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

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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

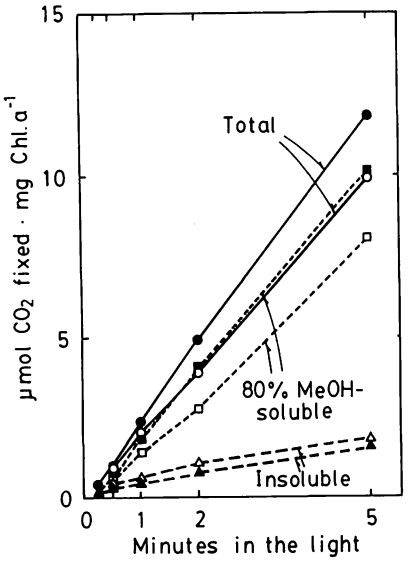

Fig. 1. Time courses of ^{14}C incorporation into 80% methanol-soluble and -insoluble fractions during photosynthetic $^{14}CO_2$ fixation in N-enriched and N-starved cells of *Heterosigma akashiwo*. Solid lines, total activity; dotted lines, 80% methanol-soluble fraction; broken lines, 80% methanol-insoluble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

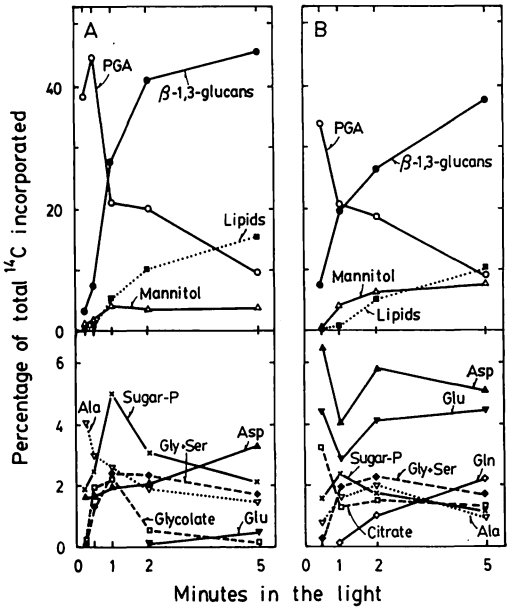

Fig. 2. Percentage distribution of ^{14}C in individual products of photosynthetic $^{14}CO_2$ fixation *versus* time in N-enriched (A) and N-starved (B) cells of *H. akashiwo*. Data are from the experiment described in Fig. 1. Symbols: Ala, alanine; Asp, aspartate; Gln, glutamine; Glu, glutamate; Gly+Ser,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

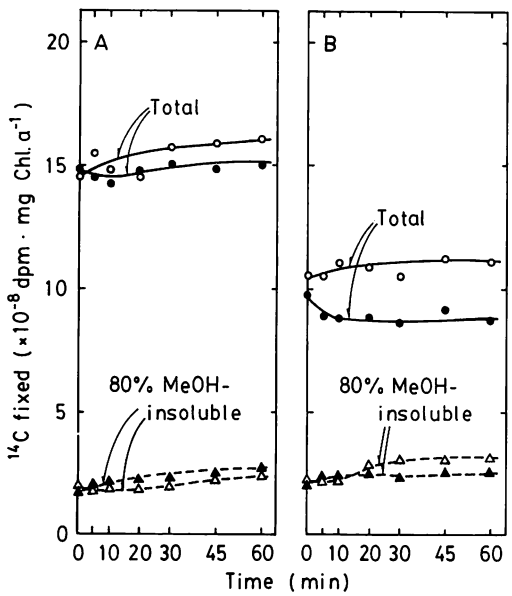

Fig. 3. Changes in the distribution of ^{14}C in total and 80% methanol-insoluble fractions in N-enriched (A) and N-starved (B) cells during the chase periods in the light and in the dark after a 5-min $^{14}CO_2$ pulse in the light. Experimental conditions are described in the text. Open symbols, chase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

