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Effects of acetate on gene expression of external carbonic anhydrase in *Chlamydomonas* (Volvocales)

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The effect of acetate on the expression of two carbonic anhydrase genes (*CAH1* and *CAH2*) was studied with a green alga *Chlamydomonas reinhardtii*. In photoautotrophically grown cells, *CAH1* transcript appeared 1 hr after the transfer from 5 to 0.04% CO_2, while *CAH2* transcript was present in 5% CO_2 and thinned out in air. In mixotrophically grown cells with acetate, *CAH1* transcript level was lowered drastically under low CO_2 concentration, while *CAH2* transcript was present both under low and high CO_2 conditions. The effect of acetate on the gene expression of *CAH1* and *CAH2* is discussed in relation to photosynthesis and CO_2 concentration.

Key Index Words: *acetate—carbonic anhydrase*—Chlamydomonas—*gene expression—mixotrophy.*

Carbonic anhydrase (CA; EC4.2.1.1) has been investigated in several microalgae. It has been shown that this enzyme plays an important role for ensuring a high apparent affinity for inorganic carbon in photosynthesis under ordinary air (Aizawa and Miyachi 1986, Tsuzuki and Miyachi 1990). In *Chlamydomonas reinhardtii*, a high level of CA activity was found in the periplasmic area (Kimpel *et al.* 1983) and it increased under low CO_2 conditions (Yang *et al.* 1985). cDNA clones for the periplasmic CA were isolated by using oligonucleotide probes raised from amino acid sequence of the enzyme subunits (Fukuzawa *et al.* 1990). Nucleotide sequence analysis revealed that the cDNA clones encoded both large and small subunits of CA.

Genes which corresponded to the obtained cDNA clones, *CAH1* and a structurally similar gene *CAH2*, were identified. Both were tandemly clustered on the nuclear genome approximately 2 kb apart from each other (Fujiwara *et al.* 1990, Fukuzawa *et al.* 1990). CA which appears in low CO_2 conditions (CA1) is derived from *CAH1*. CA purified from cells grown in 5% CO_2 condition (high-CO_2 cells) (CA2) was encoded by *CAH2*, and was not the remnants or slightly expressed CA1 (Tachiki *et al.* 1992). Most of the attention has been paid to CA1 so far, not only because CA activity in the cells grown in ordinary air (low-CO_2 cells) is much higher than that in high-CO_2 cells, but also because CA1 enhances the supply of CO_2 to photosynthetic organ from the suspending medium under low CO_2 conditions. No physiological role of CA2 has been postulated yet.

We report the effect of acetate on the gene expression of *CAH1* and *CAH2* in this paper.

Materials and Methods

Cells and culture condition

Cells of *Chlamydomonas reinhardtii* Dangeard C-9 mt$^-$ (IAM Culture Collection, Institute of Molecular and Cellular Biosciences, University of Tokyo) were cultured photoautotrophically at 30°C in 3/10 HSM medium (Sueoka *et al.* 1967) by aeration with 5% CO_2-enriched air. Mixotrophic cells were prepared in the same medium with addition of acetate (17 mM) every 12 hr in 5% CO_2. The light intensity for the culture was 18 W·m^{-2}.

In the induction experiments of CA, high-CO_2 cells were resuspended in fresh culture medium at the density of 1±0.2 ml packed cell volume per liter and CO_2 concentration in the bubbling gas was reduced from 5 to 0.04% (ordinary air).

RNA blot hybridization

Total RNA was isolated from the cells of *C. reinhardtii* by using guanidium-isothiocyanate and CsCl ultracentrifugation method (Maniatis *et al.* 1982). Ten μg each of total RNA were electrophoresed in formaldehyde containing 1% agarose gel (Maniatis *et al.* 1982) and capillary blotted to nylon membrane (Zeta-probe, BioRad). The membrane was then probed by ^{32}P-terminal labeled gene specific oligonucleotide probes, PrCAH1 and PrCAH2. The nucleotide sequences of PrCAH1 and PrCAH2 are 5'-GCC GTG CCG ACG GTG GTA GCG TGA CTA ACT ACT GGG AAG T-3' and 5'-CAG TGC TCA CAT AGT AGT TTC GAA TTC TGC CAA TCC TGT C-3', respectively. RNA ladder (BRL) was used as size markers.

