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The structure and physiological properties of the cytoplasm in intact *Valonia* cell.

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The structure and physiological characteristics of the protoplasm-layer were studied in the intact cell of *Valonia ventricosa* (*Ventricaria ventricosa*). Scanning and transmission electron microscopy revealed the three dimensional organization of the cytoplasm. The cytoplasmic matrix existed as a thin layer surrounding the chloroplasts and nuclei. Many spaces between each chloroplasts were continuous with each other and were continuous with the central vacuole. Therefore the edge of the central vacuole entered into the protoplasm-layer with intricate structure.

The turgor pressure of the intact *Valonia* cell was determined to be 3.2 atm according to the difference between the osmotic values of the vacuolar sap and of the culture medium. Ion concentrations in the vacuolar sap were determined spectrochemically. Free Ca^{2+} and Cl^- were analyzed using Ca^{2+} electrode and Ag-AgCl electrode, respectively. Significance of the each ion concentration in the vacuole is discussed.

It was observed that the loss of turgor pressure upon wounding caused the disorganization of microtubule systems which had been essential for organization of the intact protoplasm-layer and resulted in the induction of aplanospore formation.

Key Index Words: cytoplasmic matrix—ion concentration—microtubule organization—osmotic value—protoplasm-layer—stereo view—turgor pressure—vacuolar sap—Valonia.

The thallus of *Valonia ventricosa* (*Ventricaria ventricosa*, Siphonocladales) (Fig. 1) is a multinucleate vesicular cell, reaching several centimeters in diameter. A thin layer of protoplasm is located on the inner surface of the cell wall, covering a huge central vacuole. The organization of the protoplasm-layer in *V. ventricosa* is sustained by two microtubular systems, the cortical and nuclear microtubules (Shihira-Ishikawa, 1987), similar to *Ernodesmis* and *Boergesenia* (La Claire 1987, La Claire and Fulginiti 1991).

In common with several species of Siphonocladales, *Boergesenia forbesii* (Enomoto and Hirose 1972, O'Neil and La Claire 1984) and *Dictyosphaeria cavernosa* (Enomoto and Okuda 1981), *V. ventricosa* segregates its protoplasm and forms numerous protoplasts by mechanical induction (Kopac 1933). However, the mechanism of the protoplast formation has not been elucidated. Regarding the study of the mechanism of protoplast morphogenesis in *Valonia*, basic knowledge about the morphology and physiology of the cytoplasm in the intact cell has been lacking.

In this paper, to investigate the protoplasmic organization of the intact cell of *V. ventricosa*, several methods of microscopy were used

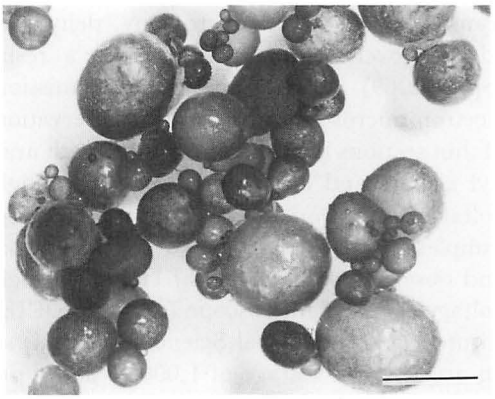

Fig. 1. Cells of *Valonia ventricosa* cultured in synthetic medium. Cells stick together by entwining rhizoids. Scale bar=5 mm.

and its physiological characteristics were discussed by measurement of the osmotic value and ion concentrations of vacuolar sap.

Material and Methods

Valonia cells

Valonia ventricosa grown in the aquarium in Kushimoto Marine Park, Wakayama, Japan, was kindly provided by Dr. S. Ui in 1989. A unialgal culture, which was started from the aplanospores, has since been maintained using Müller's synthetic sea water (Müller 1962). Cells were maintained in vessels at 22°C and under 1500 lux illumination (12: 12 hr LD) with daylight fluorescent lamps. The diameter of the cells increased about 2-3 mm per month. Cells 3-5 mm in diameter were used for the observations.

