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FREEZING STRESS AND OSMOTIC DEHYDRATION IN *FUCUS DISTICHUS* (PHAEOPHYTA): EVIDENCE FOR PHYSIOLOGICAL SIMILARITY[1]

Gareth A. Pearson[2] *and Ian R. Davison*

Department of Plant Biology and Center for Marine Studies, University of Maine, 5722 Deering Hall, Orono, Maine 04469-5722

ABSTRACT

The effects of osmotic dehydration and freezing on photosynthesis were studied in the brown alga Fucus distichus *L. The data indicated that* F. distichus *exhibits similar physiological responses to both osmotic dehydration and freezing stress and that these responses resemble those in the literature for the effect of desiccation in air. Both stresses inhibited light-limited (P_{subsat}) and light-saturated (P_{max}) photosynthesis measured immediately after plants were reimmersed in seawater. The degree of initial inhibition and subsequent recovery of photosynthesis were proportional to the severity of the dehydration or freezing treatment. P_{subsat} and P_{max} recovered completely from osmotic dehydration for 3 h in 200‰ and 3 hr at −10° C, but recovery was only partial following 3 h in 300‰ or 3 h at −15°C. In most cases, recovery was complete within 2 h following dehydration, with little further recovery occurring between 2 and 24 h posttreatment. No time-dependent recovery occurred following severe freezing. Observations using the vital stain fluorescein diacetate suggested that the lack of complete recovery might be due to severe damage or death of a proportion of cells in the thallus. There were no clear effects of either osmotic dehydration or freezing on dark respiration (R_d), although R_d was stimulated in all emersed treatments (frozen plants and 5° C controls) immediately following reimmersion. Measurement of chlorophyll fluorescence induction kinetics indicated that both osmotic dehydration and freezing reduced the ratio of variable to maximum florescence (F_v/F_m), indicating a decrease in the quantum efficiency of photosystem I. Based on these data, we suggest that there are common cellular and physiological components involved in the response of fucoid algae to a range of water stresses. This hypothesis was supported by experiments that showed that osmoacclimation in hyperosmotic seawater (51‰) for 2 weeks increased the ability of* F. distichus *to recover from freezing at −15° C. During acclimation, mannitol content increased under hyperosmotic conditions*

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[2] Author for reprint requests.

and decreased under hypoosmotic conditions. Changes in plasma membrane integrity, determined by fresh weight: dry weight ratio, and amino acid release following freezing indicated an increasing gradient of freezing tolerance from low to high salinity. However, none of these physiological changes fully explained the marked increase in the freezing tolerance of photosynthesis observed in plants acclimated under hyperosmotic conditions.

Key index words: freezing; Fucus distichus; *intertidal ecology; osmotic dehydration; Phaeophyta; photosynthesis; stress physiology*

Over 60% of the earth's surface experiences mean annual temperatures below 0° C (Sakai and Larcher 1987). On rocky shores in Maine and other regions of the northwest Atlantic, intertidal organisms are frequently exposed to subzero temperatures during tidal emersion in the winter months (Kanwisher 1957, Parker 1960, Bird and McLachlan 1974). For the dense stands of macroalgae that dominate these shores, freezing is an important stress, particularly for less tolerant, low-shore species such as *Fucus evanescens* (Davison et al. 1989, Dudgeon et al. 1989, Pearson and Davison 1993).

Previous physiological studies have demonstrated that variations occur in the ability of different species of macroalgae to recover from desiccation (Dring and Brown 1982, Smith and Berry 1986), freezing (Davison et al. 1989, Pearson and Davison 1993), and photoinhibition (Bose et al. 1988, Herbert and Waaland 1988). Upper eulittoral species exhibit greater tolerance to these stresses than lower eulittoral or subtidal species. In addition, both desiccation (Brawley and Johnson 1991) and freezing rate (Pearson and Davison 1993) have been shown to vary on the shore over small spatial scales (within the distribution of a species) and to influence the mortality and photosynthetic rates of species, respectively.

Despite the importance of stress tolerance in intertidal seaweeds, relatively little is known about the underlying mechanisms that confer tolerance, in particular whether or not tolerance to different dehydration stresses is conferred by a common set of cellular responses. There is evidence indicating that this occurs in higher plants where osmotic stress and water stress are effective in inducing freezing tolerance (e.g., Reaney et al. 1989). Nonlethal freezing (in which intracellular ice crystals do not form) is primarily a dehydration stress in common with osmotic dehydration and desiccation in air. The formation of ice in extracellular spaces increases the concentration of solutes and causes water to move out of the cells by osmosis (see reviews by Siminovitch and Cloutier 1983, Kacperska 1993).

This article investigates the possible similarity between desiccation and freezing tolerance in the intertidal fucoid alga *Fucus distichus* (L.). *Fucus distichus* is a small brown alga that inhabits upper-shore rock pools, where it forms the dominant canopy, but does not occur on emergent rock surface. Transplant experiments with adult plants have indicated that *F. distichus* is physiologically incapable of surviving outside of pools in the mid- or upper eulittoral, although transplanted juveniles survived for more than a year in the lower eulittoral (Chapman and Johnson 1990). In laboratory experiments, *F. distichus* is less freezing tolerant than other *Fucus* species (Davison et al. 1989). Adult *F. distichus* is very resistant to grazing, having a higher phlorotannin content than other fucoids, but it is slow growing and is considered to be a poor competitor in addition to being stress intolerant (Denton and Chapman 1991). It thus appears to inhabit a competitive refuge in tide pools, where grazing pressure is strong but physical stresses are less severe than on emergent rock. However, during the winter months smaller tide pools often freeze completely (pers. observ.), encasing *F. distichus* in ice at temperatures potentially reaching −20° C or below. Larger pools may develop an ice cover of several centimeters, exposing the plants below to hyperosmotic stress due to concentration of the unfrozen seawater (see also Edelstein and McLachlan 1975). Despite these stresses, *F. distichus* remains physiologically active during the winter months and during this period becomes reproductive, producing viable zygotes.