Determination of photosynthesis and respiration

Photosynthetic oxygen evolution was determined with a Clark type oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) which was illuminated from one side at 55 W·m^{-2} by a projector lamp at 30°C. The rate of *in situ* photosynthesis was measured with the cells immediately after transfer from the culture, and the capacity of photosynthesis was determined in the presence of 5 or 10 mM $NaHCO_3$. The rate of respiration was measured with the same oxygen electrode in the dark.

Determination of pcv and CA activity

Packed cell volume (pcv) was determined by centrifugation at 3,500×g for 10 min at 4°C. Extracellular CA activity was measured by the time needed for a pH change from 8.3 to 7.3 after the addition of 2 ml of CO_2 saturated water to 12 mM Veronal-H_2SO_4 buffer (pH 8.3) containing the algal cells in a total volume of 5 ml at 2°C (Yang *et al.* 1985). Enzyme activity unit was calculated according to Unit=T_0/T-1 where T and T_0 represent the time (second) needed for the pH change with and without samples.

Results

CA activity in cells grown photoautotrophically and mixotrophically with acetate

The extracellular CA activity in the high-CO_2 cells grown photoautotrophically increased upon exposure to low CO_2 concentration (Fig. 1). Under 5% CO_2, CA activity in mixotrophically grown cells was much smaller than in photoautotrophically grown cells (compare the values at time zero in Fig. 1), and stayed similar at very low level even under low CO_2 concentration. When acetate was supplied to the photoautotrophically grown cells simultaneously with lowering CO_2 concentration, CA activity increased for 4–6 hrs as was observed without acetate and then decreased to the same level as observed in mixotrophically grown cells (data not shown). These results suggest that acetate suppresses the induction of CA.

Accumulation of CA mRNAs in mixotrophically grown cells

Expression of two CA genes, *CAH1* and *CAH2*, was examined separately with both photoautotrophically and mixotrophically grown cells. When the photoautotrophically grown cells were transferred from 5% CO_2 to

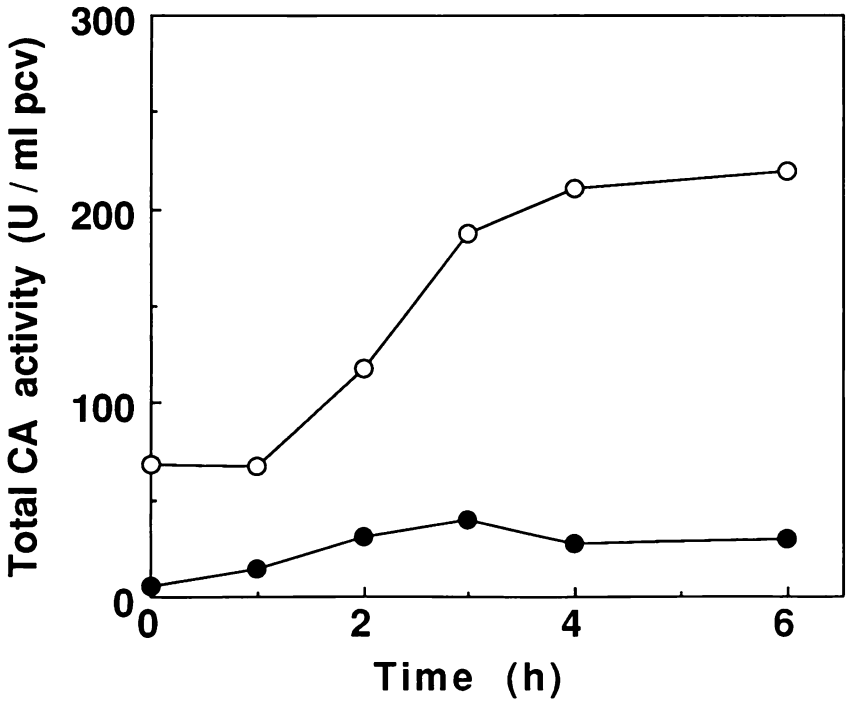

Fig. 1. Changes in the periplasmic CA activities induced by transferring the cells of *Chlamydomonas reinhardtii* grown photoautotrophically (○) and mixotrophically (●) with acetate from 5% to 0.04% CO_2 concentration at time zero. The horizontal line indicates the time (hr) after the transfer from 5% to 0.04% CO_2.

