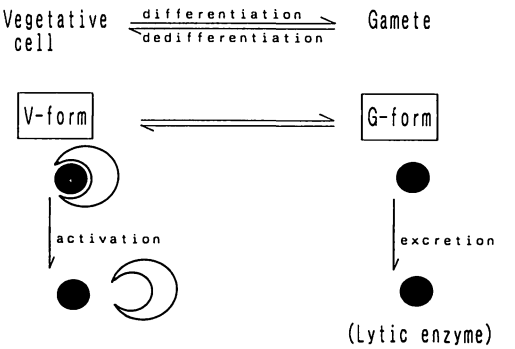

Fig. 3. Schematic diagram of the storage form of lytic enzyme in vegetative cell and gamete, and its processing during gametic differentiation and dedifferentiation.

properties are very similar to those of the G-form enzyme purified from gametic cell homogenates and lytic enzyme isolated from the mating medium. The inactive V-form enzyme was also purified by sucrose gradient centrifugation and gel filtration, and characterized as a homologous particle with a sedimentation coefficient of about 20 S (ONO *et al.* 1987). After sonication, the 20-S particle releases an active, 4-S enzyme, which coincides with the S-value of the G-form enzyme and lytic enzyme excreted into the medium (ONO *et al.* 1987). It is possible that the activity of the V-form enzyme is concealed by forming an enzyme-masking protein complex (Fig. 3) or enzyme aggregates.

It is noteworthy that the storage form of lytic enzyme shifts back and forth between the two, dependent upon gametic differentiation and dedifferentiation of the cell (MATSUDA *et al.* 1987b) (Fig. 3). When vegetative cells are starved of nitrogen, the storage form shifts from the V-form to the G-form in correlation with the ability to mate. Adding nitrogen to the gametic culture converts the G-form to the V-form, concurrently with the loss of mating ability. Therefore, we can clearly distinguish between vegetative cells and gametes, which are morphologically very similar in *C. reinhardtii*, by a simple determination of lytic activity in the cell homogenates. Without this assay, it is especially difficult to dis-

tinguish between the two cell types in agglutinin-deficient cells or flagella-less cells (SAITO *et al.* 1988; MATSUDA *et al.* 1988b).

Several pieces of indirect evidence suggest that lytic enzyme is stored in the periplasmic space (MILLIKIN and WEISS 1984; MATSUDA *et al.* 1987b). MILLIKIN and WEISS (1984) have analyzed the binding of FITC or ferritin labeled concanavalin A (Con A) to gametes by epifluorescent and electron microscopy, and proposed that Con A binding globules in the anterior periplasm of gametes represent lytic enzyme or a precursor of the enzyme. MATSUDA *et al.* (1987b) have shown that when vegetative cells and gametes are treated with exogenously added lytic enzyme, the protoplasts obtained contain little enzyme activity, and when the protoplasts are incubated further after washing out the added enzyme, lytic enzyme is again accumulated in these cells when most of the wall has been regenerated. Gametes of many wall-less mutant strains have little, if any, lytic enzyme activity in the cell homogenates and release practically no lytic enzyme into the medium during mating (MATSUDA *et al.* 1987b).

Excretion signal

A study of the release of lytic enzyme during mating is complicated by the fact that unmated gametes of some strains often release the enzyme activity, stimulated by an as yet unknown signal, and lose their walls before mixing (FRIEDMAN *et al.* 1968; GOODENOUGH and WEISS 1975; MATSUDA *et al.* 1978; MATSUDA 1980; KASKA and GIBOR 1982). Since gametes of both mating-types possess equal amounts of active (G-form) enzyme, it is possible that there is an inhibitor(s) in walled gametes (Fig. 1C, D), which acts on the cellular enzyme to keep the activity below the critical level until its release is stimulated by mixing gametes of the opposite mating-type (MATSUDA *et al.* 1987b). Some gametes might lose their walls before mixing by raising the lytic activity beyond the critical level; we have the experience that wall loss often occurs when the gametogenesis is accompanied with cell division (MATSUDA 1980) or when gametes are cultured for a long period under nitrogen-starvation.

When walled gametes of both mating-types are mixed together, lytic enzyme is released into the medium as a pulse, within 1–3 min of mixing (SNELL 1982; MATSUDA *et al.* 1987b). Both mating-types release lytic enzyme when agglutinated with either live gametes, fixed gametes, or isolated flagella of the opposite mating-type (CLAES 1971; GOODENOUGH and WEISS 1975; KASKA and GIBOR 1982). However, several reports have indicated that the mt^+ gametes release much more lytic enzyme than the mt^- gametes during flagellar agglutination (MATSUDA *et al.* 1978; SNELL 1982; MILLIKIN and WEISS 1984).

