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# The Relationship between Inorganic Nitrogen Metabolism and Proline Accumulation in Osmoregulatory Responses of Two Euryhaline Microalgae[1]

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## ABSTRACT

***Chlorella autotrophica*, a euryhaline marine alga, and *Stichococcus bacillaris*, a salt-tolerant soil alga, grow in the presence of methionine sulfoximine (MSX), an inhibitor of glutamine synthetase, by maintaining high levels of NADPH-glutamate dehydrogenase. Nitrate reductase showed no change in MSX-adapted cells. For both species, MSX-adapted cells retained their capacity to accumulate proline in response to salinity, and in *S. bacillaris* no major shift was observed in the presence of MSX toward the accumulation of sorbitol. Following transfer from 33 to 150% artificial seawater (ASW), both algae exhibited increases in organic solute levels without a lag. Within 6 h of this sudden increase in salinity, the levels of proline in *C. autotrophica* and of proline and sorbitol in *S. bacillaris* were similar to those found in steady state 150% ASW cultures. Following transfer from 33 to 150% ASW, *S. bacillaris* continued [$^{14}$C] bicarbonate photoassimilation at a normal rate and maintained active enzymes of nitrogen assimilation. The incorporation of [$^{14}$C]phenylalanine into proteins was inhibited for about 30 minutes in MSX-free cells and 90 minutes in MSX-adapted cells following transfer from 33 to 150% ASW; the recovery after these lag periods was almost complete.**

Accumulation of the imino acid proline in response to drought and salinity has been observed in many plants and microorganisms (10, 25), and it was suggested by Measures in 1975 (18) that before the evolution of homeoosmotic mechanisms in higher animals, this osmoregulatory mechanism may have been widespread in all living organisms. This emphasis on proline as a unique osmoticum prompted many workers to use proline accumulation as a quantitative expression of drought and salt resistance in crop plants. These attempts, however, have not been successful (10). Recent experiments with induced drought and salt resistance in suspension cell cultures have shown that levels of proline in cells adapted to 30% polyethylene glycol increase to a maximum of about 60 mM (15). Quantitatively, such an increase does not appear to be large enough to suggest a major osmotic contribution to cells adapted to media of 2000 milliosmoles (mOsm) (15). Granted, similarly low levels of proline have been suggested to play an osmoregulatory role in halophytes (26, 27), but proline is just one of many organic solutes accumulated in these plants. Certainly, there is not a general correlation between proline accumulation and the plant's ability to resist low external water potentials. It must be emphasized that an evaluation of the role of proline in adaptation of plants to saline and dry habitats requires the recognition that there are four distinct groups of vascular plants and green algae based on their capacity to accumulate proline and their ability to resist water deficit.

Group A: High proline accumulators with proline as the single predominant organic solute. Two of the most conspicuous members of this group are the coastal angiosperm, *Triglochin maritima* (27), accumulating about 350 mM proline at 400 mOsm (200 mM NaCl), and the marine microalga, *Chlorella autotrophica* (1), accumulating more than 1600 mM at about 3000 mOsm (300% artificial seawater).

Group B: High proline accumulators with an ability to accumulate large quantities of other organic solutes simultaneously. Examples are, sorbitol in the coastal alga, *Stichococcus bacillaris* (13), asparagine in salt-tolerant *Agrostis stolonifera* (24), and Δ-acetylornithine in the salt marsh halophyte, *Puccinellia maritima* (8).

Group C: Low proline accumulators with an ability to accumulate large quantities of other organic solutes. This group is exemplified by glycerol-accumulating marine flagellates (5, 11), glycinebetaine-accumulating salt-tolerant chenopods (28), and sorbitol-accumulating, salt-tolerant and alpine species of *Plantago* (7, 24).

Group D: Low proline accumulators with low capacity to accumulate other organic solutes. In the absence of water deficit avoidance mechanisms in slightly vacuolated cells, this group is only moderately resistant of low external water potential.

It has been argued that the accumulated proline, even at low tissue levels, can play a major osmoregulatory role in the cytoplasm, where it is proposed to be preferentially sequestered (15). However, there is not clear evidence for subcellular compartmentation of proline. Besides, the ratio of vacuole:cytoplasm volumes in most nonsucculent plants is in the range of 0.5 to 3 (20), which is not large. It follows from these observations that, although proline accumulation in response to drought and salinity is widespread among vascular plants and green algae, proline's role as a major osmoregulatory solute can be defined clearly only in a specialized group comprised mainly of angiosperm halophytes and marine algae. If one of the main objectives of studies into the mechanisms of proline accumulation is to develop this physiological trait in crop plants (group D), then there is a need to focus on the special physiological and biochemical features associated in high accumulators found in saline habitats (groups A and B).