Fucus distichus was chosen as a model in which to study the physiological effects of freezing and dehydration, in particular to determine whether or not tolerance to these factors is linked at the cellular level. Unlike stress-tolerant upper-shore fucoids such as *F. spiralis*, *F. distichus* responds to moderate degrees of stress, making it more amenable to the study of physiological responses in the laboratory. In addition, the small size of *F. distichus* and the fact that it naturally grows permanently immersed facilitates culture experiments. Plants were dehydrated osmotically, because this is a potential stress in adult plants in tide pools and because osmotic dehydration and air drying have previously been shown to have similar physiological consequences in other (red) seaweeds (Smith and Berry 1986). Osmotic dehydration also has the advantage of being much easier to control experimentally than desiccation. Two experimental approaches were used in the current study. In the first, the effects of freezing and osmotic dehydration on the following aspects of metabolism were tested: the induction kinetics of chlorophyll fluorescence, light-saturated and light-limited photosynthetic oxygen exchange (P_{max} and P_{subsat}, respectively), respiration (R_d), and plasma membrane integrity. A second approach was used to determine whether or not tolerance to osmotic dehydration and freezing are induced by similar physiological mechanisms. This was done by acclimating plants in hypo- and hyperosmotic seawater media and then determining the level of freezing tolerance relative to control plants. If a single suite of cellular re-

sponses contributes to both freezing and dehydration tolerance, acclimation to one (in this case dehydration) should confer tolerance to the other.

MATERIALS AND METHODS

Adult vegetative plants of *Fucus distichus* were collected at low tide from high-shore rock pools in the intertidal zone at Schoodic Point, Maine (44°20'N, 68°5'W) between June and September 1992. Plants were transported to the laboratory in seawater, cleaned of visible epibionts, and maintained at 5° C in plexiglass aquaria containing 6 L of aerated, filtered seawater. The photon flux density (PFD) was between 70 and 80 μmol photons·m^{-2}·s^{-1} in a 16:8 h LD cycle. Plants were used in experiments within 10 days of collection.

Photosynthesis measurements. Vegetative apices (ca. 1 cm) were cut 12–24 h before use and incubated under culture conditions to allow recovery from wound respiration (Bidwell and McLachlan 1985). For experimental treatments, replicate apices (n = 6) were exposed to either 1) osmotic dehydration in seawater containing 200 or 300‰ NaCl at 5° C or 2) freezing at −10° or −15° C. The cooling rate was 5° C·min^{-1}. Different control treatments were used for osmotic dehydration and freezing treatments. Controls for osmotic dehydration were immersed at 30‰ at 5° C, whereas controls for freezing were kept emersed in air at 5° C in closed test tubes to ensure the apices remained moist and did not experience desiccation. All treatments were carried out in darkness for 3 h. Rates of photosynthesis and respiration were measured on independent groups of apices after 0, 2, and 24 h recovery in seawater under culture conditions. Rates of net photosynthesis at saturating PFD (500 μmol photons·m^{-2}·s^{-1}; P_{max}) and subsaturating PFD (25 μmol photons·m^{-2}·s^{-1}; P_{subsat}) and dark respiration (R_d) were measured in Clark-type oxygen electrodes (Rank Bros., Botisham, UK) in 5 mL seawater at 5° C, as described previously (Davison et al. 1991). Rates of P_{max} and P_{subsat} were adjusted for respiratory oxygen consumption and are presented as gross photosynthesis.

Release of amino acids. Changes in membrane permeability due to freezing or osmotic dehydration were assessed by determining the efflux of amino acids from *Fucus distichus*, as described previously (Dudgeon et al. 1989). Amino acids released during osmotic dehydration were determined by taking a sample of the hyperosmotic medium 20 min after adding apices. Previous studies have indicated that the majority of release occurs within this time (Davison et al. 1989). In addition, release during recovery was measured in samples of seawater taken 20 min after reimmersion. For emersed (frozen) treatments, only release during reimmersion was measured. Total cellular amino acid content was determined by extracting ca. 0.5 g tissue twice with hot 80% ethanol. Amino acids in the pooled extracts and seawater samples were assayed using the colorimetric reaction with ninhydrin, as described by Rosen (1957).

Induction kinetics of chlorophyll fluorescence. In this experiment, apices were frozen and dehydrated as already described, and fluorescence kinetics were determined using a Morgan instruments CF-1000 fluorescence monitor. Measurements were made after 30 min, 2 h, and 24 h recovery in seawater under culture conditions. The actinic light source (from the fiber-optic probe) was 500 μmol photons·m^{-2}·s^{-1}, which was determined prior to the experiment to provide saturating light while maintaining the maximum F_v/F_m ratio. The shutter speed of the Morgan fluorimeter is 3 ms. This is relatively slow, and as a result initial fluorescence, F_o, may be overestimated and variable fluorescence, F_v, consequently underestimated. Fluorescence was measured for 3 min, following dark incubation for 10 min, generating the parameters of a typical Kautsky-type curve as follows: F_o, the initial fluorescence minima recorded when the pool of primary electron acceptors Q_A of photosystem II (PSII) reaction centers are oxidized (= traps open); F_m the maximum fluorescence emission when the pool of Q_A is fully reduced (= traps closed); F_v, the variable fluorescence ($=F_m - F_o$); F_v/F_m, the ratio of variable to maximum fluorescence; F_t, steady-state fluorescence after the induction of carbon fixation; and F_q, the quenching fluorescence ($=F_m - F_t$). The quenching coefficient, q ($=1 - [F_t/F_m]$), represents the degree of dissipation of excitation energy (both photochemical quenching, [qQ] and nonphotochemical quenching, [qNP]). When q = 1 there is complete fluorescence quenching, and when q = 0 no fluorescence quenching occurs.