Electron microscopy

Cells were fixed in 2% glutaraldehyde in 35 mM cacodylic acid with 1% tannic acid for 2 hr at room temperature and 1% OsO_4 for 1 hr at 4°C after washing out the glutaraldehyde. Fixed cells were cut into several pieces and dehydrated stepwise in ethanol. For scanning electron microscopy, ethanol was replaced by isoamyl acetate and samples were critical-point dried in liquid CO_2 (JCPD-5, JEOL). The mounted dry cell pieces were coated with gold-palladium using ion spatter (JFC-1100, JEOL) and observed with a scanning electron microscope JEOL F-15. For transmission electron microscopy, dehydrated cells were embedded in Spurr's resin (Spurr 1969). A Hitachi H-300 transmission electron microscope was used for observation of thin sections after double staining with uranyl acetate and lead citrate. For ultrahigh voltage electron microscopy, Spurr embedded samples were sectioned (0.5-1.0 μm thick) and observed with a Hitachi H-1250M high voltage electron microscope (at National Institute for Physiological Science, Okazaki) at an accelerating voltage of 1,000 kv and a tilt angle of $+/-8°$.

Osmotic value

Osmotic values of the vacuolar sap and the culture medium were measured with a vapor pressure osmometer (Type 5500, Wescor, USA). Before measuring the osmotic value of the vacuolar sap, culture medium on the cell surface was removed with a filter paper. The vacuolar sap was then directly squeezed out onto a small filter paper disk (6 mm in diameter) by cutting the cell. For the culture medium, 10 μl of the medium was put on the filter paper disk. Calibration of the osmometer was carried out with NaCl solution of 200 mmol/kg and of 1,000 mmol/kg.

Ion concentration

Vacuolar sap was aspirated with a syringe after the cell was washed in distilled water and the water was immediately removed from the cell surface with filter paper to avoid the contamination by water outside the cell. Both in the vacuolar sap and in the culture medium, concentrations of four ions, K^+, Na^+, Mg^{2+} and Ca^{2+}, were measured by atomic absorption spectroscopy (Seiko Atomic Absorption/Frame Spectrophotometer SAS/760 connected with a terminal computer, NEC PC9801UV). Free Cl^- concentrations in the vacuole and in the culture medium were determined with an Ag-AgCl electrode (Mailman and Mullins 1966, Tazawa et al. 1974). Electric potential of an Ag-AgCl electrode in the test solution was measured against another Ag-AgCl electrode (reference electrode) which was immersed in a saturated KCl solution and connected to the test solution through an agar salt bridge containing 100 mM KNO_3 and 2% agar. Free Ca^{2+} concentration of the vacuolar sap and external medium were measured with a Ca^{2+} electrode (Philips, Ion selective electrode Ca^{2+}).

Indirect immunofluorescence

Whole cells were immersed in chilled methanol for 10 min and immediately transfered into phosphate buffered saline (PBS). The cell was cut gently in PBS on a glass slide with microscissors (Nisshin EM) and extra PBS was removed with filter paper. A prima-

ry antibody (monoclonal anti-alpha-tubulin mouse IgG, Amersham) was applied to the fixed cell pieces on the glass slide and the sample was incubated in a wet chamber for 30 min at 37°C. After washing with 0.03% Tween 20-PBS for 15 min, an FITC-labelled secondary antibody (anti-mouse Ig, fluorescein linked whole antibody, sheep, Amersham) was applied. The sample was incubated for 30 min at 37°C and washed again with 0.03% Tween 20-PBS for 15 min. The samples were mounted with p-phenylendiamine-glycerol (1 mg/ml, pH 8.0) and were observed with a epifluorescent microscope (Olympus BH2-RFK) with 490 nm excitation and G520 absorption filter.

Results

Fine structure of protoplasm-layer

Scanning electron microscopic observation showed the intricate organization of the protoplasm-layer which covered the inner surface of the cell wall (Fig. 2). Many spaces were observed in the protoplasm-layer which were continuous with each other and were also continuous with the central vacuole. Therefore, the surface of the central vacuole, which corresponded to the tonoplast, was extremely irregular with a fine intricate structure.