Lytic enzyme is secreted only after flagellar agglutination: Deflagellated gametes neither agglutinate nor excrete enzyme. However, agglutinability recovers when about 10% of the complete flagella is regenerated, while the ability to release the enzyme is regenerated only when 50% of the flagellar length is restored (RAY *et al.* 1978). It is thought that flagellar agglutination sends a signal to the cell body that triggers the release of the lytic enzyme (GOODENOUGH 1977). Several reports have indicated that cyclic AMP (cAMP) and Ca^{2+} ions may play a role as second messengers in signalling secretion of lytic enzyme and other events required for cell fusion. A transient 10 to 30-fold elevation of intracellular cAMP levels is observed during initial agglutination in *C. eugametos* (PIJST *et al.* 1984) and *C. reinhardtii* (PASQUALE and GOODENOUGH 1987). BLOODGOOD and LEVIN (1983) have observed that the rate of efflux of Ca^{2+} from gametes also increases up to 20 times during the initial mating, but the efflux rate returns to the control level within a few minutes. The transient increase in Ca^{2+} efflux may reflect a transient increase in the cytoplasmic free-Ca^{2+} concentration released from intracellular storage sites (BLOODGOOD and LEVIN 1983).

KASKA *et al.* (1985) used X-ray microanalysis to analyze the intracellular distribution of Ca^{2+} and revealed that Ca^{2+} is sequestered in descrete granules within the gametic cell body prior to mating only to become diffuse throughout the cell during mating. The presence of calmodulin has been reported in the cell bodies and flagella (GITELMAN and WITMAN 1980), and inhibitors of calmodulin, trifluoperazine and W-7, prevent the transduction of signals for cell wall loss (DETMERS and CONDEELIS 1986). Lidocaine, an inhibitor of the movement of Ca^{2+} ion across cell membranes, also prevents cell wall loss (SNELL *et al.* 1982). More recently, PASQUALE and GOODENOUGH (1987) reported that unmated gametes, but not vegetative cells, can be induced to undergo wall loss by addition of dibutyryl-cAMP and/or cyclic nucleotide phosphodiesterase inhibitors (*e.g.* isobutylmethylxanthine).

2. *Hatching enzyme* (*sporangium wall-autolysin*)

Chemical nature

The release of daughter cells by breaking down the surrounding mother cell wall is mediated by a second type of enzyme, named "sporangium wall-autolysin" or "hatching enzyme" (SCHLÖSSER 1966, 1976; MIHARA and HASE 1975). This enzyme is excreted into the medium as the zoospores hatch, and therefore can be concentrated from the medium of synchronized cultures (SCHLÖSSER 1976). Hatching enzyme is specific for mother cell wall and will not affect vegetative or gamete cell walls (SCHLÖSSER 1976). JAENICKE's group (JAENICKE and WAFFENSCHMIDT 1981; JAENICKE *et al.* 1987) purified the hatching enzyme of *C. reinhardtii*, and characterized it as a serine protease with a molecular mass of 37 kDa (gel filtration) or 40 kDa (SDS-PAGE) and a pH-optimum at 8.2. To compare the properties of hatching enzyme with those of lytic enzyme, we recently purified and characterized the hatching enzyme (KOSEKI *et al.* 1987; manuscript in preparation) (Table 1). Our purified enzyme specifically acts on sporangium cell walls, and is an extremely basic glycoprotein which binds to Con A-Sepharose. It has a molecular mass of 114 kDa in gel filtration and 125 kDa in SDS-PAGE, a pH-optimum at around 9.0, and is inactivated by PMSF, TLCK, $HgCl_2$, iodoacetate, 1,10-phenanthroline and EDTA. Therefore, the molecular size of our hatching enzyme is much greater than that of JAENICKE and WAFFENSCHMIDT (1981) on both gel filtration and SDS-PAGE. We noted that a protein component of about 40 kDa is one of the prominent contaminants in crude and partially purified enzyme preparations; this component might be related to the hydroxyproline-rich glycoproteins which are liberated during hatching of the zoospores from the mother cell wall (VOIGT 1985a, b). To confirm that the hatching enzyme is actually a larger molecule than the lytic enzyme, the two purified enzymes were mixed and applied to a gel filtration column packed with Sephacryl-S200. The activity, as determined by use of glutaraldehyde-fixed sporangia as substrates, gave two peaks: the heavier one (PMSF sensitive, 115 kDa peak) is hatching enzyme, and the lighter one (PMSF insensitive, 65 kDa peak) lytic enzyme (Fig. 4).

