Jap. J. Phycol. (Sôrui) 33: 32-44. March 10, 1985

Cell wall regeneration of new spherical cells developed from the protoplasm of a coenocytic green alga, *Boergesenia forbesii*

Shun MIZUTA, Kazuyoshi SAWADA* and Kazuo OKUDA

Department of Biology, Faculty of Science, Kochi University, Kochi, 780 Japan

MIZUTA, S., SAWADA, K. and OKUDA, K. 1985. Cell wall regeneration of new spherical cells developed from the protoplasm of a coenocytic green alga, *Boergesenia forbesii*. Jap. J. Phycol. 33: 32-44.

The cell wall structure and processes of cell wall regeneration were investigated in spherical cells (protoplasts) developed from the protoplasm of *Boergesenia forbesii*. The protoplasts rapidly developed an amorphous matrix layer on the outer surface of the plasma membrane and then generated a randomly-oriented fibril layer underneath the former during 3 hr of culture at 25°C. When the matrix-rich layers thickened to about 0.2 μm, microfibril-rich layers formed underneath them. The formation of the first two layers was closely dependent on temperature, while that of fibril-rich layers depended not only on temperature but light irradiation as well. The fibril-rich layers consisted of many sublayers formed in accordance with cell polarity. Each such layer generated from the pole toward the equator of the cell, with its orientation shifted about 30° to the layer underneath. On the freeze-fractured plasma membrane, cellulose synthesizing enzyme complexes were observed at the terminal of the microfibril impression and it was assumed that the orientation of the cellulose microfibrils could be induced by the change in the direction of the complexes.

Key Index Words: Boergesenia forbesii; *cell wall deposition*; *freeze fracture*; *microfibril orientation*.

The new spherical cells (protoplasts) generated from the protoplasm of *Boergesenia forbesii* (HARVEY) FELDMANN, had strong stable activity for cell wall regeneration ISHIZAWA *et al.* 1979). On the outer side of the plasma membrane, the new wall materials were deposited successively and the thickened wall showed a complex polylamellate structure in which fibrils changed their orientation with regularity (MIZUTA and WADA 1982). The cells are thus suitable for studying the regeneration processes of the polylamellate cell wall.

Similar polylamellate structure have already been reported in some algal cells with definite cell polarity (PRESTON and KUYPER 1951, NICOLAI and PRESTON 1959, FREI and PRESTON 1961, ROBINSON and PRESTON 1972). In the thallus of *Boergesenia*, most of the cell wall was found to be composed of multifibrillar layers with a microfibril arrangement dependent on cell polarity; the layers were alternately oriented purpendicular and parallel to the longer cell axis (MIZUTA and WADA 1981).

In the newly-formed spherical cells of *Boergesenia*, it is still not known whether the microfibril orientation of each layer depends on cell polarity or not. Consequently, we observed the processes of the cell wall regeneration and examined the polar-dependency of the ordered microfibril arrangement of the *Boergesenia* cell wall by electron microscopy.

* Present address: Institute of Bio-Active Science, Nippon Zoki Pharmaceutical Co., Ltd. Kinashi Hyogo, 673-14 Japan.

Material and Methods

A culture of *Boergesenia* and the formation of new cells (protoplasts) from adult *Boergesenia* were reported previously (MIZUTA and WADA 1982). The protoplasts were filtered through stainless steel meshes to obtain almost equal size cells (90–120 μm in diameter) which were cultured in continuous light (4W/m^2) using white fluorescent tubes (Hitachi FL 20NL) or dark at 25°C.

The replica preparation methods were essentially the same as those of TAKEDA and SHIBAOKA (1978). The cells were cut in the culture medium with fine razors and the content was rinsed out. The inside surface of the wall was made to face upward on a tiny acethylcellulose film (2×2 mm) and then washed with distilled water, dried, shadowed with Pt and coated with carbon. The replicated samples were incubated in 70% sulfuric acid for 3 days at 60°C and after being washed three times with distilled water, they were picked up with Formvar-coated grids.

Thin sectioning to observe the cross section of the wall has already been described (MIZUTA and WADA 1981). The new cells were fixed with glutaraldehyde fixative, washed and post-fixed in osmic acid solution. The materials were then dehydrated with acetone, embedded in Spurr resin, and sectioned with a Leichert Om U2 ultramicrotome. Some sectiones about 150 nm in thickness were put on Formvar-coated grids and shadowed with Pt-Carbon after the embedded resin was removed in KOH-ethanol solution.

For freeze-replication, JEOL EE-FED B2 freeze etching apparatus and double replica-forming unit were used. At first, the cells were put into small holes in the double replica unit and rapidly frozen in Freon 12. The unit was then transferred to liquid nitrogen and loaded onto apparatus previously kept in the liquid nitrogen. Both were then placed in a vacuum evaporator in which the cells were fractured at 2×10^{-4}Pa, shadowed with Pt and coated with carbon. The replicated samples were transferred to 75% sulfuric acid, heated at 60°C for 3 days, and the cleaned replicas were picked up on Formvar-coated grids.

The samples were observed with a JEOL JEM 100U electron microscope. The nomenclature of BRANTON *et al.* (1975) was used for description of the fractured membrane faces.

Results

Processes and structural features in the early stage of cell wall regeneration: Fig. 1 shows the cell wall thickening of new cells cultured for 3, 6, 24 and 72 hr at 25°C following protoplasts formation. During 6 hr of culture, the central vacuole of the cell developed and the cell wall regenerated about the cell surface. The wall thickened continuously during this period.

In the first 4.5 hr of culture, a large number of vesicles were found to be located in the cortical cytoplasm and to open toward the outside of the plasmalemma (Fig. 2). The vesicles contained small particles, as shown in Fig. 3a. Similar size particles were also observed on the fractured membrane plane (Fig. 3a) and in the cell wall (Fig. 4). About one thousand particles in each area were selected at random and their diameters were measured. The average diameter was 11±2 nm in the vesicles, 9±3 nm in the membrane

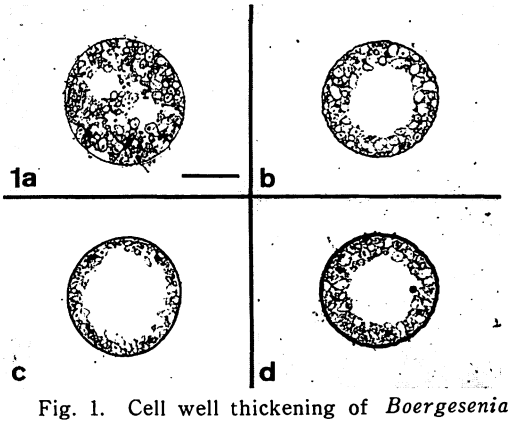


Fig. 1. Cell well thickening of *Boergesenia* protoplasts. The cells were culured for 3 hr (a), 6 hr (b), 24 hr (c) and 72 hr (d) at 25°C following protoplasts formation. Bar=50 μm.

and 11±1 nm in the cell wall. Particles and other amorphous materials within the vesicles apparently are carried outside the plasmalemma by exocytosis and are involved in the deposition of cell wall matrix substances.