Most of the protoplasm was occupied by disc-shaped chloroplasts which were spread over the plasma membrane. The edge of these chloroplasts lay one above the other (Fig. 3a). However, further overlapping was disturbed by the starch accumulated around the pyrenoids which existed at the center of each chloroplast (Fig. 3a and b). Nuclei were located at the vacuolar side of the chloroplast layer (Fig. 3a and b). Each of the chloroplasts as well as the nucleus was covered by a thin layer of cytoplasmic matrix (Fig. 3c and d) and therefore each chloroplast was attached to the other *via* the cytoplasmic matrix (Fig. 3d). Mitochondria were observed in the cytoplasmic matrix around the chloroplasts and nuclei (Fig. 3d). Ultrahigh voltage electron microscopy enabled the visualization of the three dimensional images of the localization of chloroplasts and nuclei (Fig. 4).

Turgor pressure of the cell

The osmotic value of the vacuolar sap was 1092 mmol/Kg on average, while that of the culture medium was 964 mmol/Kg (Table 1). The difference between them was about

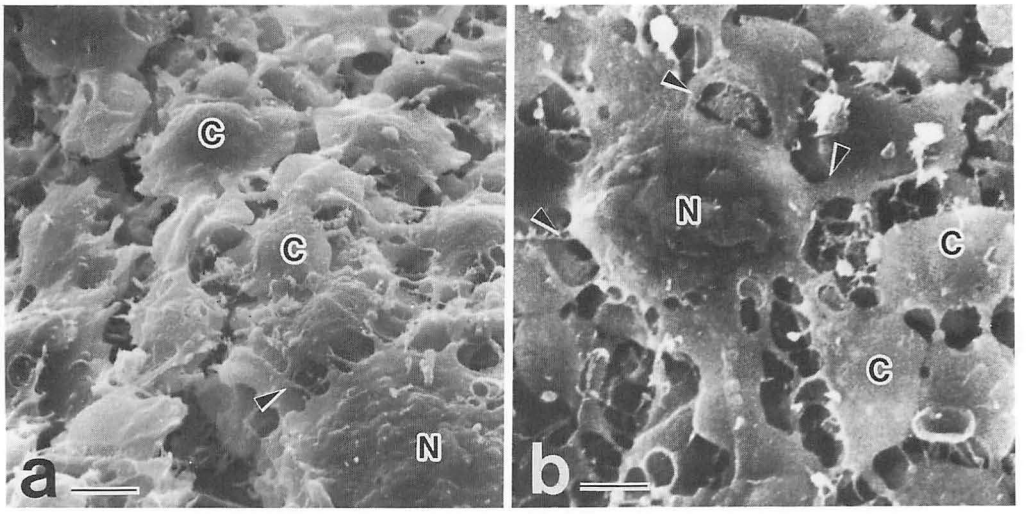

Fig. 2. Scanning electron micrographs showing the surface of protoplasm-layer facing the center of *Valonia* cell. The cell was cut into several pieces and the protoplasm-layer adhering to each piece was observed from the inside of the cell, that is, from the vacuolar side of the protoplasm-layer. Scale bars=2 μm a. tangential view. b. diametrical view. Protoplasm-layer is sponge-like and protoplasmic strands (arrowheads) are observed which are radially extending from nucleus to chloroplasts. C, chloroplast; N, nucleus.