ordinary air, the transcript of *CAH1* was accumulated 1 hr after the transfer and the level reached the maximum after 2 hrs. *CAH2* mRNA which was expressed under 5% CO_2 disappeared 1 hr after the transfer to low CO_2 concentration (0.04%) (Fig. 2). On the other hand, when mixotrophically grown high-CO_2 cells were placed to ordinary air in the presence of acetate, *CAH1* appeared 2 hrs after the transfer to reach a maximum level after 4 hrs, and then decreased to less than a half of the maximum after 6 hrs (Fig. 2). Therefore, the appearance of *CAH1* under low CO_2 conditions was delayed in mixotrophically grown cells compared with that in photoautotrophically grown cells. The level of *CAH1* in the mixotrophically grown cells was much lower than that in the photoautotrophically grown cells (Fig. 2). *CAH2* mRNA was expressed at almost the same level before and after the CO_2 shift in the mixotrophically grown cells.

Transcript stability of the CA genes, CAH1 *and* CAH2

To evaluate the stability of mRNA transcripts from *CAH1* and *CAH2* under low CO_2 conditions, the effect of actinomycin D, a potent inhibitor of nuclear transcription, on the accumulation of *CAH1* and *CAH2* mRNA was investigated. Actinomycin D was added to the culture medium at the concentration of 20 $\mu g \cdot ml^{-1}$ 2 hrs after lowering the CO_2 concentration from 5% to air. The level of *CAH1* mRNA decreased to that less than a half 1 hr after the addition of actinomycin D and paled after 2 hrs (Fig. 3, lanes 1-4). The additional bands of 4.3 kb observed in lanes 1 and 2 corresponds to the size of the precursor mRNA of *CAH1* including introns (see Fujiwara *et al.* 1990), which faded away with time. The level of 2.0 kb *CAH2* mRNA decreased to approximately 0.5 hr after addition of actinomycin D and was not detected after 2 hrs (Fig. 3, lanes 5-8).