Proline biosynthesis occurs mainly from glutamate via the synthesis and reduction of Δ-pyroline-5-carboxylate (23). In the marine microalgae *Chlorella autotrophica* (3) and *Stichococcus bacillaris* (4), the enzymology of primary glutamate formation is

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distinct in that following the inhibition of GS[2] by MSX these unicells are able to maintain normal levels of glutamate synthesis via the reductive amination reaction of NADPH-GDH. In *C. autotrophica*, the levels of both GS and NADPH-GDH have been found to change with salinity of the external media (2), indicating that these changes may in part be associated with proline accumulation. The demand for nitrogen for proline synthesis is very high; in *C. autotrophica* at 200% ASW, proline constitutes about 25% of total cell nitrogen (2) and more than 90% of C and N in soluble compounds is found to be present in this imino acid (1). We are presently investigating the consequences of proline accumulation on other metabolic functions, particularly on photosynthesis and protein synthesis, in algae both at steady state and during the transitory period of osmotic adjustment. Part of our work is concerned with the potential role of NADPH-GDH in proline-accumulating algae.

In this paper we describe the relationship between growth parameters, the relative activities of NADH-NR, GS and NADPH-GDH, osmoregulatory responses and protein synthesis in *C. autotrophica* and *S. bacillaris* grown in the presence and absence of MSX.

## MATERIALS AND METHODS

**Algal Cultures and Growth Conditions.** Axenic cultures of *Chlorella autotrophica* Shihira and Kraus (Clone 580, obtained from Dr. R. R. L. Guillard, Woods Hole Oceanographic Institution, Woods Hole, MA, culture collection) and *Stichococcus bacillaris* Naeg (UTEX 314) were grown exponentially to a cell density of 0.8 to 1.5 × $10^6$ cells $mL^{-1}$ at 18°C on a 12:12 h light-dark cycle with 24 $W \cdot m^{-2}$ cool white light at pH 7.6 in 33% ASW (132 mM NaCl, 3.3 mM KCl, 3.3 mM $CaCl_2$, 6.6 mM $MgCl_2$, and 6.6 mM $MgSO_4$) or 150% ASW (600 mM NaCl, 15 mM KCl, 15 mM $CaCl_2$, 30 mM $MgCl_2$, and 30 mM $MgSO_4$) with 2 mM $NH_4Cl$ or $NaNO_3$ as the nitrogen source and nutrient enrichments as described previously (17). In MSX studies, media containing 0.4 and 2 mM MSX were prepared for *S. bacillaris* and *C. autotrophica*, respectively, according to Ahmad and Hellebust (3, 4). Cell densities and growth constants ($\mu$) were determined as described previously (4).

**Transfer of Cultures to High Salinities (Salt Shock).** Cultures grown at 33% ASW were mixed with equal volumes of 267% ASW containing appropriate MSX concentration and nitrogen source to give a final seawater concentration of 150%. These experiments were initiated 2 to 3 h after the start of the light period, and the cultures were kept at 20°C with 24 $W \cdot m^{-2}$ cool white light.

**Harvesting.** Cells were harvested and washed twice by centrifugation as described previously (3, 4).

**Extraction and Measurement of Organic Solutes.** Cells from 200- to 500- mL-cultures were extracted twice in 2.5 mL of methanol:chloroform:water (12:5:3), and the water-soluble layer of the extract was separated by centrifugation after mixing it with 5 mL each of chloroform and water. The water-soluble fraction was then rotary evaporated at 40°C and stored in 1 mL at −20°C. Proline was determined by a modification of the method of Bergman and Loxley (12). Sorbitol was determined enzymically by the spectrophotometric procedure described by Williams-Ashman (30).

**Preparation of Cell-Free Extracts.** For NADH-NR, cells from 0.5 L culture were suspended in 2 mL of extraction buffer, pH 7.6, containing 100 mM potassium phosphate, 10 mM $KNO_3$, 1 mM EDTA, 1 mM DTT, and 3% caseine. The cell suspension was disrupted twice by passing through a French pressure cell at 200 MPa and 0°C. For GS and NADPH-GDH, cells from 1- to 4-L cultures were suspended in 4 mL of extraction buffer, pH 7.6, prepared with or without thiols according to Ahmad and Hellebust (6). The cell suspension was disrupted as described for NADH-NR extracts. Two aliquots of 250 $\mu$L were removed for Chl and protein determinations. A further 250-$\mu$L aliquot was removed from thiol-containing preparations and was clarified by 20% ammonium sulfate precipitation for GDH determination as described previously (4). The remaining preparation was clarified by centrifugation at 25,000*g* for 30 min at 0°C, followed by a passage through a 0.22-$\mu$m filter assembly, and was loaded onto a Pharmacia Mono Q anion-exchange column attached to an FPLC system to fractionate $GS_1$ and $GS_2$ activities.

**Fast Protein Liquid Chromatography.** FPLC media were prepared with or without thiols; salt gradients and fraction collection were as described previously (6).

**Enzyme Assay Procedures.** For NADH-NR, a modification of the procedure of Scholl *et al.* (22) using phenazine methosulfate during nitrite color development was adapted to avoid interference by excess NADH, which was found to give 10 to 20% less color. The synthetase activity of GS and the amination reaction of NADPH-GDH were determined as described previously (4).