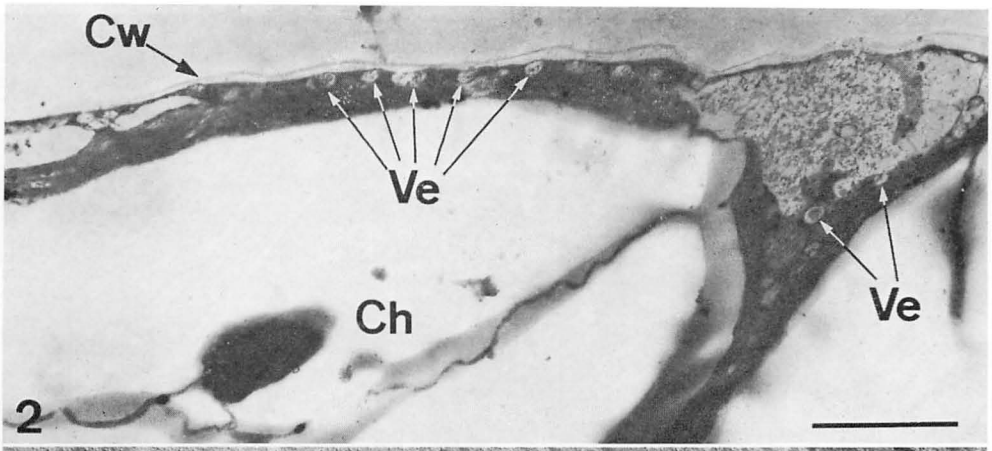
Cell wall regeneration during 4.5 hr of culture is shown in Fig. 2. The wall, about 150 nm in thickness, was assumed to be composed of fibrous materials. Microfibrils deposited about the cell surface were confirmed by freeze-replication. In Fig. 3, many fibril impressions can be seen on the fractured plane of the plasma membrane (Fig. 3a), and fibrils covered with amorphous materials on the outer surface of the wall (Fig. 3b). Some of these impressions are arranged linearly but most are randomly oriented in a curving configuration.

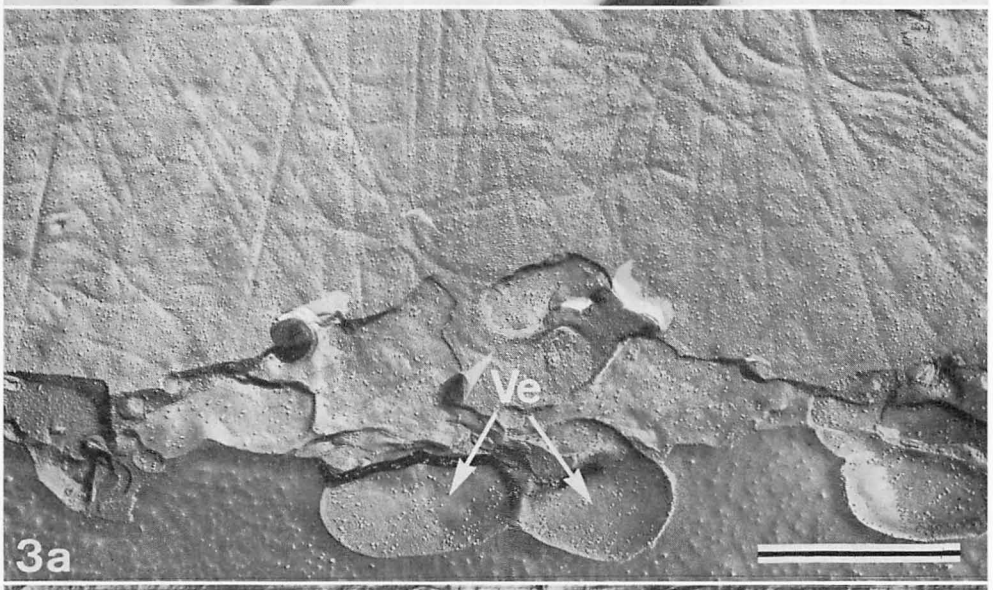
After 6 to 7 hr of culture, all of these randomly-oriented fibrils took on a linear arrangement and curved fibrils could no longer be seen (Fig. 4 and 5). The cells were expected to expand during culture and consequently, diameter measurements were made at 3, 6, 10 and 72 hr. Fig. 6 shows that the cell diameter increased by 10% during 6 hr of culture, indicating that the curved fibrils extended by expansion. After 6 to 7 hr, cell expansion gradually stopped (Fig. 6) and specifically oriented fibril groups could be seen beneath the randomly-oriented fibril layer (Fig. 4 and 5).

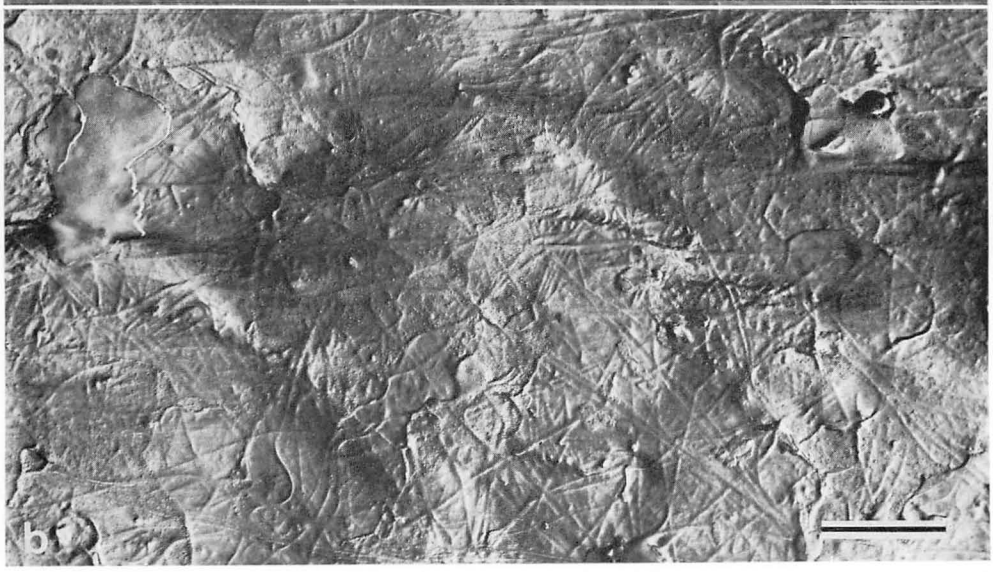
Fig. 7 shows a cross section of cells cultured for 24 hr at 25°C. Vesicles and exocytosis were not frequently observed as after 4.5 hr of culture. Since the wall structure was not clearly observable, a section was treated with KOH-ethanol solution to dissolve the embedded resin and shadowed with Pt-Carbon. The section is shown in Fig. 8. It is evident that the outer part of the wall (M) containes two sublayers, a non-fibrillar matrix layer (the outermost layer, OML) covering the outer surface of the wall and a randomly-oriented fibrillar layer (ROFL) under the former. These two layers were designated as "matrix-rich layers". Most of the wall (C) consisted of several laminated sublayers whose fibrils shifted their orientation in a regular manner. These layers were termed "constantly-oriented fibril layers" or "fibril-rich layers".

