Regulation of proline content of *Chlorella autotrophica* in response to changes in salinity

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In *Chlorella autotrophica*, proline, chlorophyll, and protein synthesis took place almost exclusively in the light. No evidence for proline degradation in the dark was obtained. An osmotic upshock from 50 to 150% artificial seawater caused a temporary inhibition of photosynthesis in this alga. An osmotic downshock from 150 to 50% artificial seawater had no effect on its photosynthetic rate. Synthesis of proline, the main osmoregulatory solute in *C. autotrophica*, showed no lag phase following an upshock and was not inhibited in the presence of cycloheximide. These results suggest that the enzymes of the proline pathway are not synthesized de novo in response to the upshock. In the dark, the rate of proline synthesis was lower, whereas the presence of acetate allowed a rate of proline synthesis similar to that of cells in the light. Synthesis of proline in the dark in the presence of acetate was dependent on the induction of isocitrate lyase. Thus the glyoxylate cycle plays a key role in furnishing carbon and reducing power for proline synthesis in the dark. Following a sudden downshock, more than 55% of the proline content leaked out of the cell in the first 5 min due to transient breakdown in membrane permeability. However, if the downshock is gradual, proline is oxidized by the cells rather than being leaking out.

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Chez *Chlorella autotrophica*, la synthèse de la proline, de la chlorophylle et des protéines a lieu presqu'exclusivement à la lumière. Aucune preuve de la dégradation de la proline à l'obscurité n'a été obtenue. Un choc osmotique ascendant par de l'eau de mer artificielle variant de 50 à 150% a causé une inhibition temporaire de la photosynthèse chez cette algue. Un choc osmotique par de l'eau de mer artificielle descendant de 150 à 50% n'a eu aucun effet sur son taux de photosynthèse. La synthèse de la proline, principal soluté osmorégulateur chez *C. autotrophica* n'a pas révélé de phase de latence suivant le choc ascendant et n'a pas été inhibée en présence de cycloheximide. Ces résultats suggèrent que les enzymes de la voie de la proline ne sont pas synthétisées *de novo* en réponse au choc ascendant. À l'obscurité, le taux de synthèse de la proline était plus bas, alors que la préscence d'acétate permettait un taux de synthèse de proline similaire à celui des cellules à la lumière. La synthèse de proline à l'obscurité en présence d'acétate était dépendante de l'induction de l'isocitrate lyase. Ainsi, le cycle glyoxylique joue un rôle-clé dans l'apport de carbone et de pouvoir réducteur pour la synthèse de proline à l'obscurité. Suite à un choc descendant soudain, plus de 55% du contenu en proline s'échappait de la cellule durant les 5 premières minutes dû à une perturbation transitoire de la perméabilité membranaire. Cependant, si le choc descendant est graduel, la proline est oxydée par les cellules au lieu d'en sortir.

[Traduit par la revue]

Introduction

Many unicellular algae occur in habitats where they are exposed to a wide range of salinities (12, 27). In response to increased salt concentration, algae often synthesize osmoregulatory solutes to counterbalance the low water potential of the environment. It has been shown that, at low salinities, *Chlorella autotrophica*, a euryhaline microalga, osmoregulates mainly by accumulation of inorganic ions (1). However, at high salinities, proline plays a key role in the osmoregulatory mechanisms of this alga (1). It has also been recognized that accumulation of proline plays an important role in response to osmotic stress in bacteria, algae, and higher plants (5). However, the influence of environmental factors on proline synthesis is still a matter of controversy. Greenway and Setter (8) concluded from their studies on osmoregulation in *Chlorella emersonii* that proline accumulation in this alga was due to de novo synthesis of enzymes involved in proline formation. This was indicated by inhibition of net proline synthesis in the presence of cycloheximide. In contrast, in tobacco leaves facing water stress, proline synthesis was not inhibited by cycloheximide (3). Also, the accumulation of proline is promoted by light in both higher plants (10, 15) and algae (8, 16, 20). It is believed that this dependence upon light is due to the need for a continuous supply of reducing power and ATP (10, 15). The requirement for light is not absolute, as proline synthesis can take place in the dark in the presence of an external source of glucose (16), sucrose, or glutamate (26). In *C. emersonii*, proline synthesis in the dark was possible as long as an external source of glutamate was supplied, but the interpretation of these results was somewhat complicated by the presence of sucrose accumulating in parallel to proline (8). In contrast, proline is the only significant organic solute accumulating in *C. autotrophica* (1). This alga is thus an ideal organism to study the effects of light and dark conditions on proline synthesis. This paper also reports on the effects of upshocks and downshocks on photosynthesis in *C. autotrophica*.