**[$^{14}$C]Bicarbonate Photoassimilation and [$^{14}$C]Phenylalanine Incorporation.** Bicarbonate photoassimilation was determined as described previously (1). For phenylalanine incorporation, cell suspensions containing 50 × $10^6$ cells $mL^{-1}$ were prepared and incubated with 10 $\mu$M [$^{14}$C]phenylalanine (0.1 $\mu$Ci $mL^{-1}$) and 1-mL samples were removed at times 0, 15, 30, 60, 90, 120, 180, and 240 min. The cells were collected by centrifugation at 9000*g* for 30 s and were suspended in 0.5 mL of 5% cold TCA. After 30 min incubation at 4°C, the sample was centrifuged at 9000*g* for 1 min to separate TCA-soluble and -insoluble fractions. The insoluble fraction was solubilized in 100 $\mu$L of NCS tissue solubilizer from Amersham and finally was dissolved in 3 mL of aqueous counting scintillant (ACS) from Amersham.

**Analytical.** Chl was extracted in 90% acetone and determined by the procedure described by Arnon (9). Protein was extracted in 1 N NaOH and determined as described previously (3, 4). Radioactivity was determined on a Beckman LS-230.

## RESULTS

**Growth Characteristics.** The results shown in Tables I and II are consistent with our previous observations (1, 3, 4) in that both *C. autotrophica* and *S. bacillaris* divide faster in the presence of ammonium than in the presence of nitrate. At 150% ASW, the inhibition in the cell division rate in *C. autotrophica* was in the range of 20 to 30% (Table I), while in *S. bacillaris* it exceeded 85% (Table II). That the cell protein content of *S. bacillaris* at 150% ASW was higher than at 33% ASW (Table II) indicates that biomass production in this alga is less sensitive to salinity than cell division. *Chlorella autotrophica* showed little change in either protein or Chl content in response to the given increase in salinity (Table I). For both algae, the division rates and Chl contents of cells growing in the presence of MSX (MSX-adapted cells) were within 75% of those growing without MSX (MSX-free cells). The estimated required nitrogen assimilation rates shown in Tables I and II were calculated from growth rates, and the estimated cell nitrogen contents derived from cell protein contents shown here in Tables I and II and cell proline contents shown later.

**Recovery of Enzymes in Cell-free Preparations.** Because of large differences found in the levels of NADH-NR, GS isoenzymes $GS_1$ and $GS_2$, and NADPH-GH between nitrate- and ammonium-grown cells of both *C. autotrophica* (Table III) and *S. bacillaris* (Table IV), an experiment was carried out with MSX-free cultures to test the recovery of each of these enzymes

[2] Abbreviations: amol, attomol; ASW, artificial seawater; FPLC, fast protein liquid chromatography; GDH, glutamate dehydrogenase; GS, glutamine synthetase; MSX, methionine sulfoximine; NR, nitrate reductase.

Table I. *Growth Parameters of MSX-Free and MSX-Adapted Cells of Chlorella autotrophica*

Cells were grown exponentially at 33 or 150% ASW. Growth constants are based on triplicate 50-mL cultures. Protein and Chl contents were determined in cell-free extracts and are presented as averages and SE of three to five determinations. The estimated required nitrogen assimilation rate was calculated as $\mu m \times$ cell nitrogen content, which in turn was calculated as cell protein/6.25 + cell proline-nitrogen shown in Table V.

| Salinity | Nitrogen Source | MSX-Free Cells | | | | MSX-Adapted Cells | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Growth constant ($\mu$) | Chl | Protein | Estimated N-assimilation rate | Growth constant ($\mu$) | Chl | Protein | Estimated N-assimilation rate |
| *% ASW* | | $h^{-1}$ | *pg cell*$^{-1}$ | | *amol·cell*$^{-1}$·*min*$^{-1}$ | $h^{-1}$ | *pg cell*$^{-1}$ | | *amol·cell*$^{-1}$·*min*$^{-1}$ |
| 33 | $NO_3^-$ | 0.0352 | 0.31 ± 0.05 | 6.1 ± 0.3 | 42 | 0.0314 | 0.35 ± 0.05 | 5.5 ± 0.06 | 33 |
| 150 | $NO_3^-$ | 0.0262 | 0.29 ± 0.03 | 6.6 ± 0.5 | 35 | 0.0267 | 0.29 ± 0.02 | 4.9 ± 0.03 | 28 |
| 33 | $NH_4^+$ | 0.0383 | 0.30 ± 0.05 | 6.5 ± 0.2 | 48 | 0.0364 | 0.35 ± 0.04 | 6.8 ± 0.04 | 47 |
| 150 | $NH_4^+$ | 0.0294 | 0.38 ± 0.04 | 6.7 ± 0.5 | 42 | 0.0275 | 0.33 ± 0.04 | 7.4 ± 0.06 | 43 |