Hatching enzyme digests α-casein. The chromatographic patterns of enzyme digests

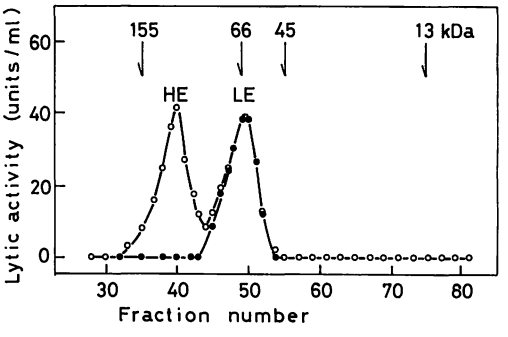

Fig. 4. Gel filtration of the mixture of the purified lytic enzyme (LE) and hatching enzyme (HE). ○, cell wall degrading activity measured by glutaraldehyde-fixed sporangia as substrates; ●, activity measured in the presence of 0.2 mM PMSF.

of α-casein, as analyzed by reversed phase chromatography, are distinctly different for hatching enzyme and lytic enzyme. Hatching enzyme hydrolyzes α-mating factor, α-neo-endorphin, dynorphin, neurotensin, mastoparan and β-endorphin, but does not act on oxidized insulin B. In general, the imino side of a Lys or Arg residue in the P_1' position is selectively cleaved, provided that the P_1 position is occupied by a basic or hydrophobic amino acid (MATSUDA *et al.* manuscript in preparation).

Intracellular enzyme

Hatching enzyme of *C. reinhardtii* seems to be formed in the young zoospores during ripening of sporangia (MIHARA and HASE 1975). Cells at later stages of the cell cycle contain the hatching enzyme, but its action is suppressed until the regular time of zoospore liberation in the cell cycle. However, if the cells are subjected to vibration, they liberate zoospores and concurrently excrete the enzyme into the medium (MIHARA and HASE 1975). In order to see the production or activation of hatching enzyme in the cells during ripening of the sporangia, we prepared cell homogenates using the French press and analyzed the activity (MATSUDA *et al.* 1988a). Unexpectedly, no activity was detected in the homogenates of sporangia even just before hatching. The homogenates were found to contain inhibitor(s) which inactivated specifically and irreversibly the activity of the isolated hatching enzyme. Therefore, it seems to be difficult to analyze the intracellular hatching enzyme through cell breakdown (MATSUDA *et al.* 1988a).

III. Systematic Studies

Many species of the genus *Chlamydomonas* have been divided into subgenera or subgroups generally based on the light-microscopically visible features of vegetative cells (PASCHER 1927; GERLOFF 1940; ETTL 1976). However, the problems of the taxonomy of *Chlamydomonas* at the species level have been presented by LEWIN (1975) who stated: 'Specific distinctions in this genus are generally based on relatively few microscopically visible features of the biflagellate (presumably haploid) cells, as found in nature or when grown in media often insufficiently characterized.··· Therefore, the taxonomy of the genus is in a mess, and little confidence can be placed on specific identifications based on published descriptions' (*cf.* TRAINOR and CAIN 1986). To overcome the confusion and artificiality of the taxonomy of this primitive green alga, several authors have considered that works on the specificity of the cell walls and their degrading enzymes might contribute a truer and more reliable assessment of natural relationships (ROBERTS 1974; SCHLÖSSER 1984; MATSUDA *et al.* 1987a).

1. *Cell wall as a taxonomic marker*

All members of the Volvocales except the family Polyblepharidaceae have cell walls or extracellular matrices which are constructed in a similar manner (BOLD and WYNNE 1985; ROBERTS *et al.* 1985a): they consist of a stable amorphous inner wall layer and a crystalline outer wall layer, and are built up of unique glycoprotein molecules with little, if any cellulose or chitin, which is abundant in most other algal cell walls. The detailed cell-wall structures and chemical compositions, however, are suggested to be different between individual algae, and could be used as an important phylogenic marker (ROBERTS 1974). ROBERTS (1974) and ROBERTS *et al.* (1982) have analyzed the glycoprotein arrays on the outer surface of cell walls in a large number of organisms of the Volvocales, and concluded that their crystal structures fall into 4 general classes (Tables 2 and 3). Eleven species of *Chlamydomonas* (*e.g. C. eugametos*, *C. moewusii*, *C. chlamydogama*) and many other members in the family Chlamydomonadaceae (*e.g. Carteria*, *Chlorogonium*, *Haematococcus*) possess the simplest cell wall crystal structure (class II). In contrast, *C. reinhardtii*, *C. cribrum*, *C. angulosa* and *C. inepta* have more complicated

Table 2. Grouping of algae in the genus *Chlamydomonas* on the basis of cell walls and their degrading enzyme specificities.