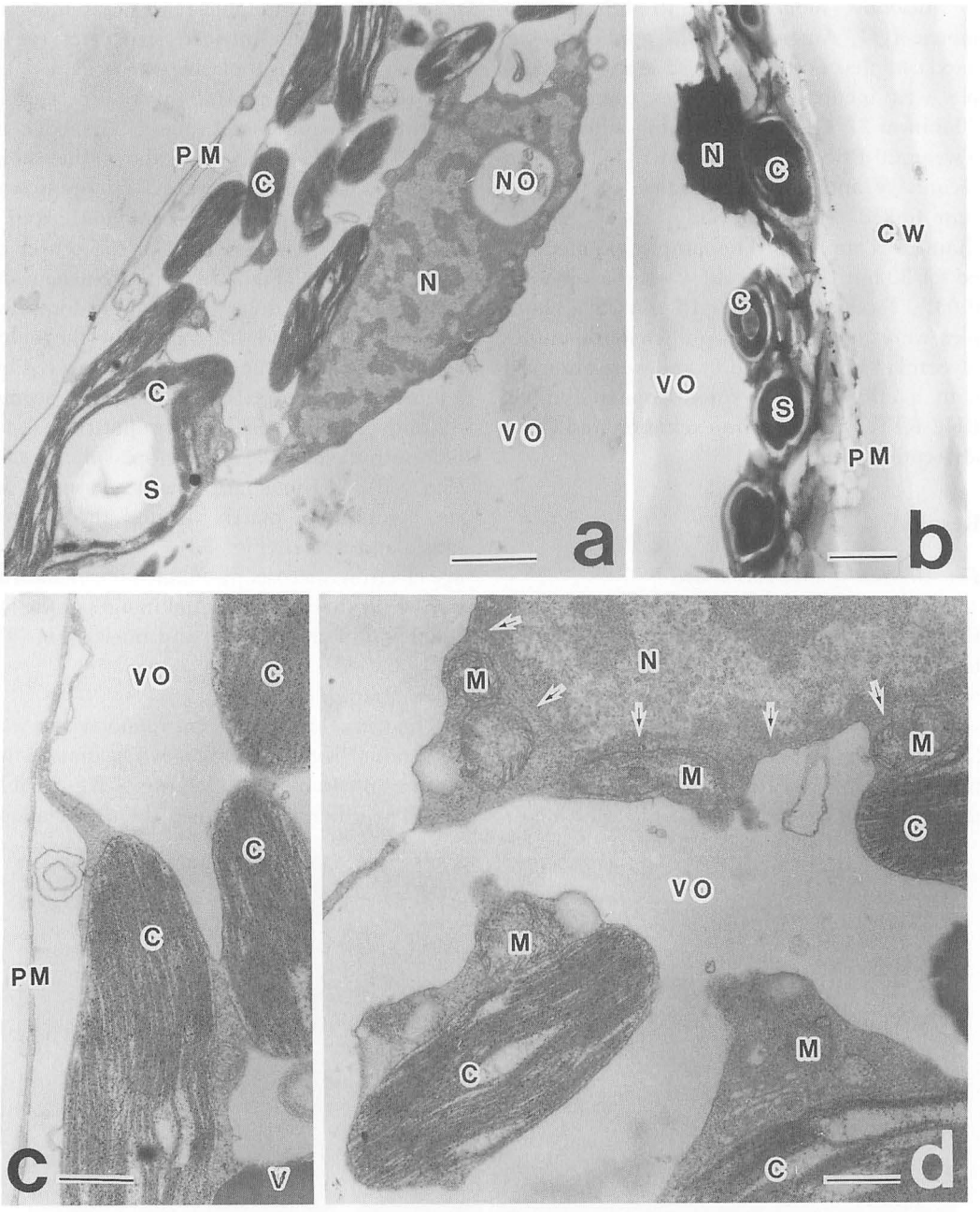

Fig. 3. Transmission electron micrographs of the protoplasm-layer. a. oblique section of the protoplasm-layer: Nucleus is situated on the chloroplast-layer, at the vacuolar side of the protoplasm-layer. The overlapping edges of chloroplasts are layered beneath the nucleus. Scale bar=2 μm. C, chloroplast; N, nucleus; NO, nucleolus; PM, plasma membrane with a layer of cytoplasmic matrix; S, starch accumulated in chloroplast; VO, vacuole. b. Thick section (1 μm thick) of the protoplasm-layer observed with high voltage electron microscope, showing the arrangement of chloroplasts. Only the edges of chloroplasts are piled up, because the overlapping of the whole chloroplast is interrupted by the accumulation of starch. Scale bar=5 μm. C, chloroplast; S, starch; N, nucleus; VO, vacuole; CW, cell wall; PM, plasma membrane. c and d. Cross sections of the protoplasm-layer. A thin layer of cytoplasmic matrix exists around the individual nucleus and chloroplast. Scale bar=0.5 μm. C, chloroplast; M, mitochondria; N, nucleus; V, vesicle; PM, plasma membrane with a layer

