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

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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 $CaCO_3$

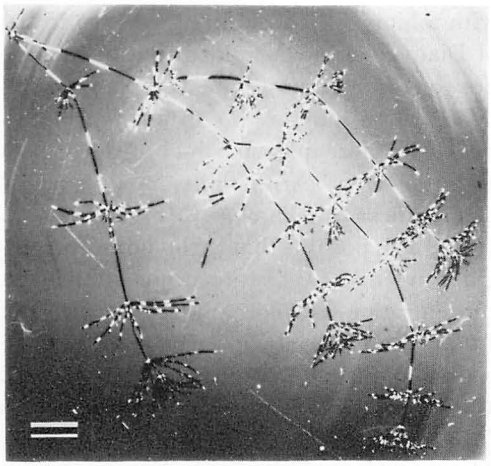

Fig. 1. Heavily calcified *Chara braunii* grown in Ca^{2+}-enriched pond water. Note white bands of $CaCO_3$ deposited on the internodal cells of main axis and wheels. Photograph was taken under polarized light. Scale=1 cm.

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-

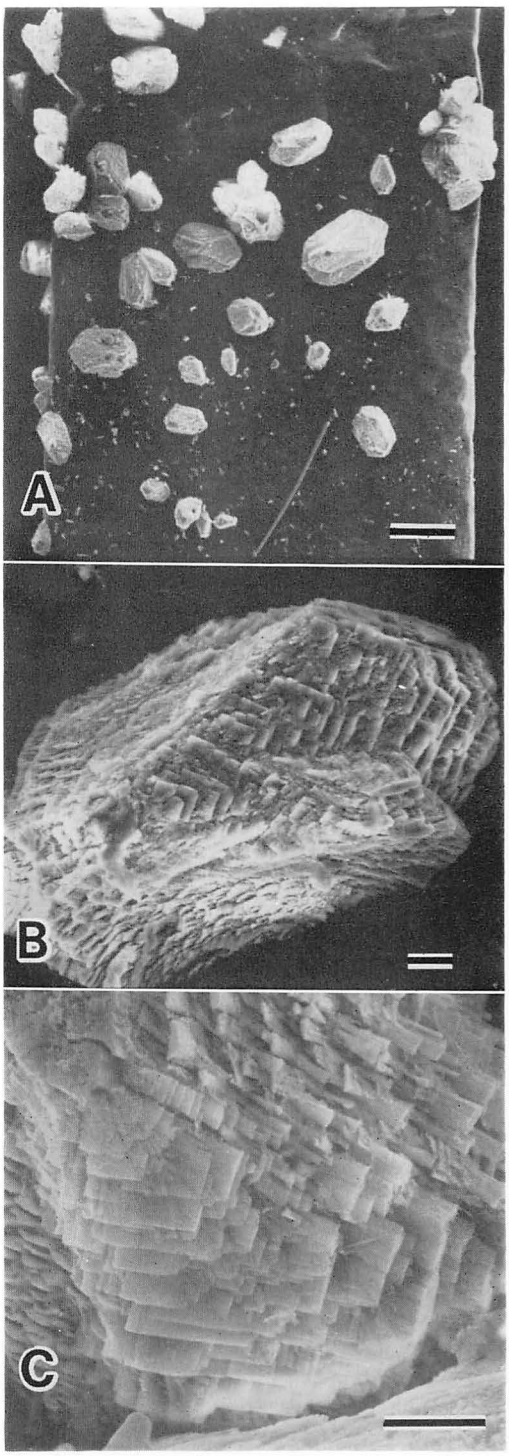

Fig. 2. Scanning electron micrographs of calcite crystals deposited in band on the fourth internodal cell from tip. Note that most crystals are hexagonal and foliated. C shows a magnified view of a crystal in B.
Scale=100 μm (A), 10 μm (B), 5 μm (C).