Table II. *Growth Parameters of MSX-Free and MSX-Adapted Cells of Stichococcus bacillaris*

Cells were grown exponentially at 33 or 150% ASW. Growth constants are based on triplicate 50-mL cultures. Protein and Chl contents were determined in cell-free extracts and are presented as averages and SE of three to seven determinations. The estimated required nitrogen assimilation rate was calculated as $\mu m \times$ cell nitrogen content, which was calculated as cell protein/6.25 + cell proline-nitrogen shown in Table V.

| Salinity | Nitrogen Source | MSX-Free Cells | | | | MSX-Adapted Cells | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Growth constant ($\mu$) | Chl | Protein | Estimated N-assimilation rate | Growth constant ($\mu$) | Chl | Protein | Estimated N-assimilation rate |
| *% ASW* | | $h^{-1}$ | *pg cell*$^{-1}$ | | *amol·cell*$^{-1}$·*min*$^{-1}$ | $h^{-1}$ | *pg cell*$^{-1}$ | | *amol·cell*$^{-1}$·*min*$^{-1}$ |
| 33 | $NO_3^-$ | 0.0301 | 0.57 ± 0.07 | 11.8 ± 1.0 | 68.3 | 0.0275 | 0.61 ± 0.07 | 10.8 ± 1.1 | 57.2 |
| 150 | $NO_3^-$ | 0.0027 | 0.61 ± 0.04 | 16.8 ± 1.4 | 9.3 | 0.0024 | 0.68 ± 0.03 | 14.9 ± 1.0 | 7.2 |
| 33 | $NH_4^+$ | 0.0334 | 0.62 ± 0.05 | 12.4 ± 1.0 | 79.6 | 0.0285 | 0.67 ± 0.04 | 11.2 ± 1.3 | 61.7 |
| 150 | $NH_4^+$ | 0.0038 | 0.58 ± 0.07 | 18.3 ± 1.5 | 14.5 | 0.0028 | 0.73 ± 0.06 | 16.7 ± 1.8 | 9.6 |

Table III. *Levels of NADH-NR, $GS_1$, $GS_2$, and NADPH-GDH in MSX-Free and MSX-Adapted Cells of C. autotrophica*

Cells grown exponentially at 33% or 150% ASW were disrupted in the extraction buffer, pH 7.6, by passing through a French pressure cell at 200 MPa and 0°C. NADH-NR activity was determined in the crude extract; the values given are averages and SE of three experiments. GS was fractionated by FPLC using -thiol buffers for $GS_1$ and +thiol buffers for $GS_2$. The synthetase activity of GS was determined colorimetrically. NADPH-GDH was determined spectrophotometrically in crude extracts clarified by 20% ammonium sulfate precipitation. The values of NADPH-GDH are averages and SE of three to five experiments.

| Salinity | Nitrogen Source | MSX-Free Cells | | | | MSX-Adapted Cells | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | NADH-NR | $GS_1$ | $GS_2$ | NADPH-GDH | NADH-NR | $GS_1$ | $GS_2$ | NADPH-GDH |
| *% ASW* | | *amol·cell*$^{-1}$·*min*$^{-1}$ | | | | | | | |
| 33 | $NO_3^-$ | 560 ± 43 | 201 | 75 | 37 ± 8 | 474 ± 53 | 14 | 8 | 86 ± 5 |
| 150 | $NO_3^-$ | 491 ± 36 | 130 | 114 | 61 ± 9 | 568 ± 41 | 10 | 9 | 97 ± 8 |
| 33 | $NH_4^+$ | <5 | 13 | 34 | 325 ± 19 | <5 | 12 | 8 | 269 ± 31 |
| 150 | $NH_4^+$ | <5 | 42 | 66 | 382 ± 41 | <5 | 7 | 9 | 298 ± 18 |

Table IV. *Levels of NADH-NR, $GS_1$, $GS_2$, and NADPH-GDH in MSX-Free and MSX-Adapted Cells of S. bacillaris*

Cells grown exponentially at 33% or 150% ASW were disrupted in the extraction buffer, pH 7.6, by passing through a French pressure cell at 200 MPa and 0°C. NADH-NR activity was determined in the crude extract. The values given are averages and SE of three experiments. GS was fractionated by FPLC using -thiol buffers for $GS_1$ and +thiol buffers for $GS_2$. The synthetase activity of GS was determined colorimetrically. NADPH-GDH was determined spectrophotometrically in crude extracts clarified by 20% ammonium sulfate precipitation. The values of NADPH-GDH are averages and SE of three to seven experiments.