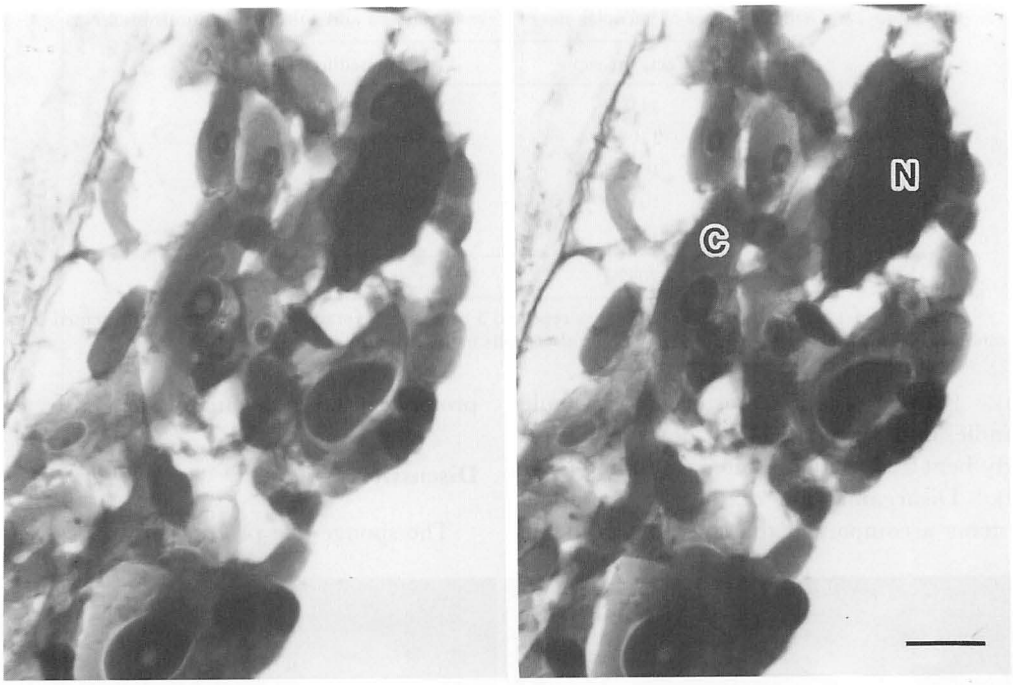

Fig. 4. Ultrahigh voltage electron micrographs showing stereo-image of the protoplasm-layer. The slant sectional views of the protoplasm-layer demonstrate three dimensional arrangement of chloroplasts and nuclli which forms a sponge-like structure of protoplasm. N, nucleus; C, chloroplast. Scale bar=2 μm.

128 mmol/Kg, which corresponds to 3.2 atm after van't Hoff's equation. Accordingly the turgor pressure of an intact cell was 3.2 atm under this culture condition.

Ion concentration in vacuolar sap

The concentrations of major cations in vacuolar sap and in culture medium, K^+, Na^+, Mg^{2+} and Ca^{2+} were determined with frame spectrophotometer and calcium eletrode (Table 2). Concentration of K^+ was about twenty times higher in the vacuolar sap than that in the culture medium, while Na^+ was about six times lower in the vacuolar sap than that in the culture medium. Free Ca^{2+} concentration in vacuolar sap which was determined with a calcium electrode was 0.5 mM, while total Ca^{2+} which was obtained by spectrochemical analysis, was 1.06 mM, showing that a considerable amount of bound Ca existed in the vacuole. Cl^- was 520 mM in vacuolar sap. Assuming that each of the major cations was a chloride form in the vacuolar sap, a solution containing 78 mM NaCl, 523 mM KCl, 26 mM $MgCl_2$ and 1 mM $CaCl_2$ was prepared. The osmotic value of this solution was determined as 1190 mmol/Kg, which was approximately the same value as that of the vacuolar sap (Table 1).

Microtubule organization in protoplasm-layer

Microtubular systems, that is, the nuclear microtubules and cortical microtubules, were disorganized simultaneously throughout the entire cell when the turgor pressure decreased upon wounding. Radially extending arrays of the nuclear microtubules in the intact cell (Fig. 5a) were lost almost 30 min after being wounded, although the ones lying close to the nuclear envelope were relatively intact (Fig.

of cytoplasmic matrix; VO, vacuole. In c, cytoplasmic matrix connecting individual chloroplast is continuous to the cytoplasmic matrix which is contiguous to plasma membrane. In d, mitochondria are located in the large masses of cytoplasmic matrix around nucleus and chloroplasts. Arrows indicate the nuclear envelope.