Freezing tolerance after osmoacclimation. Whole plants cleaned of amphipods and other epibionts were acclimated for 14 days in replicate 2.5-L culture pots containing 1.5 L of EA1 artificial seawater medium (Cosson 1973) (n = 6 per treatment). The micronutrient content (NO_3, PO_4, vitamins) and carbon source (2 mM $NaHCO_3$) were kept constant while the major ions were adjusted to give salinities of 8.5, 17, 34, and 51‰, corresponding to 25, 50, 100, and 150% full strength seawater. The media were changed every 4–5 days, and the plants were maintained in a walk-in culture chamber (5° C, 70–80 μmol photons·m^{-2}·s^{-1} irradiance and a 16:8 h LD cycle). Following acclimation, apices were cut (ca. 1 cm) 12–24 h before use to minimize the effects of wound respiration on tissue (Bidwell and McLachlan 1985) and either frozen at −15° C or emersed at 5° C (controls) for 3 h in the dark. The cooling rate for frozen treatments was 5° C·min^{-1}. Controls were kept moist to prevent dehydration. Following freezing or emersion, rates of P_{max}, P_{subsat}, and R_d were determined as already described. Rates were measured after 0, 2, and 24 h recovery using independent samples (n = 6). All photosynthetic measurements and recovery incubations took place at the acclimation salinity of the plants. Samples of medium (1 mL) were taken 20 min after reimmersion to determine amino acid release. Total tissue amino acids were also determined as already described. Additional apices taken directly from cultured plants and from plants 30 min following freezing at −15° C for 3 h were weighed before drying at 60° C for 24 h to obtain dry weight : fresh weight ratios.

Mannitol, a major compatible cytoplasmic solute in brown algae (Davison and Reed 1985), was analyzed in samples prepared by extracting ca. 1 g fresh weight tissue twice with hot 70% ethanol. The combined extracts were dried by rotary evaporation and redissolved in 1 mL of distilled water. Mannitol concentration was determined spectrophotometrically after periodate oxidation (Kremer 1981). The freezing point of tissue acclimated to each salinity was determined by differential thermal analysis (DTA) with a cooling rate of ca. 0.75° C·min^{-1}, as described previously (Pearson and Davison 1993); briefly, the large latent heat of fusion of ice is utilized to detect the temperature at which ice forms in tissue using a thermocouple probe, with a reference probe in air for comparison.

Statistical analyses. Single-factor, two-factor, or three-factor analyses of variance (ANOVAs) were used throughout to test hypotheses concerning differences between treatment means after satisfying the homogeneity of variances assumptions of the test. Comparisons of individual means within groups were performed using Ryan's *q*-test (Day and Quinn 1989).

RESULTS

Effects of osmotic dehydration and freezing on respiration, photosynthesis, membrane permeability, and fluorescence kinetics. Osmotic dehydration and freezing had similar effects on photosynthesis. Rates of light-limited and light-saturated photosynthesis (P_{subsat} and P_{max}, respectively) were inhibited in dehydrated and frozen plants, relative to controls, when measured immediately after reimmersion (Fig. 1). Rates of P_{max} showed partial or total recovery from osmotic dehydration after 2 h, and in most cases

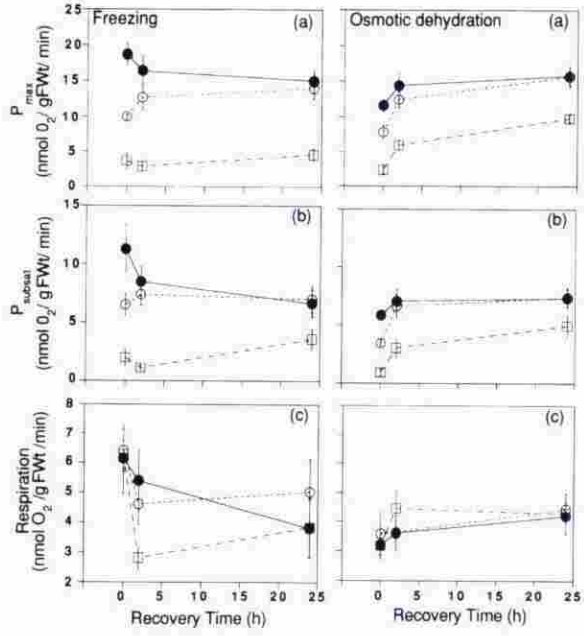

FIG. 1. Effect of freezing and osmotic shock on photosynthesis and respiration in *Fucus distichus*. Recovery of a) P_{max} (500 μmol photons·m^{-2}·s^{-1}), b) P_{subsat} (25 μmol photons·m^{-2}·s^{-1}), and c) R_d following 1) freezing, 3 h emersed at −10° C (○), −15° C (□), or 5° C (controls: ●), and 2) osmotic dehydration; 3 h immersed in seawater of 200‰ (○), 300‰ (□), or 30‰ (controls: ●). All treatments were carried out in darkness; plants were allowed to recover under culture conditions. Error bars denote ±SE (n = 6).

little further recovery occurred after 24 h. The degree of initial inhibition and the extent of recovery depended on the severity of osmotic dehydration. Photosynthetic rates did not increase over the recovery period following freezing. A significant interaction between treatment and recovery time was found ($F = 3.109$, $P < 0.005$). Multiple comparisons were performed to identify differences between means; no differences occurred between emersed and immersed controls at any recovery time. Immediately following reimmersion, rates of P_{max} of apices frozen at both −10° and −15° C were inhibited relative to (reimmersed) controls with maximum inhibition occurring at −15° C (Fig. 1a). There were no significant differences in rates of P_{max} between controls and samples frozen at −10° C after 2 and 24 h recovery. In contrast, 24 h after reimmersion samples frozen at −15° C failed to recover to levels of P_{max} of either controls or samples frozen at −10° C. Significant inhibition of P_{max} (relative to controls immersed in 30‰ seawater) occurred immediately after osmotic dehydration in 300‰, but not 200‰ seawater (Fig. 1a), although the latter did appear to show some initial depression of P_{max}. P_{max} of apices dehydrated in 300‰ seawater failed to recover to the level of controls after 2 h but were not significantly different from controls following 24 h recovery. The major difference between the two most stressed treatments (−15° C and 300‰) was that photosynthesis of frozen tissue did not recover after 24 h reimmersion whereas gradual recovery took place between 2 and 24 h following osmotic dehydration.