Materials and methods

Organism and growth conditions

Chlorella autotrophica Shihira and Krauss (clone 580 obtained from Dr. R. R. L. Guillard, Woods Hole Oceanographic Inst., Woods Hole, MA. Culture Collection) was grown axenically in an artificial seawater medium (ASW) prepared according to McLachlan (17) and enriched with nutrients and vitamins (9). For example, a 100% ASW solution contains 400 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 20 mM $MgCl_2$, 20 mM $MgSO_4$, and nutrients and vitamins for a total salt concentration of 460 mM or 890 mosM (1). The nitrogen

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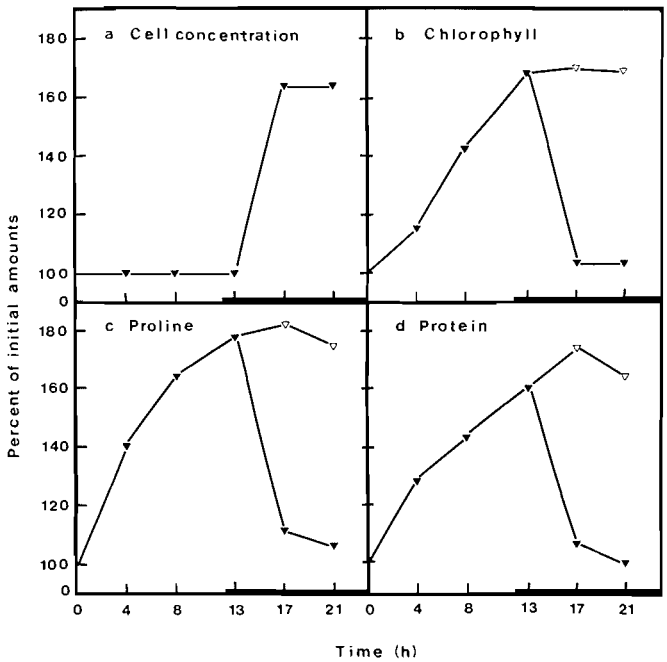

FIG. 1. Changes in (*a*) cell concentration, (*b*) chlorophyll content, (*c*) proline content, (*d*) protein content during the cell cycle of *Chlorella autotrophica* grown on a 12 h light : 12 h dark cycle in 50% ASW at 18°C and 24 W · m^{-2} cool white fluorescent light. Chemical components are expressed as percent of initial amounts on a per cell basis (▼) or per millilitre basis (▽). Levels at the beginning of the light period (100%) were as follows: (*a*) 2.1 × 10^6 cells/mL, (*b*) 0.42 pg/cell, (*c*) 2.2 fmol/cell, and (*d*) 2.9 pg/cell.

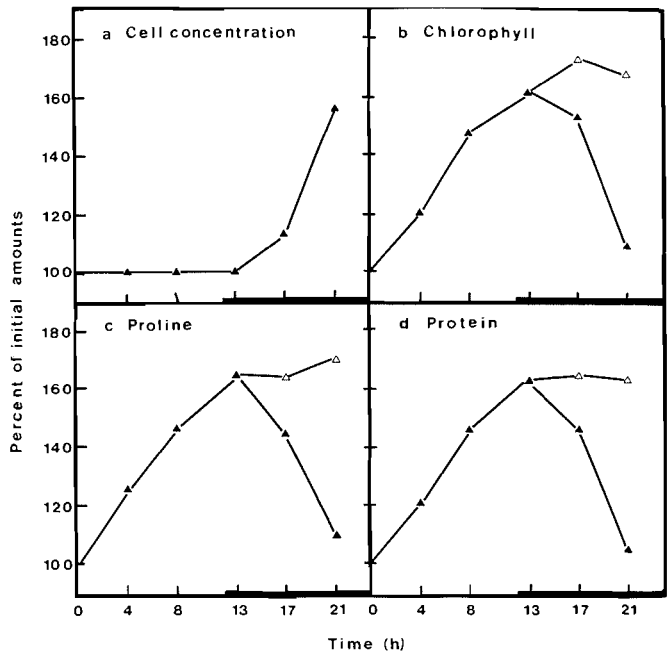

FIG. 2. Changes in (*a*) cell concentration, (*b*) chlorophyll content, (*c*) proline content, and (*d*) protein content in cells grown at 150% ASW on a 12 h light : 12 h dark cycle at 18°C and 24 W · m^{-2} cool white fluorescent light. Chemical components are expressed as percent of initial amounts on a per cell basis (▲) or a per millilitre basis (△). Levels at the beginning of the light period (100%) were as follows: (*a*) 1.62 × 10^6 cells/mL, (*b*) 0.32 pg/cell, (*c*) 9.9 fmol/cell, and (*d*) 2.8 pg/cell.

source was 2 mM NH_4Cl. Cell counting was done with a hemacytometer, and the experiments were carried out in late exponential phase of the culture. Cells were grown at 18°C on a 12 h light : 12 h dark cycle (24 W · m^{-2} from "Daylight" fluorescent tubes).