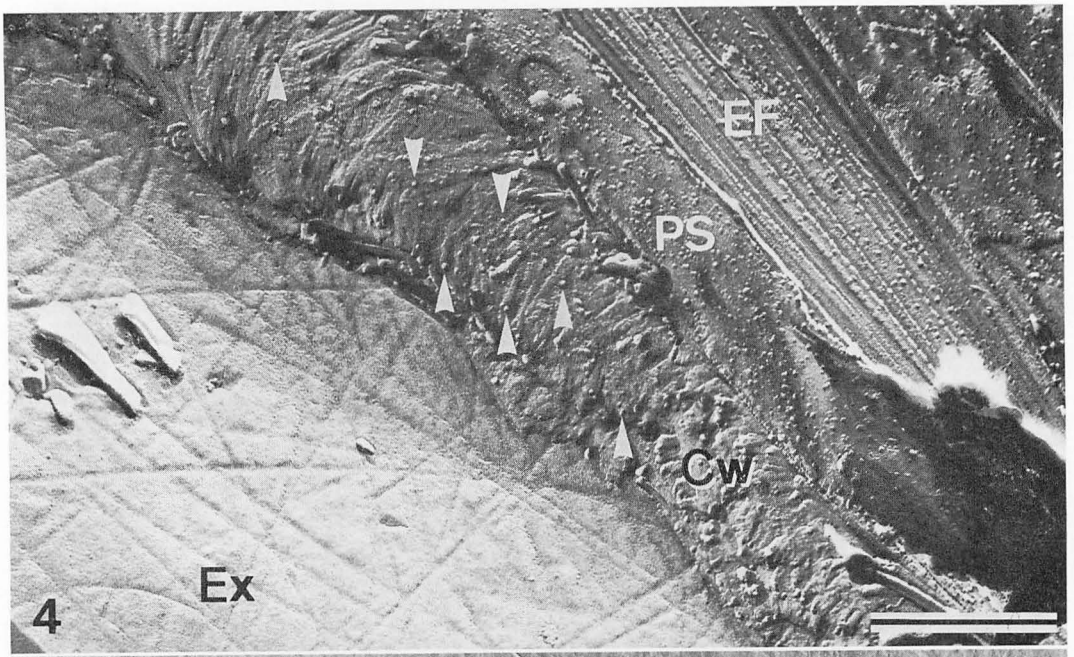
The time required for the formation of these layers is given in Fig. 9. Experiments were carried out both in the light and dark at 20, 25 and 30°C for 24 hr and the generated layers were observed with an electron microscope. Layer counting was carried out according to previously reported methods (MIZUTA and WADA 1982). Formation of matrix-rich layers (OML and ROFL) was found to closely depend on temperature. They attained a thickness of 200 nm in 3 hr at 30°C, 6 hr at 25°C and 24 hr at 20°C respectively. The constantly-oriented fibril layers formed after the matrix-rich layers had thickened to about 200 nm, and their formation depended both on temperature and light irradiation.

Polar-dependent Structure of the poly-lamellate cell wall: In Fig. 8, the cross section of the wall closely resembles that of the thallus wall whose pattern of fibril orientation is determined by cell polarity (MIZUTA and WADA 1981). To confirm whether the fibril orientation of the new cell depends on cell polarity, a replica was taken from the inner surface of the wall and its fibril orientation was examined. As apparent from Fig. 10, almost uniformly arranged fibril groups whose orientation is indicated by A, B, C and D shift counterclockwise in the direction from A to D. This shift was represented quantitatively (Fig. 11) by measuring the acute angles (θ) formed by the direction of A with the fibrils within the circle in Fig. 10. From this data, it became evident that the fibril angles (θ) of group A were distributed about 0°, B about 30°, C about 60° and D about 90°. Distribution of the microfibrils of group A was more concentrated than that of any other groups and the fibrils of group D were relatively more dense even though they were being formed on the innermost surface of the wall. Thus A and D may be considered multi-fibrillar layers and B and C, transitional layers found previously in the thallus wall