Differences in rates of light-limited photosynthesis (P_{subsat}) were found due to the interaction between treatment and recovery time ($F = 3.153$, $P < 0.005$) (Fig. 1b). Multiple comparison tests indicated that no differences occurred at any recovery time (i.e., 0, 2, or 24 h) between 1) emersed and immersed controls (except at 0 h when P_{subsat} of emersed controls was higher than that of immersed controls), 2) apices frozen at −10° C and those osmotically shocked in 200‰ seawater, and 3) apices frozen at −15° C and those osmotically shocked in 300‰ seawater. Immediately following reimmersion, rates of P_{subsat} of −15° C frozen *F. distichus* apices were lower than those frozen at −10° C, which in turn were lower than those of controls (Fig. 1b). After 2 h recovery in seawater, apices frozen at −10° C showed rates of P_{subsat} similar to controls, and in both treatments rates were higher than those of apices frozen at −15° C. After 24 h, these three treatments did not differ significantly, although P_{subsat} remained lower in apices frozen at −15° C. Statistically similar results were obtained from osmotically shocked treatments in 200 and 300‰ seawater (Fig. 1b). However, the initial degree of inhibition of apices osmotically shocked in 300‰ seawater did not differ significantly from those in 200‰ (whereas only those from 300‰ were inhibited relative to controls).

Although ANOVA revealed a significant interaction of treatment and recovery time on dark respiration (R_d) ($F = 2.128$, $P < 0.05$) (Fig. 1c), multiple comparisons failed to reveal where the differences lay. Respiration rates were higher in emersed controls and frozen tissue than in immersed controls or osmotically dehydrated apices (Fig. 1c). A similar elevation of R_d following emersion has been found in previous studies (Pearson and Davison 1993). R_d of emersed controls and frozen apices declined after 2 h recovery, and this was most pronounced in apices frozen at −15° C. However, these differences were not significant, partially due to high variability between replicates within a treatment. Respiration rates were similar between immersed controls and osmotically dehydrated apices and did not differ over time (Fig. 1c). These data indicate that respiratory responses are complex and not simply a consequence of freezing and osmotic dehydration but also that differences in the metabolic state of emersed and immersed tissue occur independently of osmotic dehydration or freezing treatment.

Plasma membrane permeability was assessed by measuring the release of amino acids by apices into seawater during recovery. For frozen apices and emersed controls, measurements were made after 20 min reimmersion whereas release from osmotically dehydrated apices and immersed controls was based on total release during immersion (treatment) and recovery in normal seawater (see Materials and

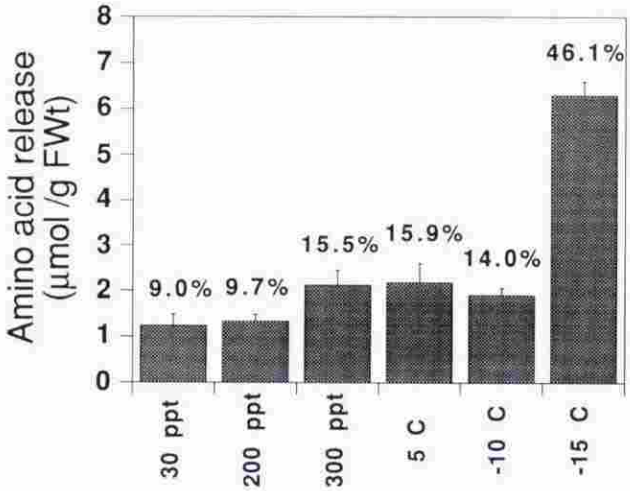

FIG. 2. Release of amino acids from *Fucus distichus* following osmotic dehydration in 200 and 300‰ seawater for 3 h (controls in 30‰) and freezing at −10° and −15° C for 3 h (controls emersed at 5° C). All treatments carried out in darkness; plants were allowed to recover under culture conditions. Error bars denote ±SE (n = 6). The numbers above each bar show the percentage of the total cellular amino acid pool released by each treatment.

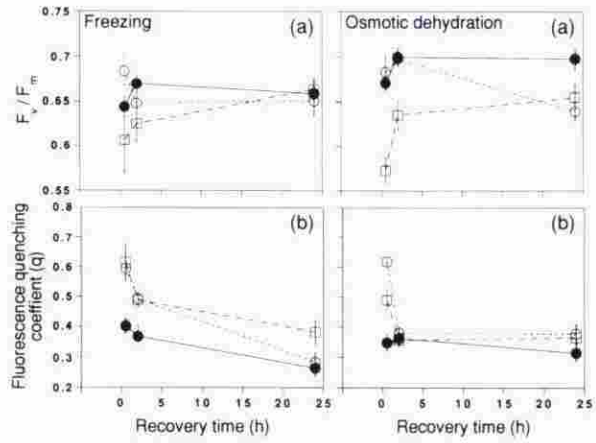

FIG. 3. The effect of freezing and osmotic dehydration on a) F_v/F_m, the ratio of variable to maximum fluorescence, and b) q, the quenching coefficient of chlorophyll fluorescence in *Fucus distichus*. Changes in F_v/F_m and q during recovery from 1) freezing, 3 h emersed at −10° C (○), −15° C (□), or 5° C (controls: ●), and 2) osmotic dehydration, 3 h immersed in seawater of 200‰ (○), 300‰ (□), or 30‰ (controls: ●). All treatments were carried out in darkness; plants were allowed to recover under culture conditions. Error bars denote ±SE (n = 12) except for those treatments in which fluorescence of some replicates was below the limits of detection. These were at 30 min, 2 h, and 24 h recovery from freezing at −15° C (n = 3, 7, and 7, respectively), at 30 min recovery from freezing at −10° C (n = 9), and at 30 min and 24 h recovery from osmotic dehydration in 300‰ (n = 7 and 9).