Measurements of photosynthetic rates

Rates of photosynthesis were estimated by the uptake of ^{14}C-bicarbonate. Aliquots of 4 mL were incubated with 2 mM NaH $^{14}CO_3$ (1 μCi · mL^{-1}; 1 Ci = 37 GBq) for 10 min at 24 W · m^{-2} cool white fluorescent light, and the assimilation of bicarbonate was determined as described previously (11).

Measurements of chlorophyll, proline, and protein contents

At the beginning of the light cycle, 30 mL of cells from the stock cultures was inoculated into 1.5 L of fresh medium. When the cells had reached the right density, 170 mL was harvested. For estimation of proline and protein contents, 150 mL of culture was centrifuged at 7000 × *g* for 5 min at 10°C and resuspended twice in 3 mL of methanol – chloroform – water (12:5:3, v/v). After 20 min, the extract was centrifuged and the supernatant poured into a test tube. To this supernatant was added 1.5 mL of chloroform and 1.5 mL of water. After centrifugation, the bottom layer of chloroform was discarded and the top layer was evaporated under air flow to a volume of 0.5 mL. This portion was used for proline determination as described by Ahmad and Hellebust (1). The pellet was resuspended for 24 h in 1 M NaOH for protein determination using the Bio-Rad Protein Assay Dye Reagent. For chlorophyll estimation, 10 mL of culture was centrifuged at 2000 × *g* for 10 min at room temperature and resuspended in 3 mL of 90% methanol. Chlorophyll was determined spectrophotometrically, as described by Holden (14).

Enzyme assay

Isocitrate lyase activity was determined as described by Goulding and Merret (7). To 1 mL of 50 mM potassium phosphate buffer, pH 7.6, containing 5 mM magnesium sulfate, 10 mM isocitrate, and 2 mM reduced glutathione, 100 μL of toluene-permeabilized cells was added. Cells were permeabilized by the addition of 10 μL of toluene to 1 mL of cell suspension containing about 1 × 10^8 cells. After 1 h incubation at room temperature, a 300-μL aliquot was transferred into a mixture of 100 μL 15% trichloroacetic acid in water and 200 μL of 0.1% 2,4-dinitrophenyl hydrazine in 2 M HCl. The reaction system was shaken frequently and, after 15 min, 0.9 mL of 2.5 M NaOH was added. The proteins were removed by centrifugation, and the absorbance of the supernatant was read at 445 nm. With all determinations, a blank containing no substrate was included.

Results

Chlorophyll, proline, and protein concentrations in C. autotrophica *at 50 and 150% ASW*

To determine the importance of the changes in proline concentrations following a transfer from 50 to 150% ASW, we investigated the changes in concentrations of proline, chlorophyll, and protein during the cell cycle at 50 and 150% ASW. As can be seen in Figs. 1 and 2, the changes in content of chlorophyll, proline, and protein during the cell cycle at either 50 or 150% ASW followed the same pattern. In each case, net synthesis took place during the light period when the cells accumulated chemical constituents. At 50% ASW, cell division took place at the beginning of the dark period (Fig. 1*a*), whereas at 150% ASW it took place throughout the dark period (Fig. 2*a*). At 50% ASW, the chlorophyll content increased during the light period from 0.42 to 0.70 pg/cell, proline from 2.2 to 3.9 fmol/cell, and protein from 2.9 to 4.6 pg/cell (Figs. 1*b*–1*d*). In comparison, at 150% ASW, the chlorophyll content increased from 0.32 to 0.51 pg/cell, proline from 9.91 to

TABLE 1. Effect of an osmotic upshock from 50 to 150% ASW on the rate of photoassimilation of CO_2

Time after transfer from 50 to 150% ASW (min)	Rate of CO_2 photoassimilation (% of control)*
0**	45±1
30	46±9
60	66±8
120	94±7

NOTE: Results expressed as mean values ± SD ($n = 3$).
*The control rate (100%) equals 3.6 ± 0.4 fmol CO_2/(cell·min) (196 μmol CO_2/(mg chl·min).
**The initial aliquot was taken within 20 s of the transfer.

16.2 fmol/cell, and protein from 2.8 to 4.5 pg/cell (Figs. 2*b*–2*d*). At either salinity, little or no accumulation of chlorophyll, proline, or protein occurred in the dark. Knowing that the water content of *C. autotrophica* grown at 50 and 150% ASW is, respectively, about 13.6 and 15.0 fL/cell (1), we calculated that, at the beginning of the light cycle, the proline concentration of *C. autotrophica* grown at 50% ASW was 160 mM and at 150% ASW was about 660 mM. It has been shown that the remainder of the cellular osmotic balance was achieved mainly by accumulation of ions (1).