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

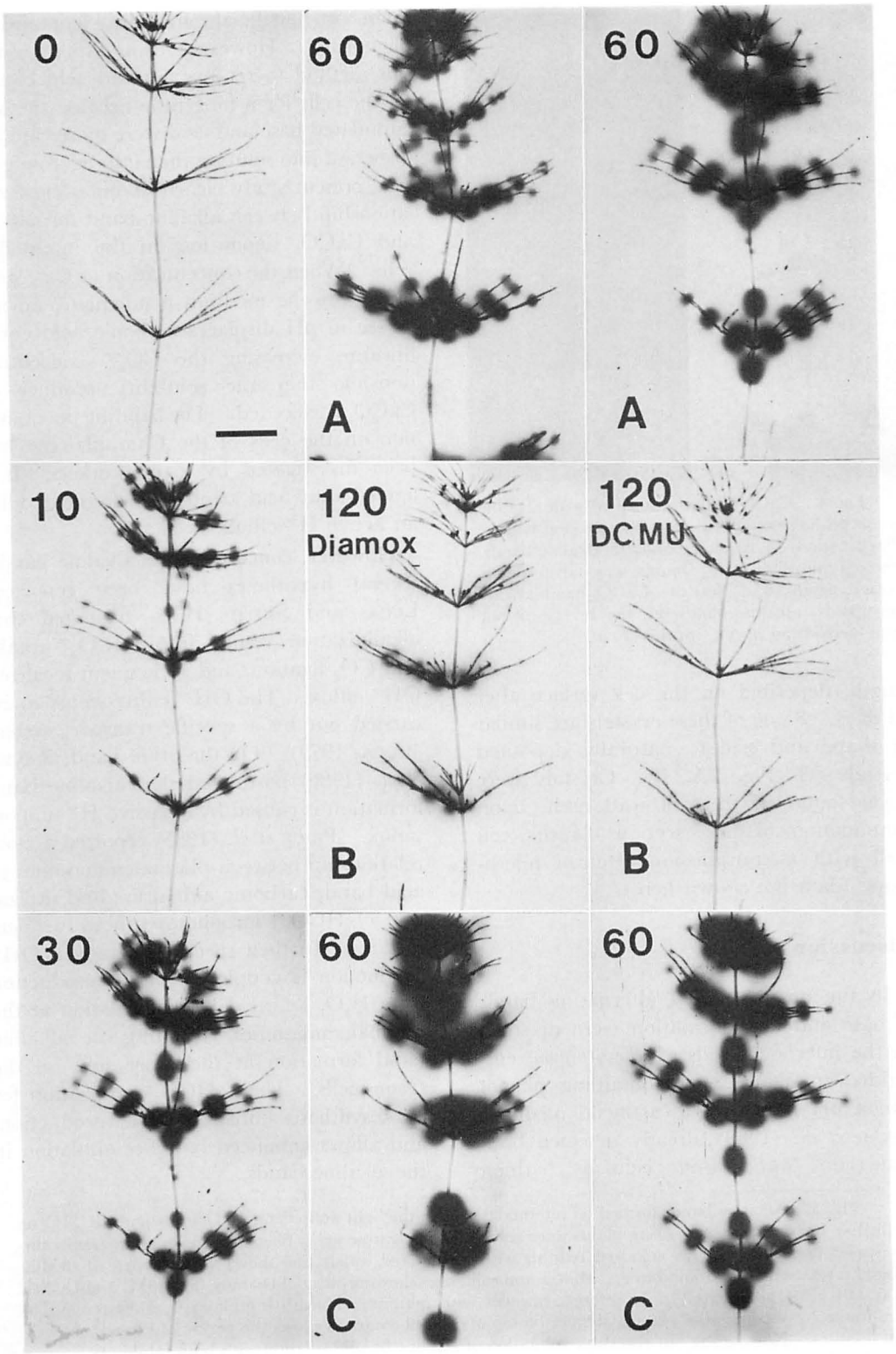

Fig. 3

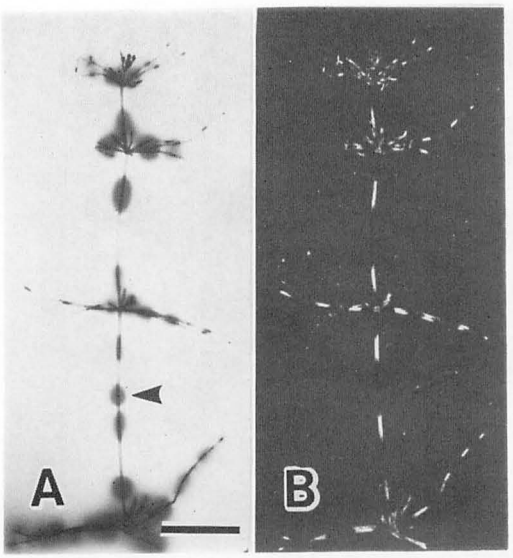

Fig. 4. $CaCO_3$ bands associated with alkaline bands on internodal cells of main axis and wheels. Black regions in A corresponds to alkaline bands. Photograph of $CaCO_3$ bands was taken under polarized light (B). Note that $CaCO_3$ bands always accompany alkaline bands but the reverse is not true (arrowhead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