| Salinity | Nitrogen Source | MSX-Free Cells | | | | MSX-Adapted Cells | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | NADH-NR | $GS_1$ | $GS_2$ | NADPH-GDH | NADH-NR | $GS_1$ | $GS_2$ | NADPH-GDH |
| *% ASW* | | *amol·cell*$^{-1}$·*min*$^{-1}$ | | | | | | | |
| 33 | $NO_3^-$ | 1141 ± 87 | 13 | 205 | 8014 ± 604 | 1285 ± 161 | 3 | 5 | 9754 ± 841 |
| 150 | $NO_3^-$ | 532 ± 45 | 25 | 336 | 3134 ± 290 | 600 ± 38 | 2 | 2 | 4567 ± 367 |
| 33 | $NH_4^+$ | <5 | 10 | 88 | 830 ± 91 | <5 | 2 | 4 | 2936 ± 127 |
| 150 | $NH_4^+$ | <5 | 20 | 145 | 945 ± 101 | <5 | 2 | 2 | 2436 ± 127 |

in cell-free preparations. For both algae, mixing cell suspensions showing high enzyme activity with cell suspensions showing low enzyme activity had no appreciable effect on the recovery of NADH-NR, GS, or NADPH-GDH in cell-free preparations. Furthermore, purified $GS_1$, $GS_2$, and NADPH-GDH were found to be not inhibited when mixed with crude extract or when added before extraction of cells containing low levels of these enzymes. These results show that the procedures used for extracting NADH-NR, GS, and NADPH-GDH were adequate for both algae. The extraction of GS from MSX-adapted cells has been addressed elsewhere (3, 4).

**Nitrate Reductase.** In both algae, nitrate-grown cells contained high levels of NADH-NR activity, while no detectable activity of this enzyme was found in ammonium-grown cells (Tables III and IV). In *C. autotrophica* the increase in salinity from 33 to 150% ASW had little effect on NADH-NR activity (Table III), while in *S. bacillaris* the levels of this enzyme at 150% ASW were reduced by more than 50% in comparison with those at 33% ASW (Table IV). The activity of NADH-NR was not influenced by the presence of MSX for either algae (Tables III and IV). The levels of NADH-NR activity in nitrate-grown cells of both species were found to be far in excess of their estimated required nitrogen assimilation rates (Tables I to IV).

**Glutamine Synthetase Isoenzymes.** The distribution of the two isoenzymes, $GS_1$ and $GS_2$, was dependent upon nitrogen source and salinity of the media. In *S. bacillaris,* $GS_2$ was the major isoenzyme in cells grown with either nitrate or ammonium (Table IV), whereas in *C. autotrophica* $GS_2$ predominated in nitrate-grown cells and $GS_2$ in ammonium-grown cells (Table III). The levels of both $GS_1$ and $GS_2$ for both algae were found to be higher in cells growing at 150% ASW than at 33% ASW, except for $GS_1$ in nitrate-grown *C. autotrophica*, which showed a decrease at 150% ASW (Tables III and IV). Consistent with our previous observations (2–4), both algae showed higher total GS ($GS_1$ + $GS_2$) activity in the presence of nitrate than in the presence of ammonium (Tables III and IV). These activities in MSX-free cultures either matched or exceeded the estimated required nitrogen assimilation rates (Tables I to IV). Only residual activities of the two GS isoenzymes were present in MSX-adapted cells, ranging from 7 to 14 amol·cell$^{-1}$·min$^{-1}$ in *C. autotrophica* and from 2 to 5 amol·cell$^{-1}$·min$^{-1}$ in *S. bacillaris.* These activities were clearly not sufficient to maintain the estimated nitrogen assimilation rates for these cells (Tables I and II).

**Glutamate Dehydrogenase.** The activity of NADPH-GDH in all MSX-free and MSX-adapted cells was in excess of the estimated required nitrogen assimilation rates, except in nitrate-grown *C. autotrophica* at 33% ASW, which showed barely adequate levels of this enzyme (Tables I to IV). In *C. autotrophica,* the levels of NADPH-GDH activity were higher in the presence of ammonium than in the presence of nitrate (Table III), whereas the reverse was true in *S. bacillaris* (Table IV). In nitrate-grown cultures of *C. autotrophica* and ammonium-grown cultures of *S. bacillaris—i.e.* the nitrogen source favoring lower levels of NADPH-GDH activity—the levels of NADPH-GDH in MSX-adapted cells were higher than those in MSX-free cells (Tables III and IV). In nitrate-grown cultures of *S. bacillaris—i.e.* the nitrogen source favoring maximum levels of NADPH-GDH activity—the levels of NADPH-GDH at 150% ASW were less than half of those found at 33% ASW (Table IV).

**Organic Solute Accumulation.** The proline content of both *C. autotrophica* and *S. bacillaris* was markedly higher in cells growing at 150% ASW than in those growing at 33% ASW (Table V). In both algae, ammonium-grown cells showed higher proline contents than those grown with nitrate. For both algae, the proline contents of MSX-free cells and MSX-adapted cells were not appreciably different. Table V also shows a marked increase in the sorbitol content of *S. bacillaris* at 150% ASW. It is interesting to note that sorbitol contents of MSX-adapted cells were about 10 to 15% higher than those of MSX-free cells (Table V).