Rates of photosynthesis following an upshock or a downshock

To determine the rate of uptake of ^{14}C-bicarbonate over a period of hours, it was necessary to distinguish between the effect of the upshock on photosynthesis and the limitation imposed on the uptake by a gradual decrease in bicarbonate concentration in the medium. From preliminary experiments, we concluded that, at 50% ASW, bicarbonate was not limiting at concentrations above 1 mM. The following experiments were, therefore, done with cell densities sufficiently low so that depletion of bicarbonate concentration below 1 mM did not occur for the duration of the experiments. Table 1 shows the effect of an upshock from 50 to 150% ASW on the photoassimilation of bicarbonate over a period of 2 h. It can be seen that the alga quickly recovered from the stress. From an initial inhibition of photosynthesis of 55%, the rate of photosynthesis went up to 66% of the control after 1 h, and it was about equal to the photosynthetic rate of the control after 2h. During the 2-h period, the bicarbonate concentration in the medium remained above 1.5 mM. When the transfer was done from 50 to 175% ASW, the alga photosynthesized at only 18% of its initial rate, and its rate went up to 33% after 2 h. Furthermore, after a transfer from 50 to 200% ASW, the cells photosynthesized at only 0.4% of the control, and there was little recovery of photosynthesis over the subsequent 2 h (data not shown). The alga conserved full photosynthetic activity after a transfer from 150 to 50% ASW. The rate of photosynthesis over a 2-h period following the downshock was never significantly different from the control (data not shown).

Proline accumulation in the light

Cells grown in 50% ASW containing 2 mM ammonium chloride were transferred to a 150% ASW medium, and the synthesis of proline was followed for 24 h. To minimize fluctuations in initial proline content due to continuous synthesis of proline in the light as seen in steady state conditions, cells were harvested 1–2 h after the beginning of the light period. Immediately after the transfer, proline synthesis commenced and no lag phase was apparent (Fig. 3; treatment 1; Table 2). There was a steady rate of proline accumulation during the first 12 h of the upshock. In that period of time, proline increased from 2.2 to 14.2 fmol/cell at a rate of 16.7 amol/(cell·min) (Fig. 3; treatment 1). During the first 4 h, the rate of proline accumulation was 22.9 amol/(cell·min). After 24 h, the alga had reached equilibrium with the new ASW concentration and contained 11.2 fmol proline/cell. The 21% decrease in proline content per cell, compared with the value obtained at 12 h, was due to cell division. The amount of proline accumulated after 7 h in cells transferred to 150% ASW containing no exogenous source of nitrogen was not significantly different from the amount accumulated in the control containing ammonium (Fig. 3; treatments 1 and 2). However, after 12 h, the proline content of these cells was 71% of the control (Fig. 3; treatment 2). Upon the addition of ammonium, 7 h after the upshock, there was a significant increase in the rate of proline synthesis (treatment 3), so that after 12 h the proline content of these cells was not significantly different from that of the control in the presence of ammonium. When photosynthesis was inhibited by the presence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), the alga accumulated very little proline after an upshock in the light (Fig. 3; treatment 4), and the rate of synthesis observed was not significantly different from that observed after an upshock in the dark (Fig. 3; treatment 6). From the absence of a lag phase in proline formation after