These results indicate that half-lives of both

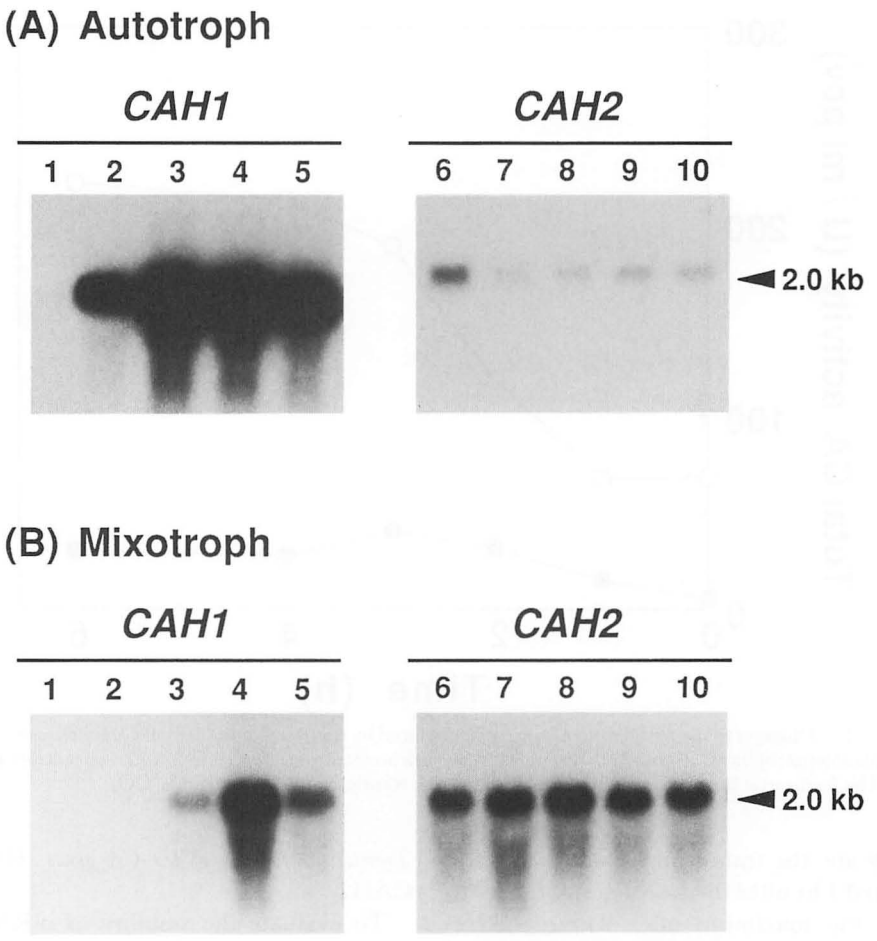

Fig. 2. Accumulation of CA mRNA induced when high-CO_2 cells of *Chlamydomonas reinhardtii* grown photoautotrophically (A) and mixotrophically with acetate (B) were transferred to ordinary air. Follwoing electrophoresis of 10 μg total RNA in each lane in denaturing agarose gel, northern blot analysis was carried out using gene-specific oligonucleotide probes, PrCAH1 (lanes 1 trhough 5) and PrCAH2 (lanes 6 through 10). The cells were transferred to air time zero (lanes 1 and 6) and kept under low CO_2 conditions (air) for 1 (lanes 2 and 7), 2 (lanes 3 and 8), 4 (lanes 4 and 9), and 6 hrs (lanes 5 and 10).

CAH1 and *CAH2* mRNAs are about 30 min. Therefore, the greater part of each blot shown in Fig. 2 consists of the trascribed product during the period after the prior sampling, but not of the remnant of mRNA shown in the prior sampling. We can also conclude that *CAH1* is transcribed continually under low CO_2 conditions, and that the rate of *CAH1* transcription is maximum at 1-2 hr after the transfer from high to low CO_2 concentration (Fig. 2).

Changes in capacity and rate of in situ *photosynthesis after lowering CO_2 concentration*

To understand the physiological conditions of the algal cells during CA induction, changes in photosynthetic activity in the cells kept in the growth medium (*in situ* photosynthesis) and the activity in the presence of saturated concentration of $NaHCO_3$ (capacity of photosynthesis) were determined. The capacity of photosynthesis in photoautotrophic cells was almost constant at 220-250 μmol $O_2 \cdot (\text{mg chl})^{-1} \cdot h^{-1}$ even when the CO_2 concentration was lowered from 5 to 0.04% (Fig. 4A). The rate of *in situ* photosynthesis, however, immediately decreased to less than a half and then recovered after a few hours. The pho-