**Response of Algae to Transfer from 33% ASW to 150% ASW.** In these experiments, cells growing exponentially at 33% ASW were transferred to 150% ASW 2 to 3 h after the start of the light period and were monitored for solute accumulation, enzyme levels, photosynthesis, and protein synthesis over a period of 2 to 6 h.

Table VI shows the time course for organic solute accumulation in the two algae following their transfer from 33 to 150% ASW (salt shock). There was no apparent lag in either proline accumulation in *C. autotrophica* or proline and sorbitol accumulation in *S. bacillaris*, and within 6 h the levels of organic solutes accumulated by these algae were similar to those exhibited at steady state (Tables V and VI). For both algae, the patterns of solute accumulation exhibited by MSX-adapted cells after their transfer from 33 to 150% ASW was not appreciably different from that shown here for MSX-free cells.

The levels of NADH-NR, GS (total), and NADPH-GDH in nitrate-grown *S. bacillaris* were monitored over a period of 4 h following the transfer from 33% to 150% ASW (results not shown). Both NADH-NR and NADPH-GDH showed little change in response to salt shock in the given period of time. However, the activity of GS in MSX-free cells showed a 30% decrease over this period.

Table VII shows the time course for [$^{14}$C]bicarbonate photoassimilation and [$^{14}$C]phenylalanine incorporation into protein in nitrate-grown cells of *S. bacillaris* either at 33% ASW (control) or following the transfer to 150% ASW (salt shock). In both MSX-free and MSX-adapted cells, the photoassimilation was found to be remarkably resistant to this salt shock. The uptake of [$^{14}$C]phenylalanine by *S. bacillaris* was not affected by salt shock (results not shown); however, its incorporation into proteins (TCA-insoluble fraction) showed a pronounced lag of about 30 min in MSX-free cells and 90 min in MSX-adapted cells (Table VII). Following these lag periods, the rate of [$^{14}$C]phenylalanine incorporation in control and salt-shocked cells was markedly similar (Table VII).

## DISCUSSION

**Salinity Tolerance and GS Levels.** The ability of *C. autotrophica* and *S. bacillaris* to adapt to the presence of MSX has been demonstrated previously (3, 4). These MSX-adapted cells have now been grown for several hundred generations in the presence of MSX. Our present study reveals that there has been no loss of vigor in these cells and that their ability to tolerate salinity is similar to that of MSX-free control cells. We have not detected any appreciable change in the residual GS levels found in MSX-adapted cells to indicate the appearance of MSX-transport mutants in these cultures. These residual GS activities in MSX-adapted cells equaled 37 to 68% of net nitrogen assimilation rate in *C. autotrophica* and 10 to 70% of that in *S. bacillaris.* Considering that the glutamate synthase cycle in these phototrophic cells may be regulated by light due to thiol control of $GS_2$ (6) and Fd-dependent activity of glutamate synthase (4), the contribution of GS in nitrogen assimilation of MSX-adapted cells appears very small. Most of the ammonium assimilation in MSX-adapted cells, therefore, must occur via NADPH-GDH, which is highly active in these cells. That NADPH-GDH is functional in MSX-adapted cells indicates that this pathway can also operate together with the glutamate synthase cycle in MSX-free cells of *C. autotrophica* and *S. bacillaris*, particularly under the conditions favoring high activities of this inducible enzyme.

**Proline Biosynthesis.** The accumulation of proline at high salinities in MSX-adapted cells of *C. autotrophica* and *S. bacillaris* at a level within 80% of their MSX-free counterparts

Table V. *Organic Solute Accumulation in MSX-Free and MSX-Adapted Cells of C. autotrophica and S. bacillaris growing at 33% and 150% ASW*

Cells from 0.5-L exponential cultures were extracted in methanol:chloroform:water (12:5:3), and the clarified water-soluble fraction was assayed for proline and sorbitol contents. Proline was determined colorimetrically, while sorbitol was determined by the sorbitol dehydrogenase assay procedure.

| Salinity | Nitrogen Source | *Chlorella autotrophica* | | *Stichococcus bacillaris* | | | |
|---|---|---|---|---|---|---|---|
| | | MSX-Free | MSX-Adapted | MSX-Free | | MSX-adapted | |
| | | Proline | | Proline | Sorbitol | Proline | Sorbitol |
| | | | | *fmol·cell*$^{-1}$ | | | |
| 33 | $NO_3^-$ | 0.50 ± 0.21 | 0.61 ± 0.18 | 1.29 ± 0.21 | 4.38 ± 0.33 | 1.54 ± 0.31 | 3.80 ± 0.41 |
| 150 | $NO_3^-$ | 6.82 ± 0.71 | 6.20 ± 0.32 | 14.10 ± 1.13 | 11.98 ± 1.29 | 12.09 ± 0.93 | 13.31 ± 1.04 |
| 33 | $NH_4^+$ | 0.71 ± 0.13 | 0.47 ± 0.23 | 1.40 ± 0.18 | 2.66 ± 0.14 | 1.96 ± 0.24 | 3.43 ± 0.17 |
| 150 | $NH_4^+$ | 9.95 ± 0.88 | 10.53 ± 0.74 | 16.04 ± 1.23 | 10.56 ± 0.59 | 14.56 ± 1.07 | 12.14 ± 0.84 |