Cw
Ve
Ch
Ve
2


Ve
3a


b



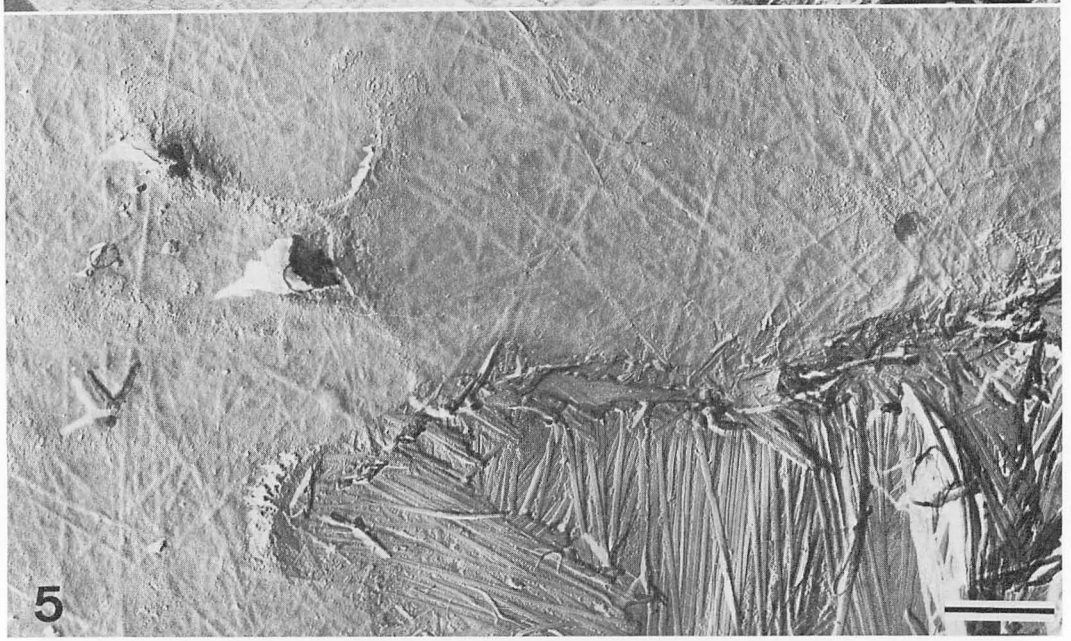


Fig. 4. Freeze-fractured cell wall and plasma membrane of the new cell. The cell was fractured after 6 hr of culture at 25°C. Arrowheads show particles existing in the wall. EF, exoplasmic fracture face of the plasma membrane; PS, protoplasmic surface of the plasma membrane; EX, impression of the outer surface of the cell wall; Cw, cell wall. Bar=0.5 μm. Fig. 5. Outer surface of new cell wall. The cell was fractured after 7 hr of culture at 25°C. Bar=0.5 μm.

← Fig. 2. Cross section of the new *Boergesenia* cell. The cells were cultured for 4.5 hr at 25°C after the protoplast had formed. Cw, cell wall; Ve, vesicle; Ch, chloroplast. Bar=2 μm. Fig. 3. P-face of the plasma membrane (a) and cell surface (b) of the new cell showing many curved fibril impressions. The cells were fractured after 4.5 hr of culture at 25°C. Ve, vesicle. Bars= 1 μm.

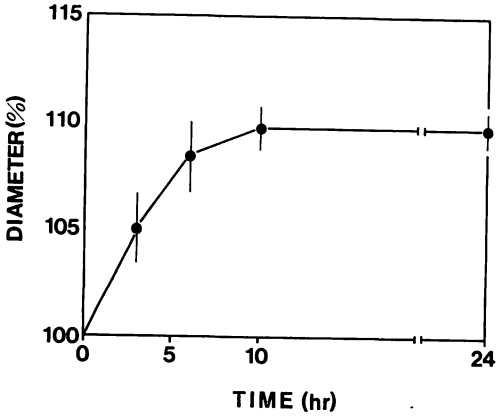


Fig. 6. Cell expansion in the early stage of cell wall development of the new cell. About 50 protoplasts were cultured at 25°C under continuous light. Cell diameter was measured after each culture period. Vertical bars indicate standard error.

of *Boergesenia* (MIZUTA and WADA 1981). This mode of shift was fundamentally similar to that of the thallus wall the multifibrillar layers of which were oriented longitudinally and transversely to the cell axis. The fibril orientation of each layer of the new cell may possibly depend on the cell polarity.

Generation of the polylamellate cell wall: Microfibril arrangement in the polar region was very complex (Fig. 12a). Some of the fibrils formed bundles and were randomly arranged. On these fibrils, the specialized structure in which fibril orientation gradually shifted to take on the shape of a fountain was frequently observed and it was termed "the fountain-like structure". Its length was about 30 μm and most of the fibrils in the structure were arranged in the direction of the arrow A on the curve.

The curved fibrils were oriented toward the equator of the cell and had many terminals (Fig. 12b). They were assumed to be generated in the direction of the arrow A and to lie at an angle of about 30° with the underlying fibril group (B group whose orientation is shown by arrow B in Fig. 12b). In group B was also oriented toward the equator. Near the equator, many opposing fibril tips could be observed (Fig. 12c), suggesting the layers to be generated from each pole toward the euqator.

Fig. 13 shows the P-face of the freeze-fractured plasma membrane of the round cell cultured for 24 hr. Many terminal complexes (TCs) are visible at the terminals of the fibril impressions lying about 30° with the underlying impressions. As reported by ITOH *et al.* (1983), the TCs were comprised of about three rows of linearly arranged particles resembling those of *Oocystis* reported by BROWN and MONTEZINOS (1976). The TCs were assumed to move within the plasma membrane with cellulose deposition, and the orientation shift of the fibrils was considered to be induced by movement modification of the complexes.

Discussion

The experiments in the present work indicate the processes involved in cell wall regeneration in *Boergesenia* protoplasts. During the first 4.5 hr of culture, a remarkable deposition of non-cellulose matrix substances occurred about the surface of the plasma membrane. These substances were apparently produced in the cytoplasm and subsequently released to the cell surface by exocytosis of the vesicles. Within the vesicles, many small particles were observed (Fig. 3) and seemed to contain enzymes relevant to the construction of non-cellulose polysaccharides since their size was essentially the same as that of particles observed in the freeze-fractured cell wall. In *Chlorella*, matrix substances may accumulate within Golgi vacuoles and subsequently be carried to the cell surface (MÜHLETHALER 1967). Although Golgi complexes could not be clearly observed in the *Boergesenia* protoplasm owing to technical difficulties in the fixation of the cytoplasm, our data support this possibility.

After 4.5 hr of culture at 25°C, cellulose microfibrils were generated within the matrix (Fig. 3). They were generally curved and randomly-oriented, but became linear after 6 hr (Fig. 4). This extension may have been due to cell expansion which was quite