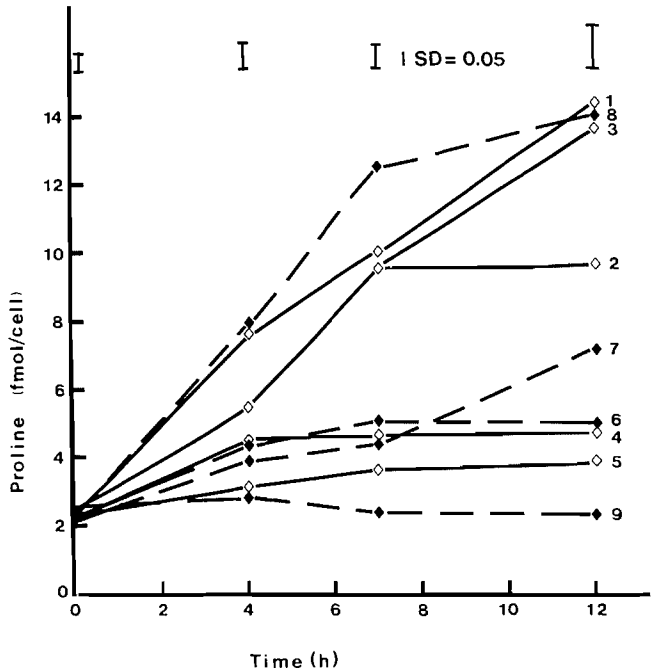

FIG. 3. Changes in proline content of cells in the light (◇) or in the dark (◆) under the following conditions: (1) upshock form 50 to 150% ASW in the presence of 2 mM ammonium chloride, (2) upshock in the absence of ammonium, (3) ammonium chloride added 7 h after the upshock, (4) upshock in the presence of 50 μM DCMU, (5) control cell suspension incubated in the light at 50% ASW, (6) upshock in the dark, (7) upshock in the dark in the presence of 0.2% sodium acetate with a cell suspension not induced for isocitrate lyase, (8) upshock in the dark in the presence of 0.2% sodium acetate with a cell suspension induced for isocitrate lyase, and (9) control cell suspension incubated in the dark at 50% ASW. Data points are means of three replicates.

TABLE 2. Effect of cycloheximide (50 $\mu g \cdot mL^{-1}$) on proline synthesis following an osmotic upshock from 50 to 150% ASW in the light

Time after transfer from 50 to 150% ASW (min)	Proline content	
	Control (fmol/cell)	Cycloheximide* (fmol/cell)
0	2.2±0.4	2.2±0.4
60	3.7±0.3	3.7±0.1
240	6.9±0.7	6.7±0.8

NOTE: Results expressed as mean values ± SD (n = 3).
*Cycloheximide was added 1 h before the upshock.

TABLE 3. Time course for induction of isocitrate lyase in cells incubated in the dark at 50% ASW in the presence of 0.2% sodium acetate

Time after addition of acetate (h)	Isocitrate lyase activity*	
	Control (amol/(cell · min))	+ Acetate (amol/(cell · min))
0	23±5	18±6
12	25±10	138±52
24	20±3	191±6
30	23±3	300±48
36	22±6	254±52

NOTE: Results expressed as mean values ± SD (n = 3).
*Isocitrate lyase activity measured as the appearance of glyoxylate from cleavage of isocitrate.

transfer to high ASW concentration, we assumed that de novo synthesis of enzymes was not required for the observed proline accumulation following an upshock. An upshock done in the presence of cycloheximide confirmed that hypothesis. Over a period of 4 h, there was never a significant difference between the proline content of control cells and cells incubated in the presence of 50 μg cycloheximide/mL of cell suspension 1 h before and during the upshock (Table 2). Preliminary experiments showed that, at this cycloheximide concentration, the induction of isocitrate lyase was completely inhibited in *C. autotrophica* (data not shown), indicating that cycloheximide was taken up by the cells.

Proline accumulation in the dark

When the transfer was done in the dark, even though very little proline accumulation took place (Fig. 3; treatment 6), after 12 h, the proline content reached 4.8 fmol/cell and was significantly different from that of cells kept in the dark without an upshock (Fig. 3; treatment 9). There was no further accumulation of proline between 12 and 24 h after the upshock. The addition of 10 mM glucose had no effect (data not shown). In the presence of 15 mM acetate, there was a significant increase in proline content after 12 h when it reached 7.1 fmol/cell (Fig. 3; treatment 7). It has been shown that the heterotrophic growth of *Chlorella vulgaris* on acetate is dependent upon the induction of the enzymes in the glyoxylate cycle (25). Thus the synthesis of proline observed after 12 h of incubation in the presence of acetate was probably dependent on the induction of glyoxylate cycle enzymes. After incubation of *C. autotrophica* in the dark in the presence of acetate, it was observed that the maximum activity of isocitrate lyase was

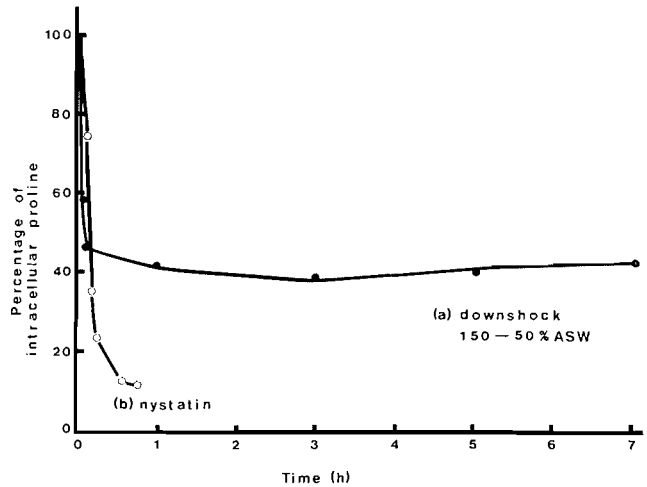

FIG. 4. Effect on cellular proline content in *Chlorella autotrophica* of (*a*) an osmotic downshock from 150 to 50% ASW and (*b*) the presence of nystatin (1 mg · mL^{-1} of cell suspension) on cells kept at 150% ASW. Proline content is expressed as the percentage of proline remaining in the cell after the treatment.