Table VI. *Time Course for Organic Solute Accumulation in MSX-Free Cells of C. autotrophica and S. bacillaris following Transfer from 33 to 150% ASW*

One-liter exponential culture growing at 33% ASW was mixed with 1 L of 267% ASW to give a final salinity of 150% ASW, and 400-mL samples were removed after 0, 1, 2, 4, and 6 h incubation in the light at 20°C. Proline and sorbitol were extracted and determined as in Table V. The values presented here were within 15% of those obtained in a separate experiment (not shown) using 0-, 2-, and 4-h incubation periods.

| Time | *Chlorella autotrophica* | | *Stichococcus bacillaris* | | | |
|---|---|---|---|---|---|---|
| | $NO_3^-$-grown cells | $NH_4^+$-grown cells | $NO_3^-$-grown cells | | $NH_4^+$-grown cells | |
| | Proline | | Proline | Sorbitol | Proline | Sorbitol |
| *h* | | | *fmol·cell*$^{-1}$ | | | |
| 0 | 0.7 | 0.9 | 1.1 | 4.1 | 1.2 | 3.2 |
| 1 | 1.7 | 2.9 | 4.2 | 6.2 | 4.8 | 5.1 |
| 2 | 2.6 | 4.4 | 7.0 | 8.3 | 7.9 | 6.9 |
| 4 | 4.2 | 7.7 | 12.4 | 11.0 | 13.9 | 9.7 |
| 6 | 5.7 | 9.5 | 16.1 | 13.2 | 17.0 | 11.9 |

Table VII. *Time Course for [$^{14}$C]Bicarbonate Photoassimilation and [$^{14}$C]Phenylalanine Incorporation in Nitrate-Grown, MSX-Free and MSX-Adapted Cells of S. bacillaris Growing at 33% ASW and following Transfer from 33 to 150% ASW*

Cells from 33% ASW growing exponentially were suspended in 10 mL of either 33% ASW (control) or 150% ASW (salt shock) and were incubated with either 2 mM $H^{14}CO_3$ (1 $\mu$Ci·mL$^{-1}$) or 10 $\mu$M [$^{14}$C]phenylalanine (0.1 $\mu$Ci·mL$^{-1}$) in the light at 20°C. For bicarbonate photoassimilation determinations, 1-mL samples removed at time 0, 15, 30, 45, 60, 90, and 120 min were collected and washed on a 0.45-$\mu$m filter paper, and the radioactivity was determined by scintillation counting. For [$^{14}$C]phenylalanine incorporation measurements, 1-mL samples were removed at time 0, 15, 30, 60, 90, 120, 180, and 240 min, and cells were extracted in 5% cold TCA. Radioactivity in the TCA-insoluble fraction obtained by centrifugation after 30 min incubation at 4°C was measured by scintillation counting. The results shown are correct for zero time radioactivity.

| Time | $H^{14}CO_3^-$ Photoassimilation | | | | Time | TCA-insoluble Phenylalanine | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MSX-free Cells | | MSX-Adapted Cells | | | MSX-free Cells | | MSX-adapted Cells | |
| | Control | Salt shock | Control | Salt shock | | Control | Salt shock | Control | Salt shock |
| *min* | *fmol·cell*$^{-1}$ | | | | *min* | *cpm·cell*$^{-1}$ × $10^4$ | | | |
| 15 | 30 | 20 | 32 | 28 | 15 | 0.5 | 0.13 | 0.43 | 0.02 |
| 30 | 62 | 55 | 59 | 61 | 30 | 1.13 | 0.18 | 1.06 | 0.10 |
| 45 | 95 | 80 | 83 | 87 | 60 | 2.07 | 0.52 | 1.95 | 0.24 |
| 60 | 137 | 114 | 119 | 130 | 90 | 3.10 | 1.49 | 2.84 | 0.63 |
| 90 | 191 | 170 | 174 | 191 | 120 | 4.16 | 2.34 | 3.87 | 1.39 |
| 120 | 229 | 200 | 210 | 238 | 180 | 6.30 | 4.13 | 5.92 | 2.94 |
| | | | | | 240 | 8.02 | 6.09 | 7.83 | 4.83 |

addresses a number of important points regarding the synthesis of imino acid in these algae. First of all, it shows that the amino acid precursor for proline biosynthesis in these high proline accumulators can be generated by NADPH-GDH. As these algae do not contain high levels of GS in media of certain nitrogen compositions, the presence of an alternative mechanism for amino acid synthesis, *i.e.* via GDH, may in part be associated with the need to ensure a rapid proline accumulation in water-stressed conditions of their natural habitats. In this respect these algae differ from proline accumulator higher plant halophytes, which do not appear to involve GDH in proline biosynthesis (25). Second, our present study raises some important questions regarding the pathway of proline biosynthesis in these algae. If proline in *C. autotrophica* and *S. bacillaris* is synthesized via the glutamate pathway, then the ability of MSX-adapted cells to accumulate proline would indicate that phosphorylation of glu-