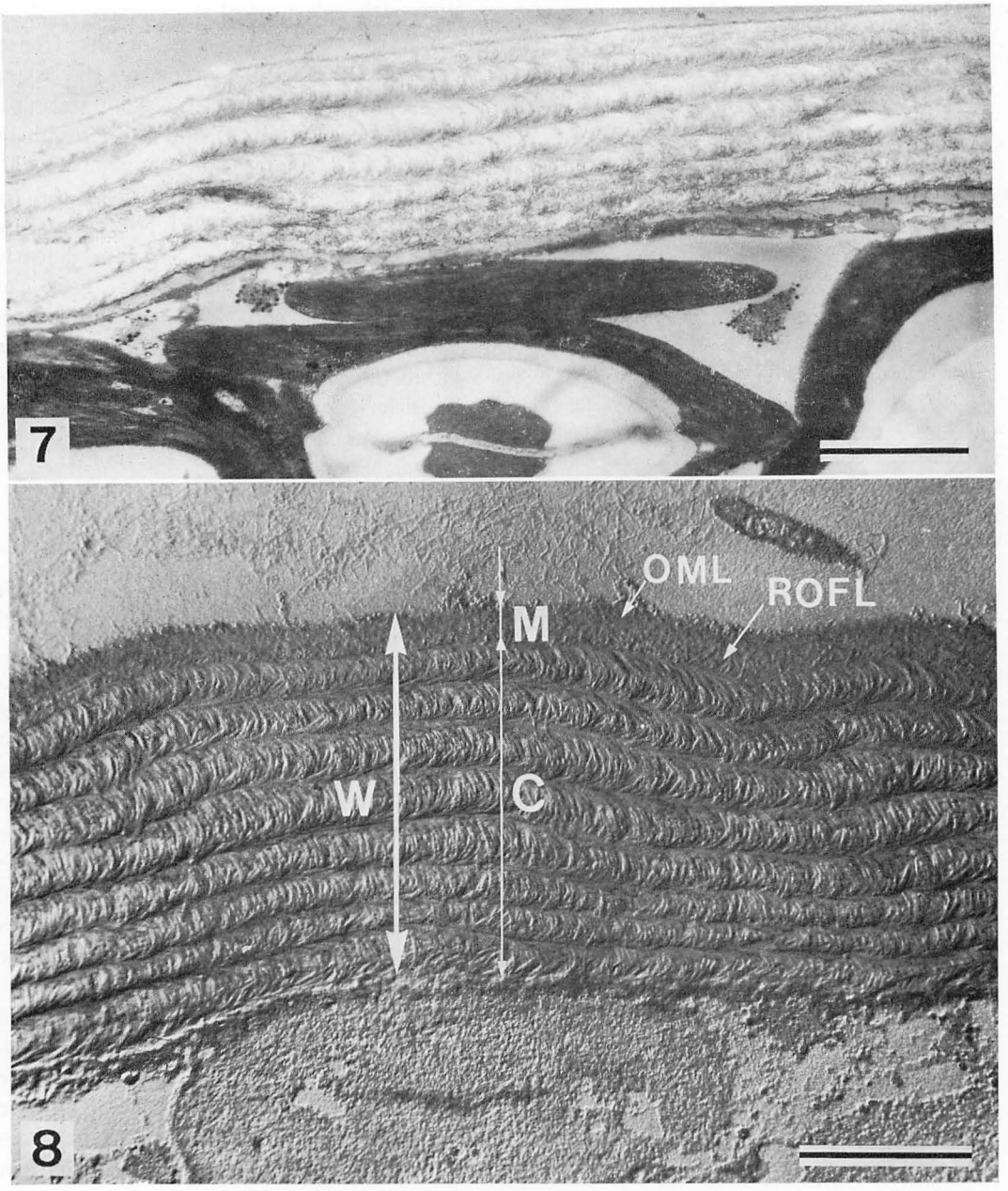


Fig. 7. Cross sectional view of new cell wall showing many fibril layers. The cell was cultured for 48 hr at 25°C under continuous light after formation of the protoplast. Bar=2 μm. Fig. 8. Cross section of new cell wall showing the regular shift of microfibril orientation. The cell was cultured for 72 hr at 25°C under continuous light after the protoplast had formed. Shadowed with Pt-Carbon after removal of embedded resin. OML, outermost layer; ROFL, randomly-oriented fibril layer; M, matrix-rich layers; C, constantly-oriented fibril layers; W, total thickness of cell wall. Bar=1 μm.

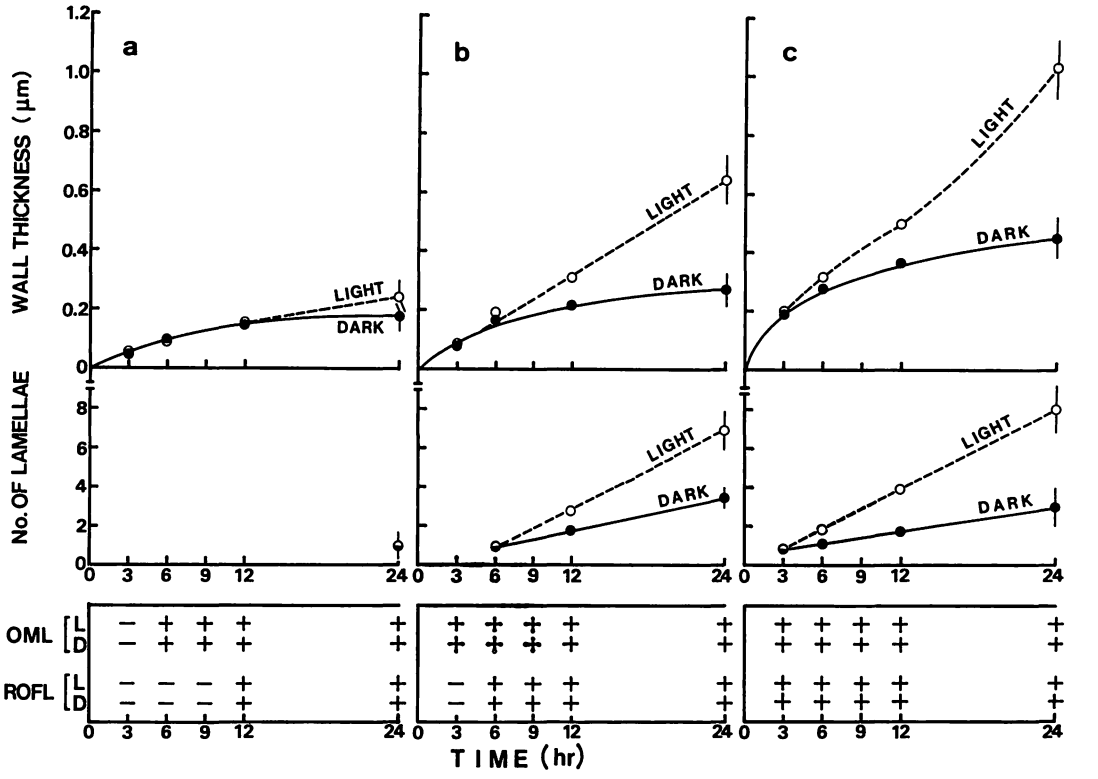


Fig. 9. Effects of light and temperature on cell wall formation. The protoplasts were cultured at 20°C, 25°C and 30°C in both light (L) and dark (D), and the number of wall layers and total wall thickness were measured with an electron microscope. The mean value from 5 cells is shown. Vertical bars indicate standard error. OML, outermost layer; ROFL, randomly-oriented fibril layer; +, recognized; −, unrecognized.

evident after 6 hr (Fig. 6). The morphological features observed in the present study suggest that acceleration of the expansion is involved in the development of the central vaculole and that this expansion is terminated as a result of wall pressure due to fibril-rich layers generated during culture. Similar cell expansion in the early developmental stages of protoplasts was observed in both *Boodlea* and *Valonia* (MIZUTA unpublished data), but the physiological significance of this is unknown.