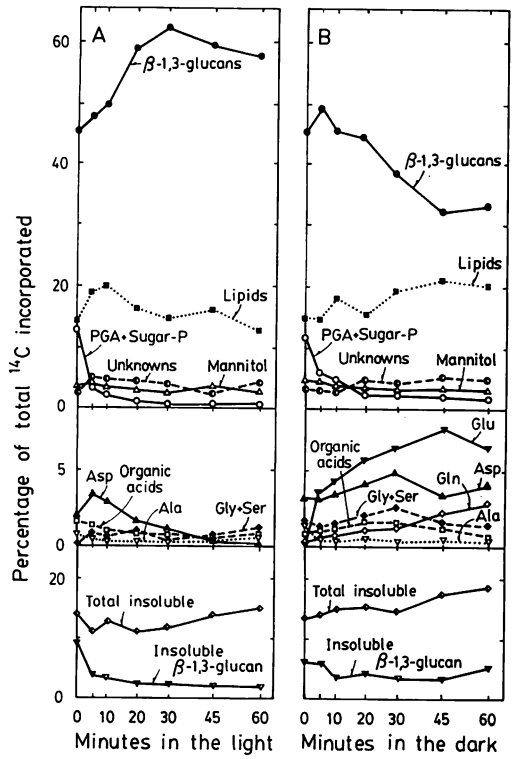

Fig. 4. Changes in the distribution of radioactivity among ^{14}C-labeled compounds in the light (A) and in the dark (B) in N-enriched *H. akashiwo* cells during pulse-chase experiments after a 5-min $^{14}CO_2$ pulse in the light. Data are from the experiment described in Fig. 3. Symbols: Total insoluble,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

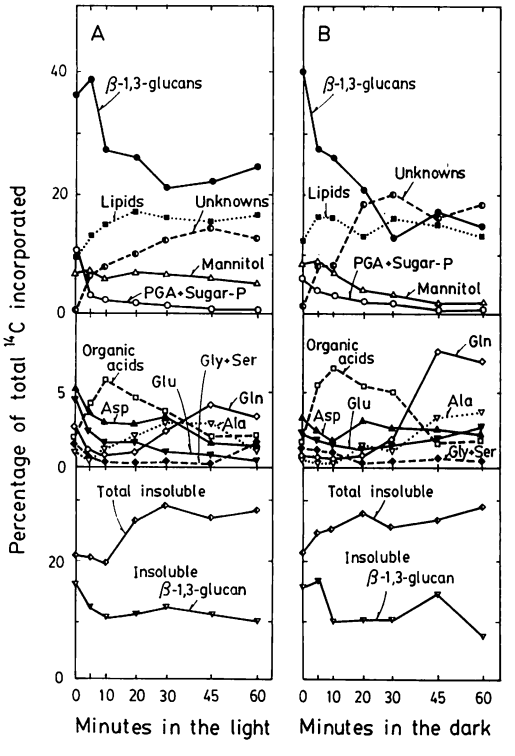

Fig. 5. Changes in the distribution of radioactivity among ^{14}C-labeled compounds in the light (A) and in the dark (B) in N-starved *H. akashiwo* cells during pulse-chase experiments after a 5-min $^{14}CO_2$ pulse in the light. Symbols are the same as those of Fig. 4.

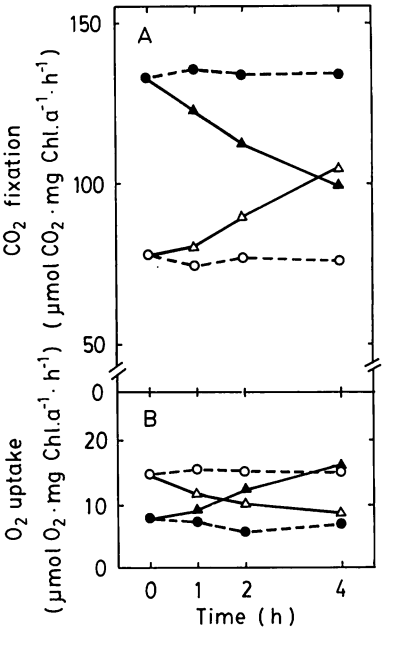

Fig. 6. Effects of nitrate on the rate of photosynthetic CO_2 fixation (A) and dark respiration (B) in *H akashiwo* cells. N-enriched or N-starved cells were collected by filtration, washed with sterile N-free culture medium and resuspended in incubation medium. N-enriched cells were incubated in either 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.

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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)