Methods). Amino acid release was similar in both groups of controls, dehydration treatments (200 and 300‰), and apices frozen at −10° C (Fig. 2). The only significant difference ($F = 43.03$, $P < 0.001$) occurred between these groups and apices frozen at −15° C, which exhibited much higher amino acid release (losing 46% of the total cellular amino acid content), indicating a major breakdown of plasma membrane integrity. The percentage of amino acids released by other treatments and controls (10–15%) was also surprisingly high, although loss from the damaged (cut) end of the apical sections may have contributed to this.

Fluorescence parameters were frequently too low to record after the more severe stress treatments, and these data are based on fewer replicates (see legend for Fig. 3). Because of the unequal numbers of observations between treatments as well as the bias introduced from ignoring the lowest measurements, no statistical analysis of this data has been attempted. The F_v/F_m ratio is proportional to the quantum yield of PSII photochemistry (Butler and Kitajima 1975) and highly correlated with the quantum yield of net photosynthesis in intact leaves (Demmig and Björkman 1987). The F_v/F_m ratio declined immediately following freezing at −15° C and osmotic dehydration in 300‰ (Fig. 3a), indicating a decrease in the quantum efficiency of charge separation in PSII, and by inference the quantum efficiency of photosynthesis. F_v/F_m of frozen treatments subsequently recovered to the level of emersed controls, but that of the dehydrated apices remained below that of immersed controls. The quenching coefficient q (a measure of the combined effect of both photochemical quenching, qQ, and nonphotochemical quenching, qNP) increased following both freezing (to −10° and −15° C) and osmotic dehydration (in 200 and 300‰) (Fig. 3b). This initial elevation is transient in osmotically shocked plants, returning to control levels in less than 2 h, but is maintained after 2 h recovery of plants frozen to −10° C and after 24 h recovery of those frozen to −15° C.

Freezing tolerance following osmoacclimation. Acclimation to salinities ranging from 8.5 to 51‰ did not influence the initial inhibition of photosynthesis of *F. distichus* following 3 h freezing at −15° C, but acclimation salinity strongly affected the ability of plants to regain levels of photosynthesis similar to those of controls (Figs. 4, 5). A three-factor ANOVA (salinity × temperature × recovery time) of the data for P_{max} revealed a significant interaction among these factors ($F = 11.478$, $P < 0.0005$). Multiple comparison tests indicated that rates of P_{max} in control plants (following 3 h emersion at 5° C) did not differ significantly, either between acclimation salinities or over recovery time. The initial inhibition of P_{max} was similar in all treatments immediately following freezing at −15° C (Fig. 4). However, while no significant recovery took place in plants acclimated to 8.5, 17, or 34‰ EA1 (Fig. 4a–c), those plants acclimated in hyperosmotic medium (51‰) recovered completely from freezing within 2 h of reimmersion (Fig. 4d).

Recovery of light-limited photosynthesis (P_{subsat})

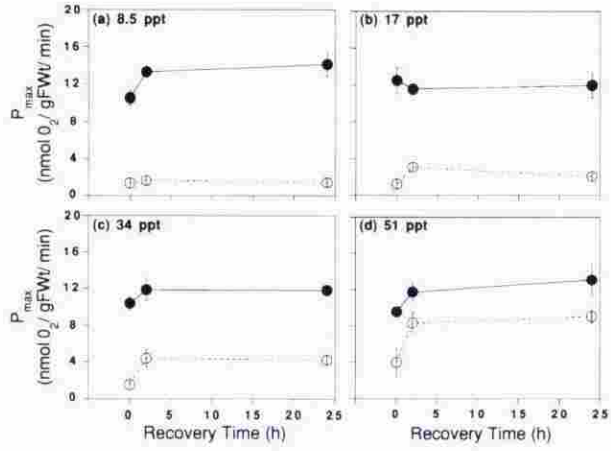

FIG. 4. The effect of osmoacclimation on freezing tolerance in *Fucus distichus*. Rates of light-saturated photosynthesis (500 μmol photons·m^{-2}·s^{-1}) during recovery in EA1 artificial seawater from 3 h emersion in air at 5° C (controls: ●) and −15° C (○). Plants had been acclimated for 14 days in 8.5 (a), 17 (b), 34 (c), and 51‰ (d) EA1. Emersion treatments were carried out in darkness; plants were allowed to recover under culture conditions, and photosynthesis was measured at the acclimation salinity. Error bars represent ±SE (n = 6).

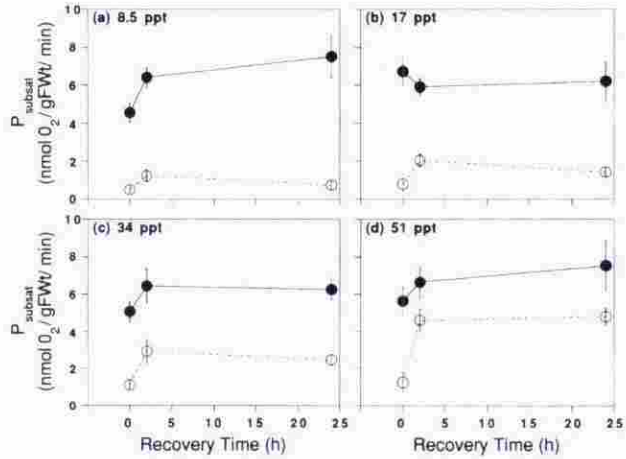

FIG. 5. The effect of osmoacclimation on freezing tolerance in *Fucus distichus*. Rates of light-limited photosynthesis (25 μmol photons·m^{-2}·s^{-1}) during recovery in EA1 artificial seawater from 3 h emersion in air at 5° C (controls: ●) and −15° C (○). Plants had been acclimated for 14 days in 8.5 (a), 17 (b), 34 (c), and 51‰ (d) EA1. Emersion treatments were carried out in darkness; plants were allowed to recover under culture conditions and photosynthesis was measured at the acclimation salinity. Error bars represent ±SE (n = 6).

was monitored following emersion at 5° C or freezing at −15° C (Fig. 5a–d). The salinity × temperature × recovery time interaction was significant at the $P < 0.0005$ level (ANOVA, $F = 5.326$). No differences in the rates of P_{subsat} due to salinity treatment (acclimation) or recovery time were found in control treatments. A similar initial level of inhibition of P_{subsat} at each salinity was observed following freezing. In plants acclimated to 8.5, 17, and 34‰ EA1 ASW no recovery of P_{subsat} took place between 0 and 24 h reimmersion, and rates of P_{subsat} remained significantly lower than those of controls even after 24 h recovery (Fig. 5a). In contrast, following freezing, plants acclimated to 51‰ showed a recovery of P_{subsat} within 2 h reimmersion (Fig. 5d) and rates did not differ significantly from those of controls.