Species	SCHLÖSSER (1976, 1984)	ROBERTS *et al.* (1982)	MATSUDA *et al.* (1987a)
Chlamydomonas reinhardtii	1	I	A
C. globosa	1		A
C. incerta	1		A
C. smithii	1		A
C. cribrum		I	A
C. komma	2		A
C. angulosa	2	I	B
C. debaryana	2		C
C. inepta	2	I	
C. asymmetrica	3	III	C
C. gloeopara	3		
C. peterfii	3		
C. oblonga	4		
C. mexicana	4		C
C. iyengarii	5		
C. sphaeroides	5		
C. callosa	6		
C. aggregata	7		C
C. humicola	7		
C. applanata	7	II	
C. dysosmos	7	II	
C. culleus	9		
C. elliptica	9		
C. frankii	9		C
C. gymnogama	10		
C. segnis	10		
C. pallidostigmatica	10		
C. gelatinosa	11		
C. eugametos	12	II	C
C. indica	12		
C. starrii	12		
C. aculeata	13		
C. pitschmannii	13		
C. geitleri	14		
C. pinicola	14		
C. hindakii	14		
C. terricola	14		
C. monoica	14		
C. noctigama	14		
C. brannonii	15		
C. texensis	15		
C. chlamydogama		II	C
C. moewusii		II	C
C. dorsoventralis		II	
C. sphaerella		II	
C. rosae		II	
C. pulsatilla		II	
C. reginae		II	
C. fimbriata		II	

Table 3. Grouping of algae in the order Volvocales on the basis of cell walls and their degrading enzyme specificities.

Family	Species	Roberts *et al.* (1982)	Matsuda *et al.* (1987a)
Astrephomenaceae	*Astrephomene gubernaculifera*		A
	A. perforata		A
Volvocaceae	*Gonium multicoccum*		A
	G. octonarium		A
	G. pectorale		A
	G. quadratum		A
	G. sacculiferum		A
	G. sociale		A
	Pandorina morum	I	B
	Volvulina steinii		B
	Eudorina elegans	I	B
	Pleodorina californica		B
	Volvox aureus	I	
	V. carteri		B
	Stephanosphaera pluvialis		C
Phacotaceae	*Dysmorphococcus globosus*		B
	Phacotus lenticularis		C
	Pteromonas angulosa		C
Chlamydomonadaceae	*Haemotococcus lacustris*	II	C
	Carteria crucifera	II	C
	C. eugametos	II	
	Chlorogonium elongatum	II	C
	C. euchlorum	II	
	Polytoma uvella	II	C
	Lobomonas piriformis	IV	C
Spondylomoraceae	*Pyrobotrys casinoensis*		C
Polyblepharidaceae	*Dunaliella salina*		C
	Pedinomonas minor		C

crystal structure (class I). Interestingly, the class I structure is typical of some colonial volvocacean algae, *Pandorina*, *Eudorina* and *Volvox* (Table 3). *C. asymmetrica* belongs to class III, while *Lobomonas piriformis* has the class IV structure. That *C. reinhardtii* and *Volvox* are more closely related than *C. eugametos* is also indicated by biochemical and morphological analyses of the cell walls (Roberts 1974; Musgrave *et al.* 1983; Goodenough and Heuser 1985; Adair *et al.* 1987). In addition, a recent hybridization experiment by Adair *et al.* (1987) showed that hybrid walls are obtained when a chaotropic salt-soluble extract from *Volvox carteri* is incubated with a salt-insoluble fraction (*i.e.* the inner wall layer) from *C. reinhardtii* and *vice versa*, whereas no interspecific assembly occurs between *C. reinhardtii* and *C. eugametos*.