That the fibril-rich layers of the cells may be generated by cell polarity is a definite possibility. The microfibrils of each layer shifted about 30° with the underlying layer (Fig. 10), and were assumed to be generated from the pole toward the equator of the cell (Fig. 12). The microfibril arrangement was essentially that of the fibril orientation of the thallus wall in both *Boergesenia* (MIZUTA and WADA 1981) and *Valonia* (PRESTON and KUYPER 1951). The polar dependency of the fibril arrangement of the new cell was apparent until the matrix-rich layers had thickened to about 200 nm (Fig. 9). As mentioned above, the matrix-rich layers were comprised of both amorphous matrix and randomly-oriented fibrils, the latters being formed prior to the generation of the fibril-rich layers. According to our data, assumption of specific orientation from a state of random arrangement on the part of the fibrils may possibly depend on cell polarity which comes about during generation of the matrix-rich layers.

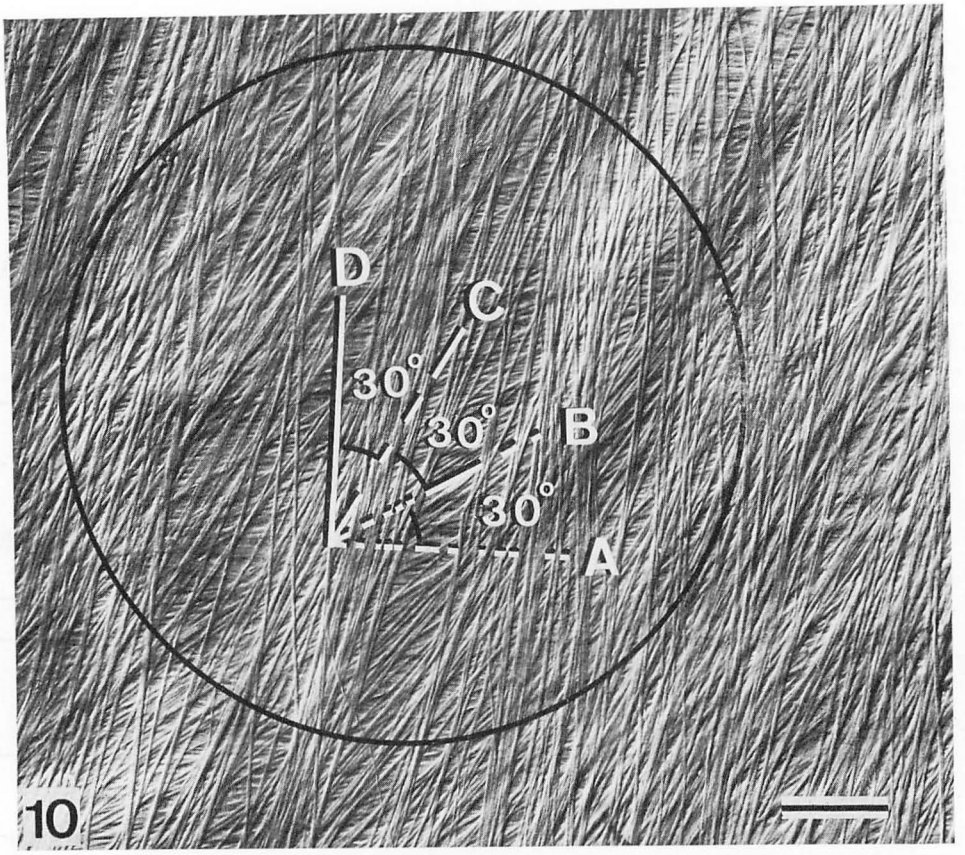


Fig. 10. Microfibril arrangement on the inner surface of the new cell wall. A, fibril from the undermost layer of the figure; B, fibril of the layer lying on that of A at an angle of 30°; C, fibril of the layer lying on that of B at an angle of 30°; D, fibril from the uppermost layer lying on that of C at an angle of 30°. Bar=1 μm.

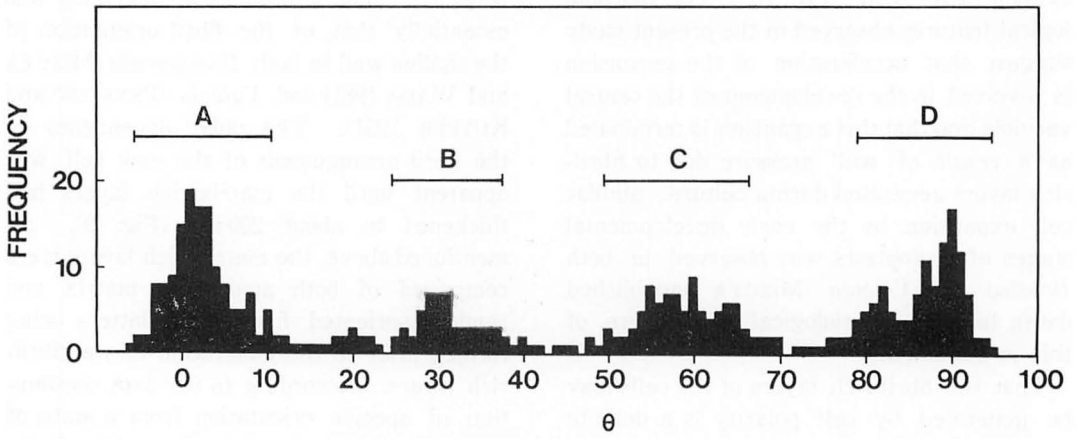
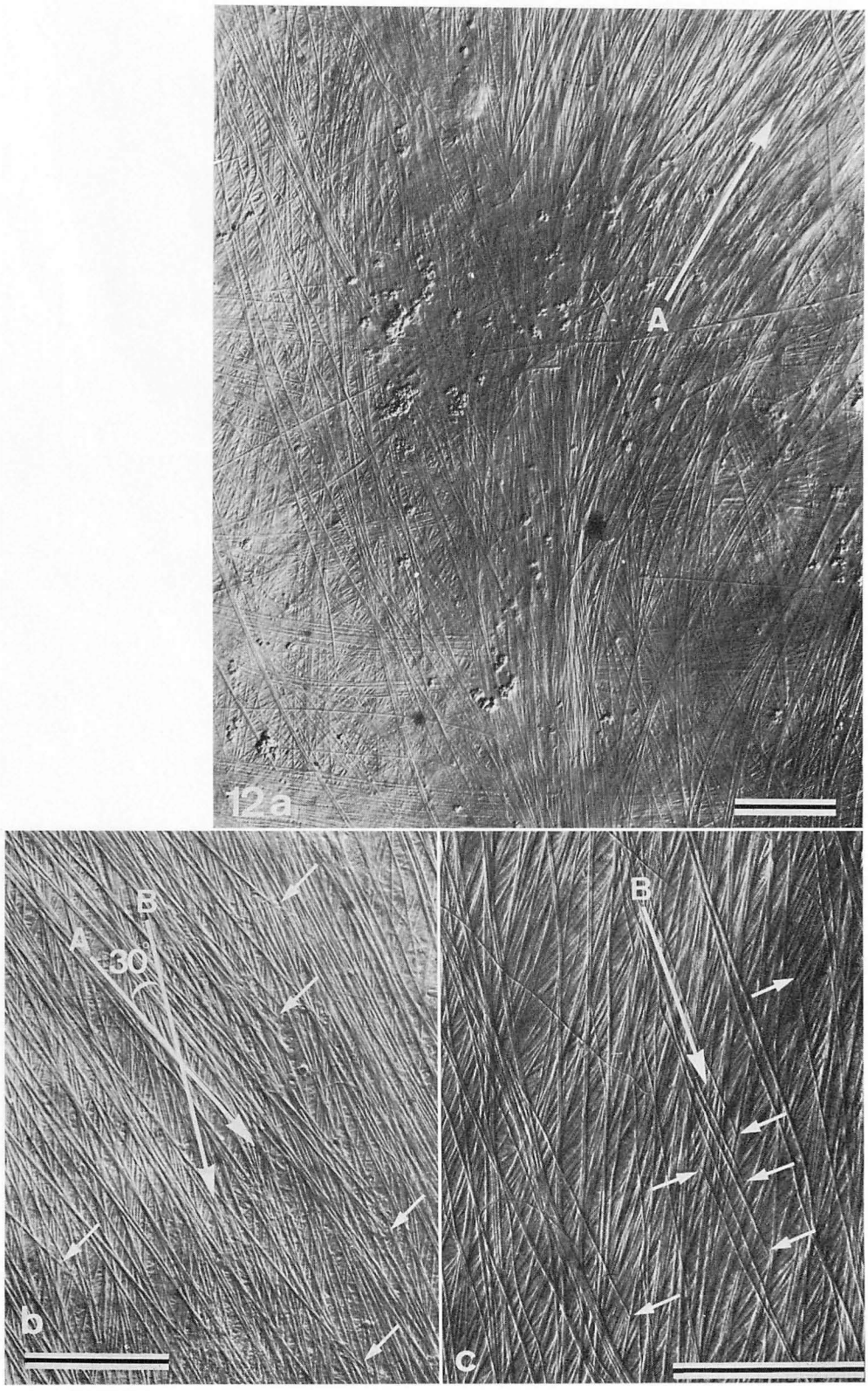