Although rates of dark respiration (R_d) were lower in apices frozen at −15° C than emersed controls in plants acclimated to 17, 34, 51‰ EA1, these differences were not significant (Fig. 6). Plants acclimated to 8.5‰ showed reduced respiration in frozen compared to control apices following 24 h recovery at the acclimation salinity ($F = 4.285$, $P < 0.001$) (Fig. 6a). Taken together, the data on photosynthesis and respiration indicate that acclimation to hyperosmotic conditions increases and acclimation in hypoosmotic seawater decreases the ability of plants to recover from freezing stress but does not affect the initial degree of inhibition.

Frozen apices released significantly more amino acids than emersed controls in plants from all acclimation salinities except 51‰ ($F = 10.63$, $P < 0.001$); the amino acid release from controls did not differ between salinity treatments (Fig. 7a). Although more amino acids were released by frozen plants acclimated to low salinity (Fig. 7a), this seems to have been a consequence of these plants having higher amino acid contents (cf. 8.5 and 17‰ acclimated plants to those at 34 and 51‰; Fig. 7b; $F = 7.114$, $P < 0.005$). When the percentage of total amino acids released by frozen tissue is compared between salinity treatments, no clear differences are evident (percentages given next to bars in Fig. 7a). Thus, the degree of plasmalemmal damage experienced by frozen plants grown under hypo- and

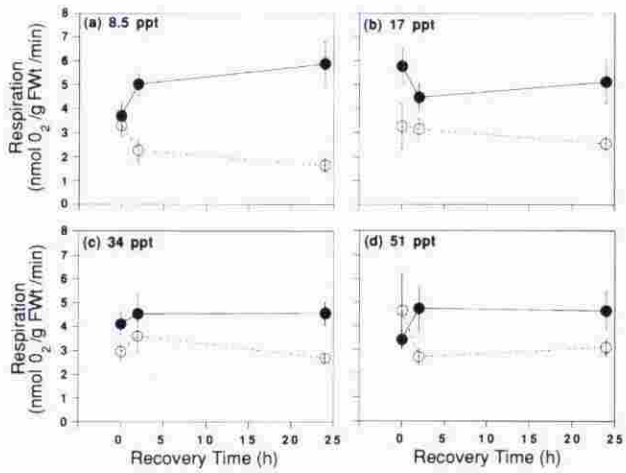

FIG. 6. The effect of osmoacclimation on freezing tolerance in *Fucus distichus*. Rates of dark respiration during recovery in EA1 artificial seawater from 3 h emersion in air at 5° C (controls: ●) and −15° C (○). Plants had been acclimated for 14 days in 8.5 (a), 17 (b), 34 (c), and 51‰ (d) EA1. Emersion treatments were carried out in darkness; plants were allowed to recover under culture conditions, and respiration was measured at the acclimation salinity. Error bars represent ±SE (n = 6).

hyperosmotic conditions appears to be similar, with plants under hypoosmotic conditions accumulating greater quantities of amino acids within the cells.

The content of mannitol (the major cytoplasmic compatible solute in brown algae; Davison and Reed 1985) increased with acclimation salinity from ca. 15 $mg \cdot g^{-1}$ (dry weight) at 8.5‰ to ca. 25 $mg \cdot g^{-1}$ (dry weight) in plants acclimated at 51‰ (Table 1). The increase in mannitol content was significant only between the lowest and highest salinities (8.5 vs. 51‰, $F = 3.243$, $P < 0.05$). The freezing points of tissue from each acclimation salinity was determined by DTA (Table 1). The mean freezing points of plants acclimated in 51‰ (−10.1° C) and 34‰ (−9.5° C) were lower than for plants kept in hypoosmotic conditions (−8.1° C for both 8.5 and 17‰). The differences between the two upper and two lower salinities were significant ($F = 13.58$, $P < 0.0004$).

The dry weight : fresh weight ratios were significantly different between treatments ($F = 6.629$, $P < 0.001$) (Table 1). The dry weight : fresh weight ratios of controls were lower in plants acclimated in salinities of 8.5 and 17‰ than those acclimated in 34 and 51‰, indicating that osmoacclimation did not completely compensate for changes in external water potential. The dry weight : fresh weight ratio was higher after freezing in plants acclimated in 51‰ than at each of the lower salinities and higher in plants acclimated in 34‰ than in 17 or 8.5‰. With the exception of plants acclimated in 51‰, the dry weight : fresh weight ratio of frozen apices was lower than that of unfrozen controls. In contrast to the data on amino acid release, these data suggest that cell solute losses due to freezing stress increased with decreasing acclimation salinity, presumably reflecting loss of cell solutes either through increased leakiness or rupture of the plasma membrane.