In order to further investigate the relationships between the cell wall glycoproteins of *Chlamydomonas* and between those of related algae, Roberts *et al.* (1985b) raised a rabbit antiserum to one (termed B2; 270 kDa) of the major glycopolypeptides of the *C. reinhardtii* cell wall. Western blotting analysis showed that the antiserum cross-reacts with the cell wall of *C. angulosa* (class I) and *Lobomonas* (class IV), but does not

bind to the glycoproteins from the cell walls of *C. moewusii* (class II) or *C. asymmetrica* (class III). An indirect immunofluorescence study showed that the cell walls of *Volvox aureus*, *Eudorina elegans*, *Pandorina morum* and *Gonium pectorale* all fluoresce brightly with the antiserum (ROBERTS *et al.* 1985b). MATSUDA *et al.* (1987a) extended the immunofluorescence study using more than 40 species belonging to six families in the Volvocales (Tables 2 and 3). Among 15 species of *Chlamydomonas*, *C. globosa*, *C. incerta*, *C. smithii*, *C. cribrum*, *C. komma* and *C. angulosa* are labeled strongly with anti-glycopeptide B2, while others show no or weak cross-reactivity with the antibody. The antibody also cross-reacts strongly with the cell walls of a unicellular alga belonging to the Phacotaceae (*Dysmorphococcus*) and many colonial algae belonging to the Volvocaceae (*Gonium*, *Pandorina*, *Volvulina*, *Eudorina*, *Pleodorina*, *Volvox*) and the Astrephomenaceae (*Astrephomene*). On the other hand, unicellular algae, *Haematococcus*, *Phacotus*, *Carteria*, *Pteromonas*, *Polytoma*, *Chlorogonium* and *Lobomonas*, and colonial algae, *Pyrobotrys* and *Stephanosphaera* all have cell walls that do not react with the antibody (MATSUDA *et al.* 1987a). A similar pattern of labeling was obtained when a polyclonal antiserum raised against deglycosylated 2BII fraction, which recognizes only the protein core of 2BII glycoprotein molecules (ROBERTS *et al.* 1985b), was used except that the cell wall of *C. angulosa* cross-reacted with the anti-deglycosylated 2BII much more weakly than the anti-glycopeptide B2 (MATSUDA *et al.* unpublished data).

2. *Hatching enzyme as a taxonomic marker*

Hatching enzyme activity can be demonstrated in all species of *Chlamydomonas* which can be grown in synchronous cultures. According to the group-specific action of hatching enzymes, SCHLÖSSER (1976, 1984) has classified 65 strains of *Chlamydomonas* into 15 groups (Table 2). For example, *C. reinhardtii* belongs to group 1; a crude solution of hatching enzyme from this alga can dissolve the sporangial walls from all algae of group 1 (*C. globosa*, *C. incerta* and *C. smithii*), and *vice versa*, but cannot act on those from algae of other groups. In most cases, the mutual lytic action is restricted to within the group, but there are some exceptions: hatching enzymes from group 2 act non-reciprocally on all strains of group 1, and those from group 3 act on group 4 (SCHLÖSSER 1976).

In *Volvox*, mature autocolonies escape from the parental spheroid through the enzymatic rupture of the peripheral somatic cell layer (JAENICKE and WAFFENSCHMIDT 1979, 1981). JAENICKE and WAFFENSCHMIDT (1981) have purified the hatching enzyme from *V. carteri* and characterized it as a protease with tryptic properties. The enzyme has limited species specificity (*V. aureus* is sensitive, *V. africanus* is less sensitive, and *V. dissipatrix* and *V. gigas* are not sensitive), and does not act on the cell wall of *C. reinhardtii* wall.

3. *Lytic enzyme as a taxonomic marker*

In conjunction with the analysis of antibody binding (see above), MATSUDA *et al.* (1987a) have analyzed the sensitivity of cell walls of a variety of members of the Volvocales to lytic enzyme of *C. reinhardtii*. In the genus *Chlamydomonas*, the lytic enzyme acts only on the cell walls of all algae of group 1 (see also SCHLÖSSER *et al.* 1976), *C. komma* which belongs to group 2 according to SCHLÖSSER (1976), and *C. cribrum* which belongs to class I according to ROBERTS *et al.* (1982) (Table 2). Among other genera tested, the cell walls of *Gonium* and *Astrephomene* are sensitive to the *C. reinhardtii* lytic enzyme: their colonial structures are broken into individual cells by exposure to the enzyme, and protoplasts are then formed. Formalin-fixed colonies are also sensitive to the enzyme, suggesting a direct action of lytic enzyme on the cell wall lysis. Hatching enzyme of *C. reinhardtii* appears to be unable to break up the colonial structures of *Gonium* and *Astrephomene* (MATSUDA *et al.* 1987a).