Table 1. Osmotic values of vacuolar sap of *Valonia ventricosa* and culture medium (mmol/Kg)

	Vacuolar sap	Culture medium
	1119[1)]	
	1109[2)]	965 (fresh medium)
	1068[3)]	
	1053[4)]	963 (used medium)
	1113[5)]	
Ave.	1092	964

[1)–5)] Different cells. Each measurement was repeated 3 times, the average value of which is presented in the table. Used medium, the medium in which *Valonia* cells had been cultured for a week.

5b). Parallel arrays of cortical microtubule bundles in the intact cell (Fig. 5c) were irregularly bent about 30 min after wounding (Fig. 5d). Disorganization of these microtubule systems accompanied the corrugation of the protoplasm-layer throughout the entire cell.

Discussion

The sponge-like protoplasm in *Valonia ven-*

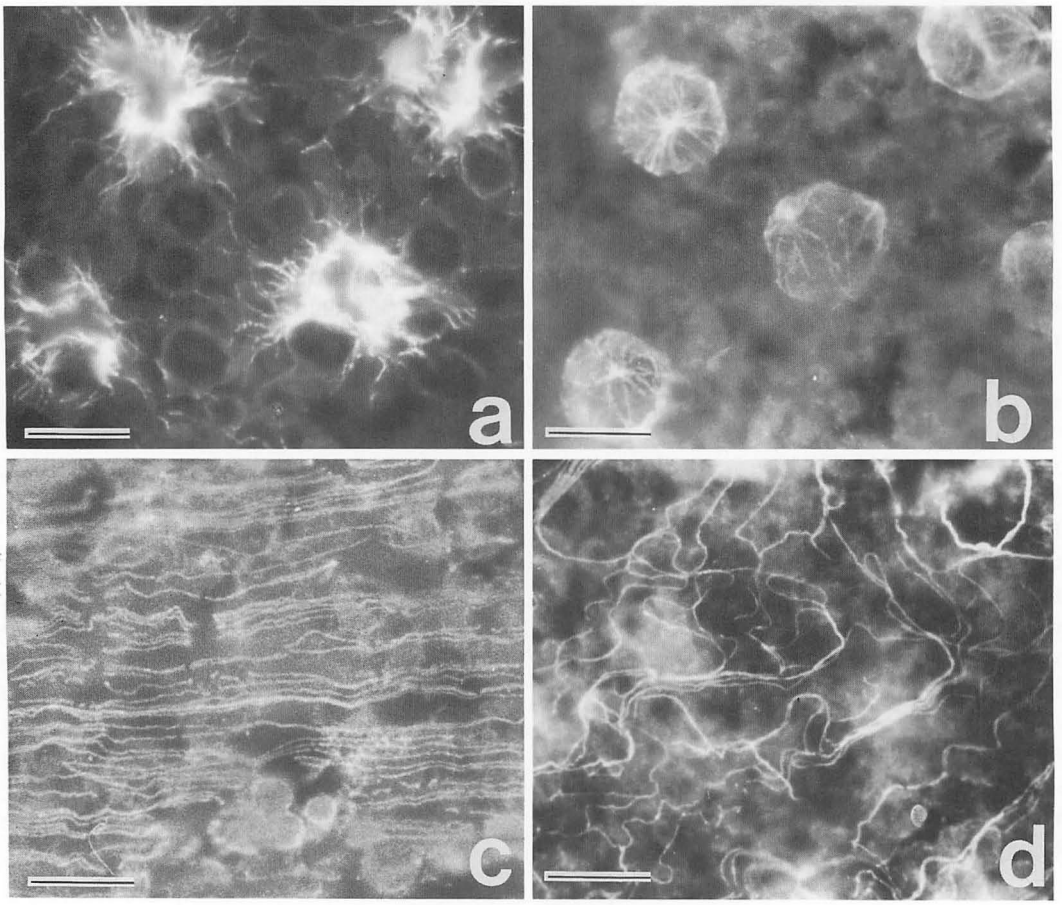

Fig. 5. Microtubule organization observed by indirect immunofluorescence. Scale bars=10 μm. a and b, nulcear microtubule systems; c and d, cortical microtubule systems; a and c, microtubule systems in intact cell; b and d, microtubule systems in wounded cell, 30 min after the reduction of turgor pressure.