reached after 30 h (Table 3). Accordingly, when cells preincubated for 30 h in the dark in the presence of acetate were transfered to a medium of higher ASW concentration, proline accumulation occurred in the dark. (Fig. 3; treatment 8). The proline content of these cells was equal to that of cells subjected to an upshock in the absence of acetate in the light (Fig. 3; treatment 1).

Proline depletion after a downshock

When cells were transferred from 150 to 50% ASW, a rapid decrease in intracellular proline occurred during the 1st h (Fig. 4). By measuring the proline content inside the cell and in the medium 10 s after the downshock, we observed 42% of the intracellular proline leaking out. During the next 5 min, an additional 12% of intracellular proline leaked out; over the ensuing 7 h, the amount of endogenous proline remained at around 43% of the initial proline content. After 24 h, the alga contained 2.9 fmol/cell. The leakage could to some extent be mimicked by the presence of nystatin in the medium. Nystatin, by the formation of channels through the membrane, permitted the leakage of proline as shown in Fig. 4. After 30 min, more than 80% of the proline content of the cell was lost to the medium. However, leakage of proline did not occur when the downshock consisted of 20% ASW decrements over a 10-h period. In this case, no proline was detected in the medium, and the rate of decrease in cellular proline content was equal to 8.3 amol/(cell · min) (Fig. 5).

Discussion

Our results show that, in *C. autotrophica*, an active synthesis of chlorophyll, proline, and protein occurs during the light period and that the levels of these constituents are fairly constant during the dark period (Figs. 1, 2). In contrast to what was found in *C. emersonii* (22), there was little net decrease of proline content in the dark. These contradictory results can be explained by the fact that, in *C. autotrophica*, proline is the main osmoregulatory solute synthesized; in *C. emersonii*, the osmotic balance is maintained by proline and sucrose. In *C. emersonii*, the degradation of proline in the dark is balanced

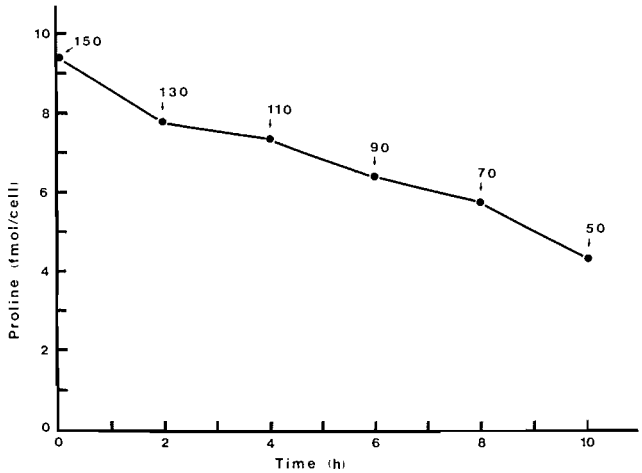

FIG. 5. Effect of a gradual series of osmotic downshocks from 150 to 50% ASW on cellular proline content. The salinity of the cell suspension was decreased in successive 2-h steps of 20% ASW, as indicated. The mean slope of decrease in proline content over the 10-h experimental period was 8.3 amol/(cell · min).

by the synthesis of sucrose, so throughout the cell cycle the sum of the concentrations of proline plus sucrose remained constant (22). Because at high salinities more than 90% of the organic carbon in the soluble fraction of the cell is confined to proline in *C. autotrophica* (1), any loss of proline due to oxidation in the dark will be detrimental to the osmotic balance of the cell. This also indicates that the regulation of proline oxidation in *C. autotrophica* differs from that observed in *C. emersonii*.

Chlorella autotrophica tolerates rapid changes in salinity in terms of its photosynthetic capacity. Photosynthesis is not substantially impaired after an upshock from 50 to 150% ASW, and part of the photosynthetic energy can be channelled for the immediate synthesis of proline. However, compared with other microalgae such as *Dunaliella tertiolecta* or *Chlamydomonas pulsatilla* that can still photosynthesize at about 40% of the control grown at 50% ASW upon transfer to 200% ASW (13), *C. autotrophica* shows a narrower range of salinity tolerance, since an upshock of the same amplitude resulted in 82% inhibition of photosynthesis. Even though *C. autotrophica* has been shown to photosynthesize at salinities as high as 400% of seawater (1), the increases in salinities have to be very gradual for the alga to tolerate them. *Chorella autotrophica* also exhibited good resistance to osmotic downshock. Its photosynthetic capacity was not impaired by a transfer from 150 to 50% ASW. This high resistance to osmotic downshock is often found in strong-walled algae such as *Nannochloris bacillaris* (4) and *Stichococcus bacillaris* (13). The strength of the cell wall is clearly important for an alga to be able to tolerate the increase in turgor pressure following a downshock.