If the sensitivity to lytic enzyme is combined with the labeling with anti-cell wall glycopeptide B2, the algae in the Volvocales can be divided into three classes (Tables 2 and 3): class A organisms (six species of *Chlamydomonas* including *C. reinhardtii* (*C. reinhardtii* group), six species of *Gonium* and two species of *Astrephomene*) whose cell walls are sensitive to the enzyme and show a strong cross-reactivity with the antibody, class B organisms (*C. angulosa. Dysmorphococcus*, *Pandorina*, *Eudorina*, *Volvulina*, *Pleodorina* and *Volvox*) whose cell walls are resistant to the enzyme, but show a strong cross-reactivity with the antibody, and class C organisms (many other species of *Chlamydomonas*, *Carteria*, *Chlorogonium*, *Polytoma*, *Haematococcus*, *Lobomonas*, *Phacotus*, *Pteromonas*, *Stephanosphaera* and *Pyrobotrys*) whose cell walls are resistant to the enzyme and show no or weak cross-reactivity with the antibody. Since lytic enzyme digests the inner wall layer of the *Chlamydomonas* cell wall while antibody will recognize the outer layers (see above), the cell walls of the class A organisms might be similar in chemical composition and arrangement of components to those of *C. reinhardtii* itself.

Taken together, all lines of evidence suggest that the members in the genus *Chlamydomonas* are composed of phylogenically diverse groups. For example, *C. reinhardtii* which belongs to group 1 appears to be closely related to other members in the same group and group 2, but distantly related to many other algae (*e.g. C. eugametos*) in other groups. Moreover, *C. reinhardtii* and its relatives (the *C. reinhardtii* group) are on the evolutionary line leading to the multicellular members of the Volvocales (Roberts *et al.* 1982; Matsuda *et al.* 1987a). Matsuda *et al.* (1987a) have proposed that there is an evolutionary sequence from a *C. reinhardtii*-like ancestor to the colonial algae, *Gonium* and *Astrephomene* (Fig. 5). There might also be a line of evolution from an ancestor of the *C. reinhardtii* to the volvocacean algae (except

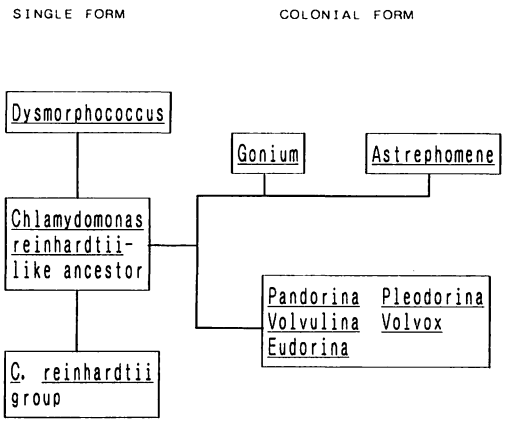

Fig. 5. Schematic representation of evolutionary lines from a *C. reinhardtii*-like ancestor to unicellular and colonial algae in the Volvocales.

Stephanosphaera) because of the similarities of the structure and chemical composition of their cell walls (Fig. 5). These phylogenic relationships agree well with those suggested from microscopically visible features (Fulton 1978a; Nozaki 1986). The pattern of cell cleavage in *Gonium* (specifically, *G. pectorale* and *G. octonarium*) and *Astrephomene* is a parallel-type, whereas that in *Pandorina*, *Volvulina*, *Eudorina*, *Pleodorina* and *Volvox*, which undergo inversion during the colony formation, is a rotational-type (Pocock 1953; Stein 1958a, b; Gerisch 1959; Goldstein 1964; Starr 1969; Fulton 1978b; Nozaki 1983, 1986). *Stephanosphaera* shows a radial-type cleavage (Hieronymus 1884). Furthermore, every cell in a colony of *Gonium* and *Astrephomene* is surrounded by the inner and outer layers of cell wall, whereas in other volvocacean algae, the outer layers do not cover over the individual cells but surround the colonial surface as an extracellular matrix (Fulton 1978a; Nozaki 1986; Nozaki *et al.* 1987).

The cell walls and their degrading enzymes will constitute very important taxonomic keys in future studies to investigate more detailed phylogenic relationships between chlamydomonads and other members of the Volvocales.