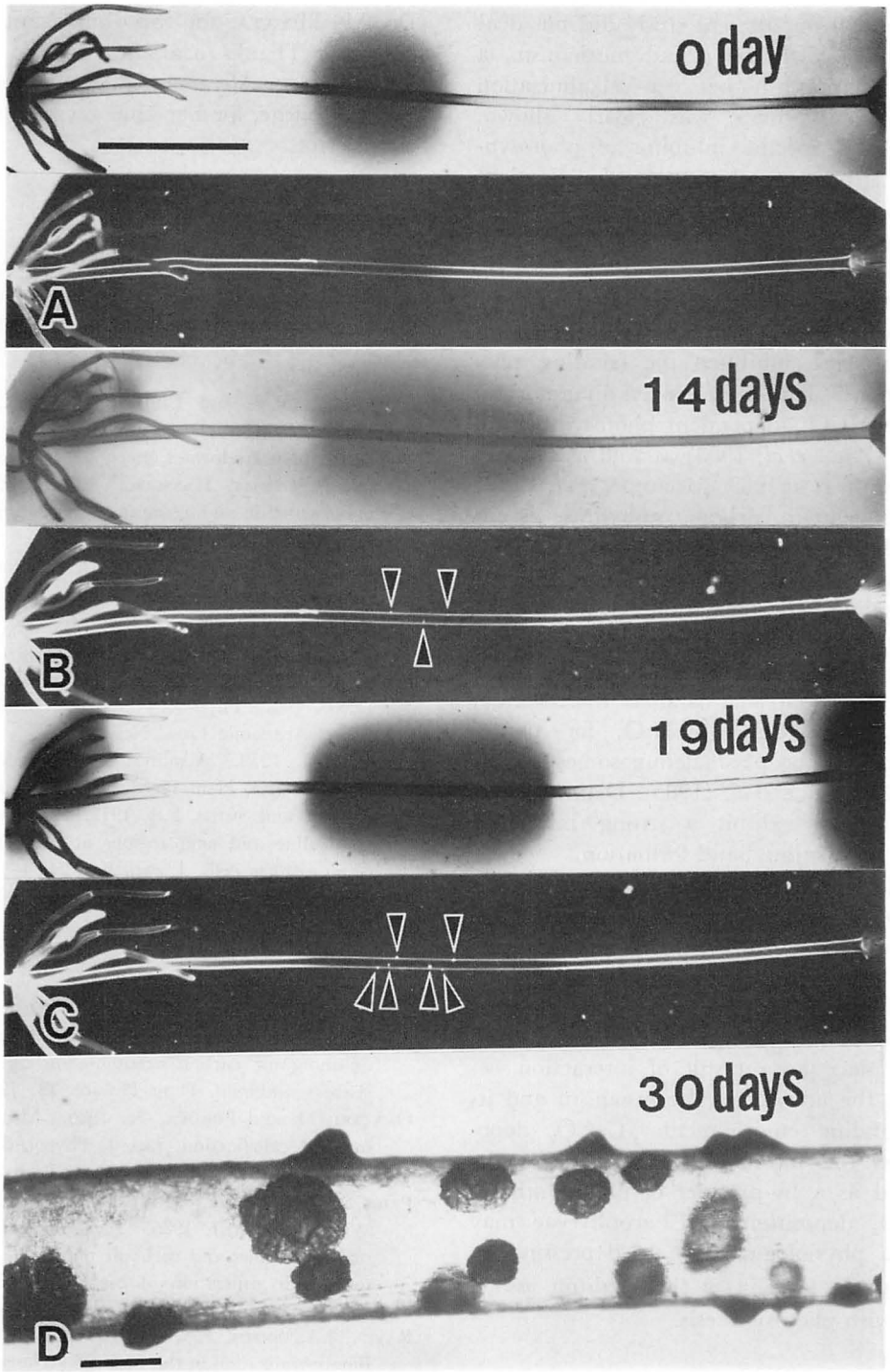

Fig. 5. $CaCO_3$ crystals induced in an alkaline band on internodal cell.
Young internodal cells free of $CaCO_3$ deposits were embedded in agarose gel mounted on a glass slide. A black region in A, B and C corresponds to alkaline band coloured reddish with phenol red. In A, B and C, photographs were 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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References

BOROWITZKA, M.A. 1982. Mechanisms in algal calcification. p. 137–177. *In* F.E. ROUND and D.J. CHAPMAN (ed.) Progress in Phycological Research, vol. 1. Elsevier Biomedical Press, Amsterdam.

DEVIPRASAD, P.V. and CHOWDARY, Y.B.K. 1981. Effects of metabolic inhibitors on the calcification of a freshwater green alga, *Gloeotaenium loitlesbergarianum* HANSGIRG. Effects of some photosynthetic and respiratory inhibitors. Ann. Bot. **47**: 451–459.

KITANO, Y. and HOOD, D.W. 1962. Calcium carbonate crystals forms formed from sea water by inorganic processes. J. Oceanogr. Soc. Japan. **18**: 141–145.

LEWIN, J.C. 1962. Calcification. p. 457–465. *In* R.A. LEWIN (ed.) Physiology and Biochemistry of Algae. Academic Press, New York.

LUCAS, W.J. 1979. Alkaline band formation in *Chara corallina*. Plant Physiol. **63**: 248–254.

LUCAS, W.J. and SMITH, F.A. 1973. The formation of alkaline and acid regions at the surface of *Chara corallina* cells. J. exp. Bot. **24**: 1–14.