Fig. 11. Fibrillar angles with the direction of A, measured in the circle shown in Fig. 10. A, group of fibrils belonging to the undermost layer in Fig. 10; B, group of fibrils lying on A at an angle of about 30°; C, group of fibrils lying on B at an angle of 30°; D, group of fibrils in the uppermost layer of the figure. The ordinate frequency indicates the number of fibrils counted.


A
12 a
B
A
30°
b
B
c

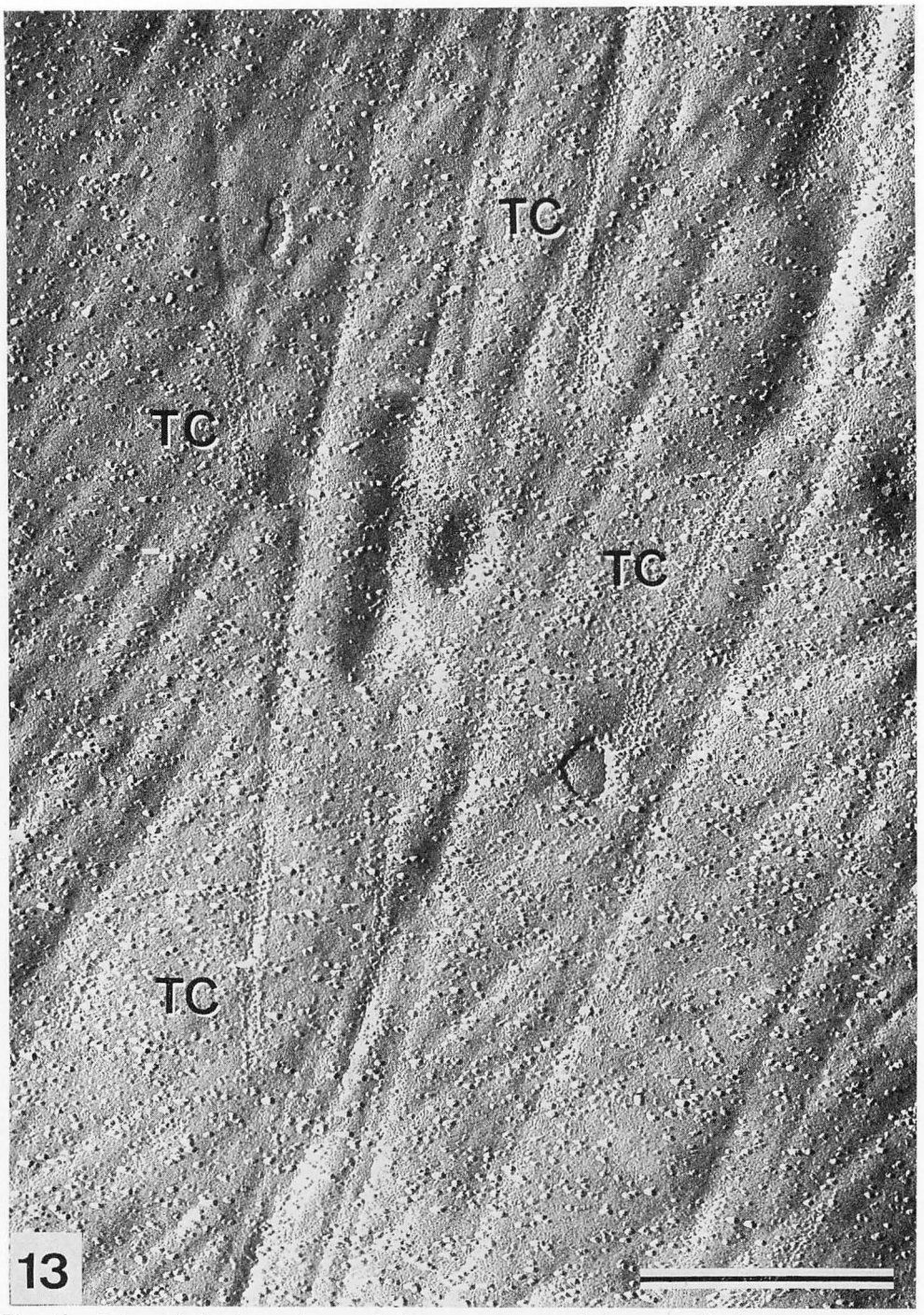


Fig. 13. P-face of the freeze-fractured plasma membrane of the new cell showing many terminal complexes (TCs). Bar=0.5 μm.