Immediately after a transfer from 50 to 150% ASW, proline accumulation begins without lag phase and is not inhibited by cycloheximide. In contrast, in *C. emersonii* a lag phase of 15 min in proline synthesis was observed after a transfer from low to high salinity, and net proline synthesis was inhibited by about 70% in the presence of cycloheximide (8). The discrepancy could be due to the fact that *C. autotrophica* is a marine microalga that normally grows at high salinities, whereas *C. emersonii* is a freshwater alga that can only tolerate a relatively narrow range of salinity. It is costly for a freshwater alga to maintain a biosynthetic pathway for use in the relatively rare event of an increase in salinity. It would be advantageous for the alga to induce the enzymes needed for the synthesis of the principal solute for osmoregulation only in the presence of an osmotic stress. On the other hand, an alga living in marine environments, such as estuaries or tide pools, frequently faces extensive osmotic stress. In that case, the alga has to respond quickly to the osmotic fluctuations by preferential activation and inhibition of the enzymes synthesizing the osmoregulatory solutes (8). Our data are consistent with this model. In steady state growth at 50% ASW, proline accumulated in the light at a rate of 2 amol/(cell · min). However, immediately after an upshock, the rate of proline accumulation increases by a factor of 10, likely by activation of the enzymes synthesizing proline. In the absence of light during the upshock, the rate of proline accumulation is much slower, and the cells are unable to synthesize enough proline for osmoregulation. If the principal source of proline accumulating in osmotic stress is synthesis from glutamate, as it is believed to be in many higher plants and algae, then it requires ATP and NAD(P)H for the accumulation. In the light, they are furnished by photophosphorylation and photoreduction. In the dark, the reducing power and ATP requirement can be obtained from the glycolytic pathway. However, in *C. autotrophica*, soluble carbohydrates are present in relatively low concentrations (1) and even though the starch content is unknown, the starch evidently does not provide sufficient energy and carbon for rapid proline biosynthesis in darkness (Fig. 3; treatment 6).

Inhibition of synthesis of proline in the dark has been suggested as being due to a low availability of reducing power and ATP in a variety of organisms, such as in wilted, starved barley leaves (10, 24), in *Cyclotella cryptica* transferred from 33 to 80% ASW (16), in *Phaeodactylum tricornutum* exposed to 0.9 M NaCl (20), and in *C. emersonii* transferred from 1 to 125 mM NaCl (8). More recently, Joyce *et al.* (15), in an attempt to determine the nature of the response of proline synthesis to light, looked at the enhancement of proline accumulation under different conditions of irradiance in excised segments of barley leaves subjected to water stress. They concluded that continuous or at least prolonged illumination is required for proline accumulation and that below a saturation point the rate of accumulation is proportional to the level of irradiance. Increase in proline was thus not induced by a triggering mechanism, but was stimulated by the continuous presence of light. This conclusion agrees well with the need of a continuous supply of reducing power and ATP for proline synthesis. It is further corroborated in *C. autotrophica* by the similarity in the rate of proline accumulation of cells confronted with an osmotic stress in the dark (Fig. 3; treatment 6) or in the presence of DCMU in the light (Fig. 3; treatment 4). In both cases, the low rate of proline synthesis observed points to a need for reducing power derived from photosynthesis. These results are similar to data for *C. emersonii* transferred from 1 to 125 mM NaCl in the dark or in the presence of DCMU in the light (8). In both treatments, proline formation was almost completely inhibited. If the upshock of *C. autotrophica* took place in absence of nitrogen in the medium, even though the rate of proline synthesis was slower than that of the control in the first 4 h, the cell accumulated 10 fmol of proline after 12 h; this was sufficient to counterbalance the water potential of the medium, as shown by the proline content in cells maintained at

150% ASW (Fig. 2*c*). However, the cells have to rely on nitrogen provided by protein degradation for proline synthesis. This will obviously be damaging to the cells on a long-term basis, and it would explain why *C. autotrophica* is unable to grow at high salinities in a medium of low ammonium concentrations (2). The dependence of the rate of proline synthesis on nitrogen was also shown by its increase upon the addition of ammonium 7 h after the upshock (Fig. 3; treatment 3).