Table 2. Ion concentration in vacuolar sap of *Valonia ventricosa* and the culture medium (mM)

	Cl^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	Free Ca^{2+}
Vacuolar sap	520	78	523	26	1.06	0.53
Culture medium	420	500	25	48	15.9	4.8

1. Na^+, K^+, Mg^{2+} and Ca^{2+} were determined by atomic absorption spectroscopy.
2. Cl^- was determined with Ag-AgCl electrode.
3. Free Ca^{2+} was determined with calcium electrode.

tricosa lies between the cell wall and the large central vacuole, forming a thin layer less than 10 μm in thickness. The two distinct components of the protoplasm-layer are chloroplasts and nuclei. The chloroplasts are tightly arranged on the inner surface of the plasma membrane and the nuclei are distributed evenly on the inner surface of chloroplast layer (Fig. 6). Chloroplasts and nuclei are individually surrounded by a thin layer of cytoplasmic matrix and are connected each other *via* this layer in which mitochondria and vesicles were detected (Fig. 6). The radial arrays of cytoplasmic matrix extending from nucleus to chloroplasts were observed by scanning electron microscopy. These arrays apparently corresponded to the radially extending microtubule organizations which were observed with immunofluorescence as nulcear microtubules, suggesting the latter lies in the arrays of cytoplasmic matrix. The microtubule bundles in these arrays possibly connect the nucleus to the chloroplasts and sustain the protoplasm-layer.

Actin cytoskeleton was investigated with immunofluorescence, but no filaments were observed (data not shown). La Claire (1984, 1989) reported that actin bundles were disassembled in the intact cells of *Ernodesmis* and *Boergesenia*, though they were revealed during the process of wound healing. Possibly in *V. ventricosa* the disassembled actin bundles were present in the cytoplasm, although actin bundles were not detected.

The outer surface of the protoplasm-layer adheres to the cell wall via the plasma membrane, while its inner surface meshes with the outer surface of the central vacuole in an intricate fashion (Fig. 6). This sponge-like protoplasm is equilibrated with the turgor pressure

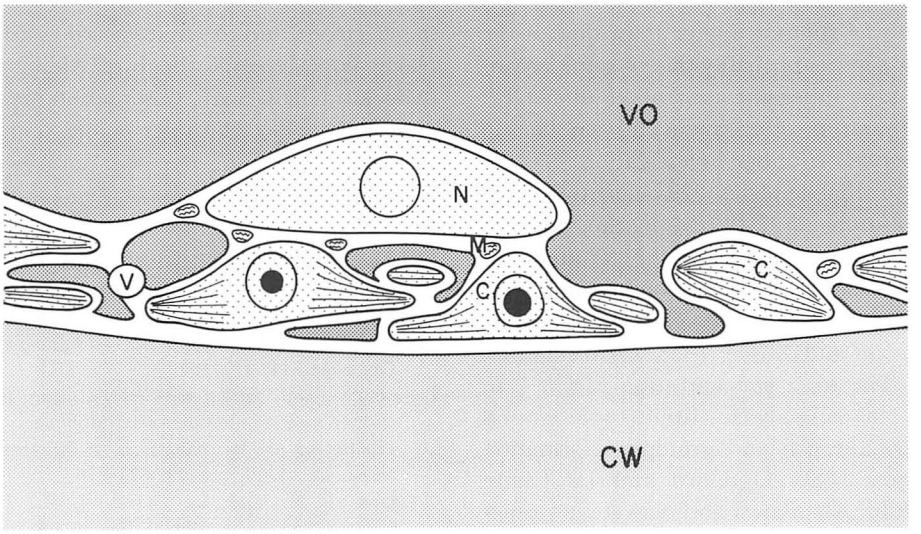

Fig. 6. Illustrated cross section of protoplasm-layer. Edge of central vacuole enters into protoplasm-layer with intricate structure. Each chloroplast and nucleus is surrounded by a thin layer of cytoplasmic matrix (white area). VO, vacuole; N, nucleus; C, chloroplast; V, vesicle; M, mitochondria; CW, cell wall.

of the central vacuole. The microtubule organization likely sustains this fragile structure of the protoplasm-layer.