← Fig. 12. Inner surface replica of the new cell wall, showing the formation of the new layers (A layer and B layer) from the polar area toward the equator. Arrows A and B indicate the main direction of fibril orientation in the A and B layers respectively. **a**, polar area. The figure shows the fountain-like structure. **b**, intermediate area between the pole and equator. The figure shows the formation of the A layer, lying at an angle of about 30° with the underlying older layer (B layer). **c**, near the equator. Deposition of the B layer can be seen. Short arrows, microfibril terminals; Bars= 0.5 μm.

The controlling mechanisms of the shift of microfibril orientation are very important. In our previous report, there was evidence that antimicrotubule agents may be ineffective for bringing about the shift in orientation (MIZUTA and WADA 1982). On the freeze-fractured plasma membrane, many TCs were observed and the microfibril deposition is considered to be induced by the complexes (Fig. 13). The orientation shift of the fibrils is also considered to be due to the movement direction of the TCs, but the specialized structure reported relevant to the control of the direction of TC movement in freeze-fractured plasma membrane of *Oocystis* (BROWN and MOTENZINOS 1976) was not observed on the membrane of *Boergesenia*. However, the orientation of the fibrils of the fountain-like structure observed in the polar area (Fig. 12) closely resembled the specialized fibrillar arrangement which was reported to be affected by probable membrane flow in the freeze-fractured plasma membrane of maize (MUELLER and BROWN 1982). The mechanisms of fibril orientation control by membrane flow are now being investigated.

Acknowledgements

The authors wish to express their sincere thanks to Dr. Shunji WADA, Tohoku University, and to Professor R.M. BROWN Jr, University of Texas, for their valuable advice and criticism and also to Dr. O.M. MAIRH, Central Salt and Chemicals Research Institute, India, for his helpful discussion.

References

BRANTON, D., BULLIVANT, S., GILULA, N.B., KARNOVSKY, K.J., MOOR, H., MÜHLETHALER, K., NORTHCOTE, D.H., PACKER, L., SATIR, B., SATIR, P., SPETH, V., STAEHELIN, L.A., STEERE, R.L. and WEINSTEIN, R.S. 1975. Freeze etching nomenclature. Science **190**: 54-56.

BROWN, R.M. and MONTEZINOS, D. 1976. Cellulose microfibrils: visualization of biosynthetic and orienting complexes in association with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **73**: 143-147.

FREI, E. and PRESTON, R.D. 1961. Cell wall organization and wall growth in the filamentous green algae *Cladophora* and *Chaetomorpha* I. The basic structure and its formation. Proc. Roy. Soc. B. **150**: 70-94.

ISHIZAWA, K., ENOMOTO, S. and WADA, S. 1979. Germination and photoinduction of polarity in the spherical cells regenerated from protoplasm flagments of *Boergesenia forbesii*. Bot. Mag. Tokyo **92**: 173-186.

ITOH, T., O'NEIL, R. and BROWN, R.M. 1983. The assembly of cellulos microfibrils in selected siphonocladalean algae. J. Cell Biol. **97**: 416a.

MIZUTA, S. and WADA, S. 1981. Microfibrillar structure of growing cell wall in a coenocytic green alga, *Boergesenia forbesii*. Bot. Mag. Tokyo **94**: 343-353.

MIZUTA, S. and WADA, S. 1982. Effects of light and inhibitors on polylamellation and shift of microfibril orientation in *Boergesenia* cell wall. Plant Cell Physiol. **23**: 257-264.

MUELLER, S.C. and BROWN, R.M. 1982. The control of cellulose microfibril deposition in the cell wall of higher plants. I. Can directed membrane flow orient cellulose microfibrils ? Indirect evidence from freeze-fractured plasma membrane of maize and pine seedlings. Planta **154**: 489-500.

MÜHLETHALER, K. 1967. Ultrastructure and formation of plant cell walls. Ann. Rev. Plant Physiol. **18**: 1-24.

NICOLAI, E. and PRESTON, R.D. 1959. Cell wall studies in the Chlorophyceae III. Differences in structure and development in the Chlorophoraceae. Proc. Roy. Soc. B. **151**: 244-259.

PRESTON, R.D. and KUYPER, B. 1951. Electron microscopic investigations of the walls of green algae I. A preliminary account of wall lamellation and deposition in *Valonia ventricosa*. J. Exp. Bot. **2**: 247-255.

ROBINSON, D.G. and PRESTON, R.D. 1972. Plasmalemma structure in relation to microfibril biosynthesis in *Oocystis*. Planta **104**: 234-246.

TAKEDA, K. and SHIBAOKA, H. 1978. The fine structure of the epidermal cell wall in azuki bean epicotyl. Bot Mag. Tokyo **91**: 235-245.

水田　俊・澤田和好・奥田一雄：　多核緑藻マガタマモの原形質から誘導したプロトプラストの細胞壁形成

マガタマモ (*Boergesenia forbesii*) から誘導したプロトプラストにおける細胞壁の形成及び構造を電顕で調べた。細胞壁の形成過程は形態的に異なる二つの段階，すなわち (1) 細胞壁マトリックスを多く含む表層部の形成，及び (2) これに続く交叉多層構造性細胞壁の形成，に分けられる。(1) は球形化後約3時間で開始し，温度に依存する。(2) の開始は，(1) における壁厚が約 0.2 μm 以上であることを必要とし，温度及び光によって制限を受ける。(2) における各層の細胞壁微繊維は規則的に30°単位で配向を変換し，その形成方向は球の極部から赤道方向へ向う極性依存性を示した。(2) の時期における原形質膜の凍結裂開面には，セルロースの形成に関係する多くの顆粒構造（TC）が観察された。TCは約3列から成る微小顆粒が直線状に並ぶ構造を成し，移動しながら細胞壁微繊維の合成と配向変換を誘導すると考えられた。(780 高知市曙町 2-5-1　高知大学理学部生物学教室)

Change of Office and Editor

The new Editor of the Japanese Journal of Phycology for 1985-1986 is Hiromu Kobayasi of Tokyo Gakugei University. Starting in January 1985, manuscripts for publication should be submitted directly to the Editor, **Prof. H. Kobayasi, Department of Biology, Tokyo Gakugei University, Koganei-shi, Tokyo, 184 Japan.**

Membership dues should be sent to **The Businese Center for Academic Societies Japan, 4-16, Yayoi 2-chome, Bunkyo-ku, Tokyo, 113 Japan** and all other inquiries should be made to **The Japanese Society of Phycology, c/o Institute of Biological Sciences, University of Tsukuba, Sakura-mura, Ibaraki-ken, 305 Japan.**