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Note Added in Proof: Recently, LEMIEUX *et al.* (BioSystems 1985, **18**: 293–298) and JUPE *et al.* (BioSystems 1988, **21**: 223–230) have analyzed the chloroplast DNA and nuclear rRNA genes of *Chlamydomonas reinhardtii, C. moewusii* and *C. eugametos* for the potential application to the systematics of *Chlamydomonas,* and suggested a large phylogenetic distance between *C. reinhardtii* and *C. moewusii* or *C. eugametos* (the latter two strains are interfertile and similar in molecular comparisons).

松田吉弘：クラミドモナスの細胞壁とその溶解酵素

オオヒゲマワリ目は，クラミドモナスのような単細胞性藻からオオヒゲマワリのような群体性藻まで多彩な藻類で構成されているが，これらの細胞壁や外被は，いずれも糖蛋白質より成り，高等植物や他の藻類でみられるポリグルカンを殆ど持たない。細胞壁の構造，生化学，構成成分の合成と分泌，人為的分解と再構成，さらに突然変異については，とくに *Chlamydomonas reinhardtii* を材料として研究が進められており，ここでは，まずこれらの最近までの知見を概説した。また，*C. reinhardtii* では，プラス型，マイナス型両配偶子が接合する時に分泌される細胞壁溶解酵素（リティックエンザイム）と無性的増殖時に母細胞壁を破って娘細胞が放出されるために必要な酵素（ハッチングエンザイム）の両者が，最近単離精製された。これらのプロテアーゼ性細胞壁溶解酵素の分子性状，細胞壁の分解様式，細胞内存在形態および存在場所，分泌シグナルなどをまとめて論議した。これまで *Chlamydomonas* の種レベルでの分類は，主として光学顕微鏡下のごく僅かな形態的特徴の違いに基いたものであり，問題が多い。そこで，最後に，*Chlamydomonas* 属内での種のグルーピングや，オオヒゲマワリ目内での単細胞性藻と群体性藻の間での系統的類縁関係を論議するためになされた，細胞壁と二種類の溶解酵素の特異性を利用したいくつかの研究を紹介した。（657 神戸市灘区六甲台1-1　神戸大学理学部生物学教室）

―学　会　録　事―

日本学術会議第14期会員の会員及び補欠の会員として推薦すべき者の決定について

昭和63年7月14日付で，日本学術会議会員推薦管理会委員長久保亮五氏より日本藻類学会（登録学術研究団体代表者）宛に，第4部生物科学・植物科学の会員として推薦すべき者として，竹内郁夫（京都大学理学部），補欠の会員として推薦すべき者として梅崎勇（京都大学農学部）に決定した旨の通知がありました。

日本藻類学会主催第3回ワークショップ・海藻採集会参加記

学会終了直後に学部専用のバスで一路志摩半島にある三重大学生物資源学部付属の水産実験所に直行した。途中1回の休憩をはさんで陸路約2時間で英虞湾に面する和具の町内に到着。ついで今回のワークショップのお世話をしてくださった前川さんが，10数名乗りのボートを操縦して，すでに真っ暗闇の湾内を真珠

写真1．実験所前の舟着場にて，出発前の総勢17名

写真2．矢取島での採集風景

養殖のいかだをすりぬけながら，我々を実験所のある座賀島に運んでくれました。その夜は世話人である喜田教授の三重県沿岸の海藻植生についての講義のあと，総勢17名の参加者による大変にアットホームな雰囲気の第1回目の懇親会が開かれた。

翌日，英虞湾をはさんで対岸にある浜島町矢取島に今度は大きな舟で出かけた。好運にも晴れで，採集にもってこいの日和であった。ここは小規模ながら大変に採集しやすい磯で，素潜りで採集する人，ヒジキを見て感動する人，さまざまな採集風景が見られた。その日の収穫物を話題にして磯で食べた弁当はなんとも美味であった。食後，希望者を募って喜田・前川両氏の研究フィールドである大飛佐多（オビシャタ）へ舟で向い，今回の採集会の呼び物であるスキューバ潜水による〝アラメ・カジメ群落見学会〟を行なった。水面はまったく波が立たず潜水には最高のコンディションであった。ここでは水深 5―8m 付近にアラメ群落が発達しており，伊豆半島で見られる水深 1―3m にアラメ，それ以深ではカジメという住み分けとはすっかり違った様子であった。場所による植生の差を肌身で感じとれたのは有意義であった。採集品を実験室に持帰り種類を調べていると思ったよりも多くの種類が採集されていた。さすがに海藻の研究者がこれだけ集まると思いもかけないような種類が取れる物だと感心した。その日の採集疲れもなんのその，夜の海の幸による第2回の懇親会はまことに盛大なものであった。