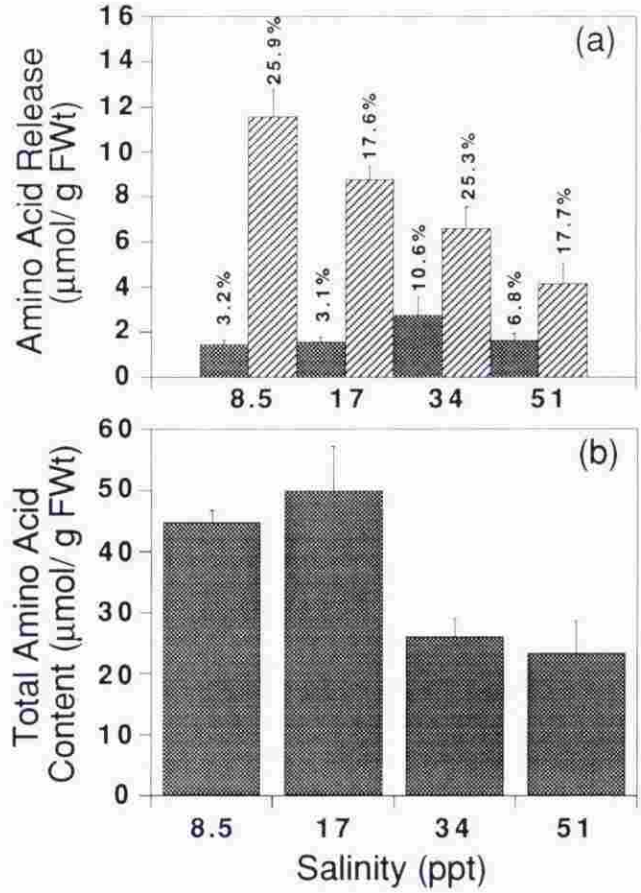

FIG. 7. Release of amino acids and amino acid content of *Fucus distichus* acclimated for 2 weeks at different salinities. a) Release of amino acids during the first 20 min of reimmersion following 3 h emersion at 5° C (controls; solid bars) or −15° C (frozen; hatched bars). Numbers above bars indicate the percentage of total cellular amino acid pool released by each treatment. Plants were emersed in darkness and then returned to culture conditions. b) Cellular amino acid content of plants. Error bars denote ±SE (n = 6).

DISCUSSION

In nature, *Fucus distichus* experiences a variety of stresses involving changes in the water potential of its rock pool habitat (Edelstein and McLachlan 1975). During the winter months, freezing of plants (in smaller rock pools during very cold weather) or exposure to hyperosmotic conditions in larger, partially frozen pools, both result in cellular dehydration. Our data suggest that the physiological consequences of freezing and osmotic dehydration are similar; both result in an initial inhibition of photosynthesis, with the majority of recovery occurring within 2 h of reimmersion. Several species of fucoids, following desiccation in air, have been shown to behave in a similar way (Dring and Brown 1982). As in our previous studies (Pearson and Davison 1993), the inhibition of photosynthesis reported here cannot be explained by changes in dark res-

TABLE 1. *Freezing temperature (n = 4), mannitol content (n = 6), and fresh weight : dry weight ratio (n = 6) of* Fucus distichus *plants acclimated at different salinities of EA1 artificial seawater media. Freezing temperatures were determined by differential thermal analysis. Fresh weight : dry weight ratios were measured from plants emersed 3 h at 5° C (control) and −15° C (frozen). Values are means ± SD. Means with the same letter are not significantly different (see text for details).*

Acclimation salinity (‰)	Freezing temperature (° C)	Mannitol content	Fresh weight : dry weight ratio	
			Control	Frozen
8.5	−8.05 ± 0.24a	14.85 ± 5.64c	0.194 ± 0.005e	0.149 ± 0.009f
17	−8.05 ± 0.78a	18.98 ± 7.38cd	0.185 ± 0.007e	0.157 ± 0.023f
34	−9.50 ± 0.64b	23.52 ± 4.67cd	0.217 ± 0.013g	0.196 ± 0.013h
51	−10.10 ± 0.46b	24.52 ± 5.64d	0.228 ± 0.024g	0.226 ± 0.009g

piration. Whereas light-limited and light-saturated photosynthesis were inhibited to a similar extent by both freezing and osmotic dehydration, dark respiration was only affected by freezing. In addition to the response of R_d, freezing and osmotic dehydration differentially affected plasma membrane permeability and chlorophyll fluorescence, indicating that some components of the two stresses are not equivalent.

A direct evaluation of the relative severity of freezing and osmotic dehydration on the physiology of *F. distichus* is not possible here, because the degree of cellular dehydration caused by incubation in 200 or 300‰ EA1 and freezing to −10° or −15° C was not determined. It would have been possible to measure the cellular dehydration caused by our experimental treatments using established techniques (Wright and Reed 1988, Dudgeon et al. 1989). However, we did not do so because the physiological response to cellular dehydration depends not only on the final degree of water loss but also on the rate at which this occurs (Pearson and Davison 1993). The latter would have been difficult to measure. While no direct comparison of the data from freezing versus osmotic dehydration can be made, when taken together with the results showing that freezing tolerance increases following osmoacclimation to high salinity, they support the hypothesis that the ability of *F. distichus* to tolerate osmotic dehydration and freezing are linked and therefore may involve many of the same physiological and cellular mechanisms.

A consistent pattern is apparent in the effects of water stresses (desiccation, osmotic dehydration, and freezing) on photosynthesis in *Fucus*. There is an initial inhibition of photosynthesis followed by a rapid (complete or partial) recovery. In those cases in which control levels of photosynthesis are not regained within 2 h, only a limited subsequent recovery occurs between 2 and 24 h (Dring and Brown 1982, Pearson and Davison 1993, this article). The failure to regain initial levels of photosynthesis following dehydration stress and the very slow recovery that occurs between 2 and 24 h poses an interesting question: is there a prolonged partial impairment of photosynthesis in all cells in the thallus, or do some cells recover rapidly, while others are severely damaged or killed? We have used the vital stain fluorescein diacetate to distinguish between these possibilities in *F. distichus*. These studies indicated that both osmotic dehydration and freezing result in the death of some cells. Osmotic dehydration in 300‰ and freezing at −10° C killed cells in and adjacent to the apical meristem. These cells were also killed by severe freezing at −15° C, together with many cells in the epidermal layer of the entire thallus (data not shown). These observations suggest that the photosynthetic data reflect two different effects of dehydration stress: in highly tolerant species such as *F. spiralis* (see Pearson and Davison 1993) and in all species following mild stress, dehydration causes a temporary inhibition of photosynthesis from which the plants rapidly recover. In more susceptible species subjected to severe stress, a proportion of cells are severely damaged and recover slowly (>24 h) or are destroyed. In the present study, the partial recovery of photosynthesis that occurs within 2 h reflects the proportion of cells that escape major damage. This aspect of the effect of environmental stress on seaweed physiology clearly has important ecological implications and deserves further study.