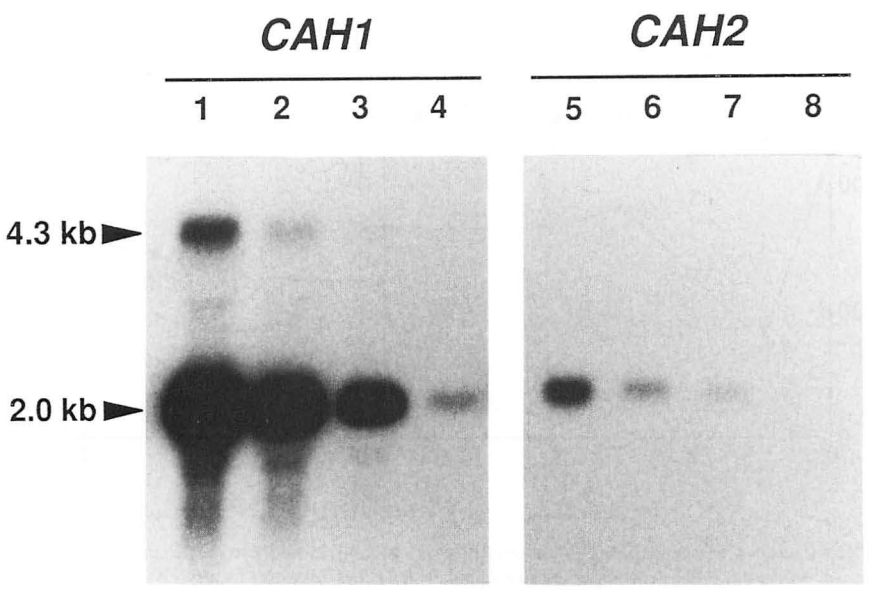

Fig. 3. The effect of actinomycin D on *in vivo* CA mRNA accumulation in *Chlamydomonas reinhardtii*. Northern blot analysis of total RNA from *Chlamydomonas* cells was carried out by using gene-apecific oligonucleotide probes. In each lane 10 μg of total RNA was electrophoresed in denaturing agarose gel, blotted to nylon membranes and probes PrCAH1 (lanes 1 through 4) and PrCAH2 (lanes 5 through 8). Photoautotrophically grown cells in 5% CO_2 were kept in ordinary air for 2 hrs (lanes 1 and 5), then 20 μg of actinomycin D per ml was added. The cells were further kept under the same low CO_2 condition for 0.5 (lanes 2 and 6), 1 (lanes 3 and 7) and 2 hrs (lanes 4 and 8). Radioactive spots of *CAH1* and *CAH2* transcripts were detected by autoradiography for 1 day and 1 week, respectively.

tosynthetic capacity in mixotrophic cells was about a half that of photoautotrophic cells (Fig. 4B). The rate of *in situ* photosynthesis was also lower than the photoautotrophic cells. The photosynthetic capacity and the rate of *in situ* photosynthesis in mixotrophically grown cells did not shown significant change for 4 hrs after the CO_2 shift. The growth rate of the cells was enhanced by the addition of acetate (data not shown). Therefore, these results indicate that the algal cells did not depend so much on photosynthesis under mixotrophic conditions as under photoautotrophic conditions.

Changes in the rate of dark respiration

Rates of dark respiration in photoautotrophically and mixotrophically grown cells was both about 60 μmol $O_2 \cdot (\text{mg chl})^{-1} \cdot h^{-1}$ under 5% CO_2 (Fig. 5). The rate in photoautotrophically grown cells decreased to 20 μmol $O_2 \cdot (\text{mg chl})^{-1} \cdot h^{-1}$ 2 hrs after lowering CO_2 concentration, whereas it was not affected in mixotrophically grown cells.

Discussion

It was shown in this paper that the trascription of *CAH1* in low CO_2 condition was suppressed in mixotrophically grown cells with acetate (Fig. 2). Coleman *et al.* (1991) reported that the accumulation of CA mRNA in low CO_2 condition was reduced by acetate. They used 2.5 kb genomic CA clone which could hybridize mRNAs of both CA genes. Since the maximum level of *CAH1* mRNA was much higher than that of *CAH2* mRNA, the above result is consistent with that of Coleman *et al.* We, further, found that the transcription of *CAH2* was enhanced in the mixotrophic conditions (Fig. 2).

Gene expression of *CAH1* and *CAH2* is strongly regulated by CO_2 concentration (Fujiwara *et al.* 1990). Therefore, one might assume that the regulation by acetate is caused by the increase in intracellular CO_2