翌日は朝から大雨。もし1日ずれていたら実に成果の上がらない採集会であったろうと思われるが，主催者である喜田教授や前川さんのほっとした顔が印象的であった。午前中採集品の整理をし，昼食後に喜田教授のヒトエグサ養殖に関するスライドを見せていただいて昼過ぎに解散した。

この実験所の宿泊棟，実験棟共に大変に立派なもので，とても快適に過ごせた。機会があれば何回でも利用したい施設である。

藻類学会主催のワークショップも今回で三回目であり，いよいよ定着した感がある。是非とも来年以降もいろいろな企画でワークショップが開催されることを期待している。今回大変に貴重な経験をさせていただいた関係者一同に御礼申し上げる。

参考までに今回採集された種類をあげておく。[三重県志摩郡浜島町，矢取島および大飛佐多（オビシャ

タ）で採集された海藻（昭和63年4月1日）]（分類順不同）

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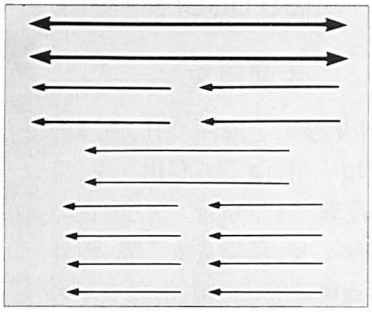

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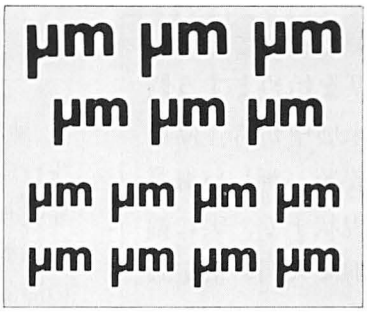

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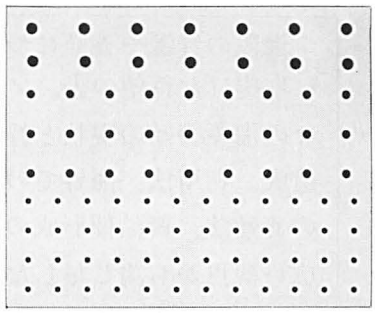

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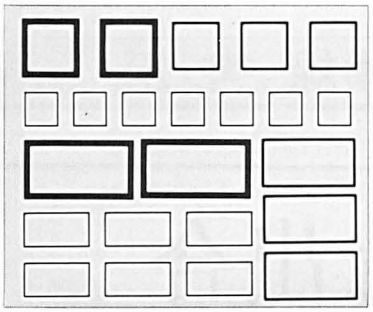

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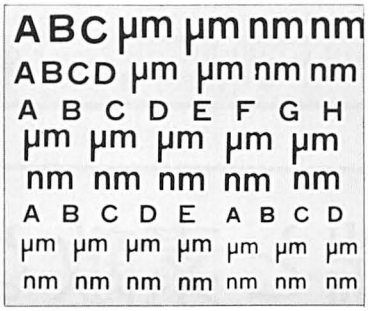

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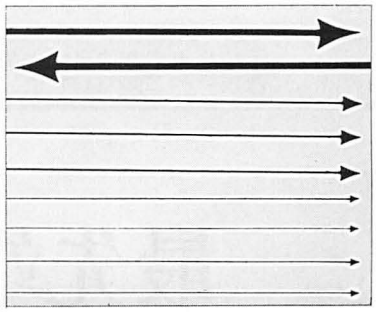

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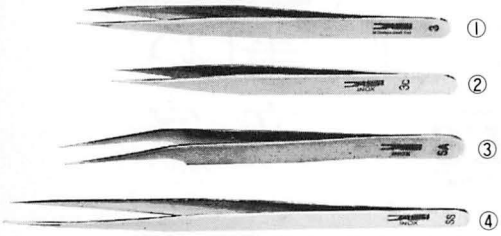

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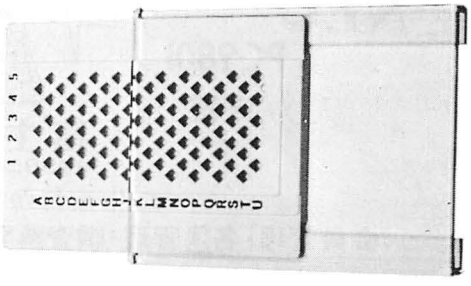

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