The difference of osmotic pressures between the culture medium and the vacuolar sap was about 128 mmole/Kg which generates the turgor pressure of 3.2 atm in the intact cell.

The concentrations of K^+, Na^+, Mg^{2+} and Ca^{2+} in the vacuolar sap were measured by atomic absorption spectroscopy. The concentrations of K^+, Na^+ were approximately the same as those of K^+ and Na^+ which had been reported by Gutknecht and Dainty (1968). Extremely high concentration of K^+ was obtained in the vacuolar sap and it is likely correlated with the turgor pressure. Cl^- concentration was measured using Ag-AgCl electrode and was higher in vacuolar sap than that in culture medium. A solution containing NaCl, KCl, $MgCl_2$ and $CaCl_2$ prepared as the same concentrations as each of these cations found in the vacuolar sap (Table 2) showed approximate osmotic value to that of vacuolar sap. This suggests that the majority of the major cations in vacuolar sap is the chloride form. The facts that the total concentration of the major cations was higher in some degree than the concentration of Cl^- in the vacuolar sap and the osmotic value of this solution was slightly higher than that of the vacuolar sap are probably due to some organic anions or inorganic ions other than Cl^-. The preparation of an artificial vacuolar sap in *Valonia* cell is under investigation.

The protoplasm lies between two high Ca^{2+} solutions; 5×10^{-4} M in the vacuolar sap and 5×10^{-3} M in the culture medium. The cytoplasm must be maintained in a state of low Ca^{2+} concentration, less than 10^{-6} M (Williamson and Ashley 1968, Okazaki et al. 1987). The high concentration of Ca^{2+} in the vacuolar sap may contribute to the wound healing of the cell and protoplast formation (La Claire 1982, Goddard and La Claire 1991, Shihira-Ishikawa and Nawata in preparation).

The turgor pressure is lost immediately when the cell is wounded, as part of the cell sap is ejected through the wound. More than 30 min after wounding, the protoplasm-layer is partially detached from the cell wall and becomes corrugated, along with the destruction of microtubule organization. It is not known whether the disorganization of microtubule bundles is the cause of the corrugation of the protplasm-layer or the result. However, distortion of the cortical microtubule systems and the loss of the radially extending nuclear microtubules suggest that the mechanical destruction of the microtubule systems occurs after the corrugation of the protoplasm-layer. In any case, the microtubule systems were disorganized after the loss of turgor pressure upon being wounded. It is undoubted that the loss of turgor pressure and the following disorganization of microtubule systems were essential factors for aplanospore induction in intact *Valonia* cells.

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石川依久子・繩田　利寿：多核緑藻バロニアの細胞質構造と生理学的特性

巨大単細胞性多核緑藻オオバロニア *Valonia ventricosa* (*Ventricaria ventricosa*) の原形質は細胞壁直下にスポンジ状の薄層をなしている。この原形質層を走査型および透過型電子顕微鏡で三次元的に観察した。細胞基質は個々の葉緑体や核を個別に薄く包み，それぞれの葉緑体や核は，この細胞基質の薄い層を介して接している。また核から放射状にのびる細胞基質は，核と葉緑体を連結している。細胞基質の外縁は液胞と接しているので中心液胞の縁辺部は原形質層の中に複雑に入り込んでいることになる。液胞液の主イオンの濃度を原子吸光，カルシウム電極および銀—塩化銀電極で測定し，各イオン濃度の生理学的意義を考察した。液胞液と外液（合成培地）の浸透価の差から算出したバロニア藻体の膨圧は3.2気圧であった。藻体に傷をつけることによって膨圧が減少すると，原形質層の構造を保持していた微小管系が崩壊し，原形質が凝集し不動胞子形成が誘導される。（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）