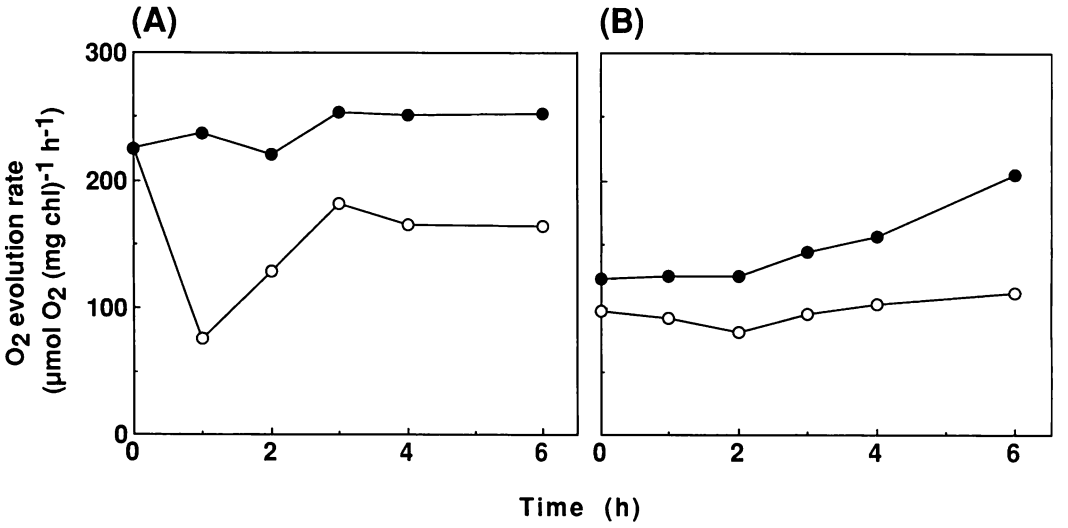

Fig. 4. Changes in the capacity of photosynthesis (●) and in the rate of *in situ* photosynthesis (○) in *Chlamydomonas reinhardtii* cells grown photoautotrophically (A) and mixotrophically (B) which were induced by lowering CO_2 concentration from 5 to 0.04%. For mixotrophically grown cells acetate was freshly added at the CO_2 shift. The horizontal line indicates the time (hr) after the transfer from 5% to 0.04% CO_2.

concentration, since rate of dark respiration is elevated and that of photosynthesis is lowered by adding acetate under low CO_2 conditions. There are, however, some antagonistic results: (1) The level of transcript of *CAH2* under the saturated CO_2 concentration (5% CO_2) was increased by acetate (compare lane 6 of (A) and (B) in Fig. 2). (2) *CAH1* mRNA was fairly accumulated in autotrophically grown cells 1 hr after the trasfer of CO_2 concentration from 5% to ordinary air, while the rate of dark respiration in these cells remained as high as that in mixotrophic cells. These results indicate that CO_2 respired in the dark would not be the major factor for *CAH1* transcription. (3) The experiments of CA induction have been carried out under the continuous light where CO_2 is incessantly fixed by photosynthesis. The genes responsible for photosynthesis such as *rbc* S (gene encoded for small subunit of ribulose 1,5-bisphosphate carboxylase) (Goldschmidt-Clermont and Rahire 1986, Steinbis and Zetsche 1986) and *cab* II (gene encoded for light harvesting chlorophyll-binding protein) (Kindle 1987) were also suppressed by acetate.

Both under photoautotrophic and mixotrophic conditions, the capacity of photosynthesis was not affected by the transfer from ordinary air to high-CO_2 conditions (Fig. 4). The rate of *in situ* photosynthesis, however, decreased drastically and transcription of *CAH1* was induced under this condition in photoautotrophically grown cells. Since photosynthesis is suppressed by CO_2 shortage during this period, following states can be considered as the factors which induce *CAH1* transcription: (1) a large amount of excited photosynthetic pigments; (2) a high redox state of the reaction centers of PS I and II and of components of electron transport; (3) much amount of oxygen radicals; and (4) greater carbon flow in photorespiratory pathway. In mixotrophically grown cells the capacity of photosynthesis was much smaller than in photoautotrophically grown cells, and the rate of *in situ* photosynthesis was not reduced even when CO_2 concentration was lowered (Fig. 4). CO_2 would not be a limitation factor in mixotrophically grown cells even under low CO_2 conditions.