The inhibition of proline synthesis in the dark is not absolute and can be released by supply of an external source of organic carbon, such as glucose, glutamate, or glutamine (8, 20). However, *C. autotrophica* is unable to grow on glucose or on amino acids in the dark, and the only compound that has been reported to permit growth in darkness is acetate (23). From this information we decided to look at the effect of a supply of sodium acetate on proline synthesis in the dark. Growth on acetate poses a unique problem because the only component of the glycolysis to which it is readily converted is acetyl-CoA under the action of aceto-coenzyme A kinase. The solution to this problem is by induction of the enzymes of the glyoxylate cycle. Among these enzymes, isocitrate lyase is largely responsible for the control of the cycle in *Chorella* (25). Under its action, isocitrate is converted to glyoxylate and succinate, and the latter can be incorporated into the tricarboxylic acids cycle for amino acids synthesis. When *C. autotrophica* grown on acetate is fully induced for isocitrate lyase, it accumulates proline in the dark, showing that this alga is able to utilize acetate as a source of carbon and reducing power to synthesize proline in the dark. If isocitrate lyase is not induced before the transfer from low to high salinity, proline synthesis takes place only after about 10 h (Fig. 3; treatment 7), showing that the accumulation is dependent on a minimum activity of isocitrate lyase. Because the enzymatic activity of isocitrate lyase after 12 h of incubation of the cells in the dark was only 30% of that after 30 h, the enzyme was unable to furnish enough carbon to the Krebs cycle to permit optimum synthesis of proline. Interestingly, in *Chlamydomonas mundana*, the incorporation of carbon from acetate into glutamate and proline was shown to increase 5.9- and 3.3-fold, respectively, in cells kept in the dark compared with cells in the light (6). Citrate could thus be a source of carbon and reducing power for the synthesis of proline in the dark.

Upon a transfer from 150 to 50% ASW, more than 40% of the proline content leaks out of the cell (Fig. 4). This process is very fast and is certainly completed in less than 10 s. In the following 5 min, an additional 12% of the proline leaked out of the cell. Over the next 7-h period, the amount of endogenous proline remained fairly constant, so that most of the loss in intracellular proline was explained by the initial leakage. It is reasonable to assume that this leakage is produced by the transient formation of pores in the plasmalemma under the rapid increase in turgor pressure. In 1980, Schobert (21) reported a similar phenomenon in *Phaedactylum tricornutum* where 70% of the intracellular proline leaked out following the relaxation to the osmotic stress. However, the first measurement of proline content was taken 30 min after the upshock, precluding any conclusion about how fast the leakage was. The suggestion that the restoration of the selective permeability of the plasmalemma was due to an alteration of the lipid composition cannot apply to *C. autotrophica* because no evidence of leakage of proline was found 30 min after the downshock. When permanent channels were artificially created in the membrane under the action of nystatin, more than 80% of the proline content rapidly leaked out, indicating that in a downshock situation the pores formation is a transient phenomenon. This is further confirmed by the absence of a significant effect of a downshock on the rate of photosynthesis of *C. autotrophica*. Reed (19) showed that a transient breakdown in the selective permeability of the plasma membrane occurred during the initial phase of a hyperosmotic shock in *C. emersonii*. Thus the phenomenon of transient membrane disruption in the presence of upshocks and downshocks may be widespread among microalgae when the transfer into a medium of a different salinity is too abrupt. Presumably, the loss of proline from *C. autotrophica* will occur only when the turgor pressure reaches a certain threshold. Below that threshold, the plasma membrane can overcome the stress, and the alga can compensate for the sudden decrease in water potential of the medium by oxidizing proline. A similar situation occurs with *C. emersonii* where a transfer from 335 mM NaCl to about 140 mM resulted in 39% loss of proline from cells, whereas only 6.5% of proline was lost after a transfer from 335 to 264 mM NaCl (18). In *C. autotrophica*, no loss of proline happened following a gradual downshock, and the rate of decrease observed, 8.3 amol/(cell · min), was sufficient to bring down the amount of proline to the level of the control over a 10-h period.

In conclusion, our results have shown that *C. autotrophica* is well adapted to salinity. A sudden increase in salinity of the medium has a moderate effect on its photosynthetic rate, and the alga quickly responds to the stress by synthesizing proline. Accumulation of proline shows no lag phase and is not inhibited by cycloheximide. In the dark, synthesis of proline is dependent upon the presence of acetate. *Chlorella autotrophica* also shows a great plasticity in relation to hypo-osmotic stress. To reestablish osmotic equilibrium with the medium, the alga can either oxidize proline over a long period of time or quickly leak it out in a matter of minutes. These responses probably take place after activation of some of the enzymes implicated in the biosynthesis and degradation of proline. Studies are in progress to purify and characterize these enzymes.

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