tamate by glutamate kinase, *i.e.* the first step in this pathway, is not inhibited by MSX. This would be consistent with the view of MSX as a selective enzyme inhibitor (19). Alternatively, MSX may inhibit glutamate kinase in these algae, and MSX-adapted cells may have circumvented its effect by synthesizing it via the ornithine pathway (23, 29). Certainly, there is a need to investigate the relative role of the two potential pathways of proline biosynthesis in these algae.

**Glutamine and Glutathione Metabolism in MSX-Adapted Cells.** Apart from GS,γ-glutamyl-cysteine synthetase is also inhibited by MSX (19). Growth of MSX-adapted cultures of *C. autotrophica* and *S. bacillaris* suggests that these algae are able to meet their glutamine and glutathione requirements in the presence of MSX. The residual GS activities found in MSX-adapted cells of the two algae appear to be sufficient to provide glutamine for protein synthesis and other glutamine-dependent reactions. At present, no information is available about glutathione metabolism of these algae. An investigation of glutathione levels and the distribution of enzymes of this tripeptide synthesis in both MSX-free and MSX-adapted cells is needed urgently.

**Ammonium Modulation of Nitrate Reductase.** Recently, Rigano *et al.* (21), working with the thermophilic alga, *Cyanidium caldarium*, reported that following the inhibition of GS by MSX, nitrate reductase in ammonium-grown cells was fully active. It was suggested that the presence of an active GS is a necessary prerequisite for the ammonium-stimulated inactivation of nitrate reductase. In *C. autotrophica* and *S. bacillaris*, the presence of MSX had no effect on ammonium inhibition of NADH-NR activity. If this inhibition in MSX-adapted cells is associated with the GS product, then it must be assumed that the residual GS activity found in these cells is sufficient to maintain a normal glutamine pool. However, our present study does not exclude the possibility that ammonium modulation of nitrate reductase may be independent of GS activity.

**Choice of Osmoregulatory Solute.** It is striking that MSX-adapted cells of *S. bacillaris* accumulated high levels of proline and did not show a major shift toward the accumulation of the other non-nitrogenous osmoregulatory solute, the polyol, sorbitol. What advantages have these algae with multiple organic osmoregulatory mechanisms over those with only a single osmoregulatory solute? The pattern of sorbitol and proline accumulation in *S. bacillaris* in response to salinity is interesting. Sorbitol in these cells is always present in large amounts: about 100 mM under freshwater conditions and increasing to more than 500 mM at high salinities (13). In the absence of data on cell inorganic ion content, it is difficult to determine the nature of cellular osmotic balance in this alga. However, it would appear that constitutively high levels of sorbitol in this soil and coastal alga will tend to avoid complete turgor loss during rapid declines in external water potentials. As far as the accumulation of proline is concerned, both *C. autotrophica* and *S. bacillaris* can grow at salinities of up to 50% ASW with very little free proline. It is only at salinities approaching upper limits of salt tolerance of *C. autotrophica* and *S. bacillaris* that proline becomes the major osmoregulatory solute in these algae (1, 13). Thus, it would appear that the accumulation of proline in these algae may be associated less with growth under optimal conditions and more with the survival at inhibitory salt levels.

**Solute Accumulation following Sudden Increase in Salinity.** A lag period apparently associated with *de novo* synthesis of enzymes prior to proline accumulation has been reported in salt-shocked cells of *Chlorella emersonii* (14). The absence of lag for proline accumulation in salt-shocked cells of both *C. autotrophica* and *S. bacillaris* as well as sorbitol accumulation in *S. bacillaris* indicates that the enzymic component for organic solute synthesis present in these algae at low salinities is adequate. The stimulation of organic solute accumulation in these cells at high salinities is therefore likely to be associated with the regulation of enzymes activities. We are currently investigating the regulatory characteristics of some of the enzymes involved in the synthesis of osmoregulatory solutes in *C. autotrophica* and *S. bacillaris*.

**Long-term Adaptation to Salinity.** Our studies into the metabolic status of salt-shocked *S. bacillaris* revealed that these cells are able to carry out normal biocarbonate photoassimilation and to maintain fully active enzymes of nitrogen assimilation. Protein synthesis in these cells was disrupted only for a short period, and the eventual recovery of the rate of protein synthesis was almost complete. It is paradoxical, therefore, that this alga with such a marked capacity to tolerate salt shock showed severe growth inhibition at high salinities. This points toward some specific effects of long-term growth at high salinities not manifest during the initial period of change in salinity. *Stichococcus bacillaris* shows marked morphological changes when grown at high salinities forming multicellular filaments (16), the size of which appears to increase with salinity (results not shown).

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