Since *CAH2* mRNA is accumulated under the conditions which suppressed transcription of *CAH1* in the mixotrophically grown cells, CA activity is always present under any condi-

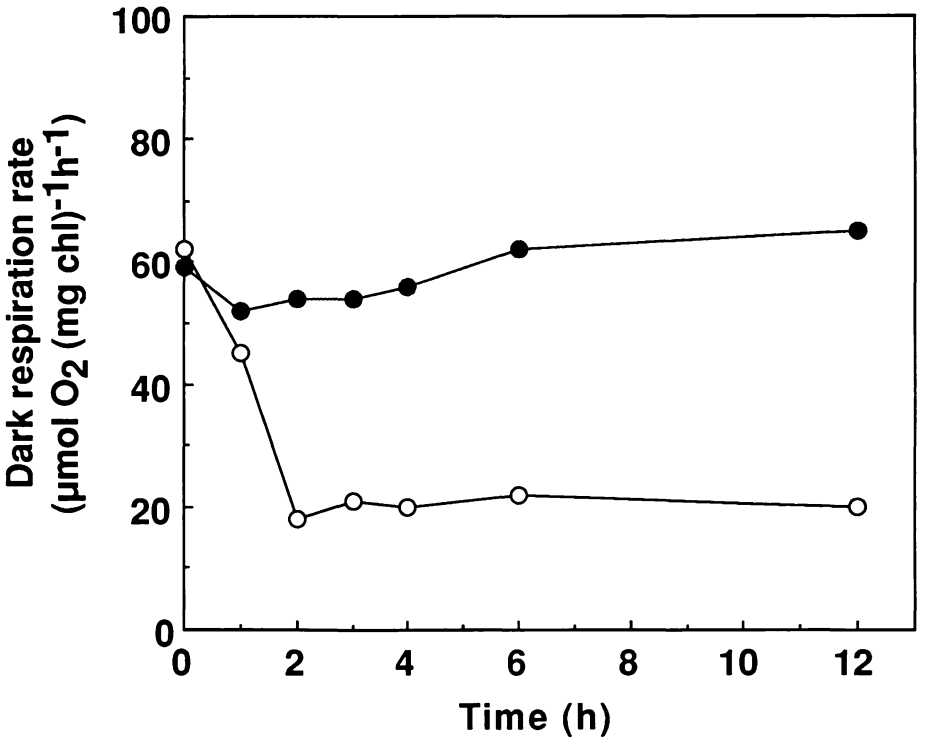

Fig. 5. Changes in the rates of dark respiration in cells of *Chlamydomonas reinhardtii* grown photoautotrophically (○) and mixotrophically (●) which were induced by lowering CO_2 concentration from 5 to 0.04%. The horizontal line indicates the time (hr) after the transfer from 5% to 0.04% CO_2.

tion, though its activity changes. So far CA has been considered as playing a role which facilitates CO_2 supply in photosynthesis (Tsuzuki and Miyachi 1990), and is known to have esterase activity (Bundy and Cote 1980). Gene products of *CAH2* should play some other role than photosynthesis, but it remains as the subject for future investigation.

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立木　光*・Maribel L. Dionisio-Sese**・藤原祥子***・都筑幹夫****・福澤秀哉*****・宮地重遠******：クラミドモナス細胞表層カーボニックアンヒドラーゼの遺伝子発現に及ぼす酢酸の影響

単細胞緑藻クラミドモナスの細胞表層カーボニックアンヒドラーゼは，*CAH1* と *CAH2* の2つの遺伝子にコードされている。光独立栄養細胞では，*CAH1* の転写産物は CO_2 濃度を5％から0.04％に移すと1時間後に発現した。*CAH2* の転写産物は5％ CO_2 条件で存在し，0.04％ CO_2 下ではわずかになった。酢酸を有機源とする光従属栄養細胞では，*CAH1* の転写産物は低 CO_2 条件でも非常に減少し，*CAH2* の転写産物は高低両 CO_2 条件共に存在した。*CAH1* と *CAH2* の転写に及ぼす酢酸の影響について光合成と CO_2 濃度の点から議論する。（*211 川崎市井田1618　新日鐵㈱先端技術研究所，**424 清水市袖師町　海洋バイオテクノロジー研，***305 つくば市東　生命工学工業技術研，****113 文京区弥生　東大分生研，*****606-01 京都市左京区　京大農学部，******113 文京区本郷　海洋バイオテクノロジー研）

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