MIYACHI, S., TSUZUKI, M. and AVARMOVA, S.T. 1983. Utilization modes of inorganic carbon for photosynthesis in various species of *Chlorella*. Plant and Cell Physiol. **24**: 441–451.

MORONEY, J.V., HUSIC, H.D. and TOLBERT, N.E. 1985. Effect of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii*. Plant Physiol. **79**: 177–183.

OKAZAKI, M. and FURUYA, K. 1985. Mechanisms in algal calcification. Jap. J. Phycol. **33**: 328–344.

PRICE, G.D., BADGER, M.R., BASSETT, M.E. and WHITECROSS, M.I. 1985. Involvement of plasmalemmasomes and carbonic anhydrase in photosynthetic utilization of bicarbonate in *Chara corallina*. Aust. J. Plant Physiol. **12**: 241–256.

RAVEN, J.A., SMITH, F.A. and WALKER, N.A. 1986. Biomineralization in the Charophyceae *sensu lato*. p. 125–139. *In* B.S.C. LEADBEATER and R. RIDING (eds.) Biomineralization in Lower Plants and Animals. Clarendon Press, Oxford.

SIMKISS, K. 1986. The processes of biomineralization in lower plants and animals-an overview.

p. 19–37. *In* B.S.C. LEADBEATER and R. RIDING (eds.) Biomineralization in Lower Plants and Animals. Clarendon Press, Oxford.

SPEAR, D.G., BARR, J.K. and BARR, C.E. 1969. Localization of hydrogen ion and chloride ion fluxes in *Nitella*. J. gen. Physiol. **54**: 397–414.

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）