Chlorophyll fluorescence has previously been used to study photoinhibition in seaweeds (Bose et al. 1988, Henley et al. 1992), but this is the first study in which it has been used to characterize the effects of freezing in brown algae. Reductions in F_v/F_m such as those that were observed after severe freezing (−15° C) or osmotic dehydration (300‰) are indicative of a reduction in the quantum yield of PSII photochemistry (Butler and Kitajima 1975) and have been shown empirically to be correlated with a decrease in the quantum yield of photosynthesis in intact leaves (Demmig and Björkman 1987). It is clear in this study that the decline in F_v/F_m is coincident with a decrease in both P_{max} and P_{subsat} in severely frozen or osmotically stressed apices. This relationship may possibly have been even more clear if the lower limits of fluorescence detection had not been exceeded by some replicates, particularly after freezing. A decline in F_v/F_m has been used as an indicator of a range of environmental stresses (see Bolhàr-Nordenkampf et al. 1989 for a review), including freezing (Strand and Öquist 1985). However, a decline in F_v/F_m may arise in one of two distinct ways. 1) By an increase in F_o (the fluorescence emission by PSII antenna chlorophylls when all reaction centers are oxidized or "open"). This is characteristic of destruction of PSII reaction centers (Bolhàr-Nordenkampf et al. 1989). 2) By a decrease in variable fluorescence, F_v, which may reflect an increase in nonphotochemical and/or photochemical quenching. Unfortunately, the chlorophyll fluorescence data collected in this study do not allow an unambiguous resolution of this question. The slow shutter speed of the fluorimeter used (and as a consequence a possible overestimation of F_o, see Strasser and Govindjee 1991), as well as the absence of a detectable fluorescence signal in several replicates under severe stress, mean that confident interpretation of F_o data is not possible. This is particularly true because F_o is a low signal and therefore has a low signal : noise ratio. A sustained increase in the fluorescence quenching coefficient q (relative to controls) took place in plants frozen at −15° C. A similar increase was shown by plants frozen at −10° C, although this returned to control levels within 24 h. In contrast, the increase in q was transient in osmotically shocked plants (<2 h). Chlorophyll quenching is a complex, multicomponent phenomenon involving both photochemical (qQ) and non-

photochemical quenching (qNP). The contribution of qQ to q depends on the reoxidation of Q_A so that electrons can be accepted from the reaction centers (Geider and Osborne 1992), whereas qNP is a composite term encompassing 1) energy-dependent quenching (qE) related to the formation of the transthylakoid H^+ gradient (Krause et al. 1982) and/or cycling of excitation energy through xanthophylls (Demmig et al. 1987), 2) state-transition-dependent quenching resulting in redistribution of excitation energy to PSI, and 3) photoinhibition-dependent quenching (Horton and Hague 1988). The type of fluorescence monitor used in this study does not allow the resolution of qQ and qNP. More detailed fluorescence measurements are required to test whether the decreases in F_v/F_m and concomitant sustained increase in q found following freezing represents a stress response distinct from that in osmotically shocked plants, where F_v/F_m declined and only a transient increase in q was observed.

The data from the osmotic acclimation experiment clearly indicate that tolerance to freezing at −15° C was enhanced by acclimation in hyperosmotic medium. The converse was also true. Although acclimation to lower salinities (8.5 and 17‰) did not cause a decreased tolerance of P_{max} to freezing relative to plants acclimated in normal salinity seawater (34‰), plants acclimated to 8.5‰ did exhibit a greater reduction in R_d following freezing than other groups. The recovery of P_{subsat} in plants acclimated in 8.5, 17, and 34‰ was statistically lower than controls 24 h after freezing. It is possible that differences between lower salinity treatments may have been masked by the severity of the freezing treatment; further experiments would be required to test whether or not this is the case. Although we will continue to use the term "freezing tolerance" to describe the changes that occur during osmoacclimation, the parameter that changes is the ability to recover from a freezing event, because the initial degree of photosynthetic inhibition observed following freezing was similar at each salinity. In this respect, these results are similar to those found in interspecific comparisons of desiccation (Dring and Brown 1982) and freezing tolerance (Pearson and Davison 1993); the ability to recover from a stress appears more important than stress avoidance.

The mechanisms responsible for the increase in freezing tolerance of plants acclimated in 51‰ are unclear. The freezing point, dry weight : fresh weight ratio, and content of amino acids and mannitol were significantly different between plants acclimated at 8.5 or 17‰ and those from 51‰. However, these factors do not appear to be involved in the changes in freezing tolerance because they did not differ between plants acclimated in 34 and 51‰, although these groups of plants clearly differed in freezing tolerance. The evidence for the involvement of the plasma membrane in changes in freezing tolerance is equivocal. The percentage of loss of the total amino acid pool following freezing was the same in all acclimation salinities, suggesting a similar agree of plasma membrane damage. However, a second indicator of the loss of cell contents (the dry weight : fresh weight ratio) was affected by freezing in all groups except for plants acclimated in 51‰. All other treatments exhibited a reduction in the dry weight : fresh weight ratio after freezing, which suggests they had lost cell contents. Furthermore, the absolute amount of amino acid release following freezing was inversely proportional to the acclimation salinity. This observation could be explained by assuming that osmoacclimation in hyperosmotic media does in fact induce changes in the plasma membrane that confer increased freezing tolerance but that this is not reflected in the percentage of loss of amino acids because, in plants acclimated in low salinities, a larger fraction of the amino acid pool is compartmentalized in regions of the cell (e.g. the vacuole) where it is not lost following a transient breakdown in plasma membrane integrity. Changes in plasma membrane composition are known to occur during cold acclimation in many plant species increasing membrane stability at low temperatures and, hence, freezing tolerance (Somerville and Browse 1991, Palta and Weiss 1993). The possibility that similar changes occur following osmoacclimation in *Fucus* deserves further investigation.

Several studies have demonstrated at the molecular level that changes in gene expression occur during cold acclimation, i.e. during exposure to cold hardening temperatures (Guy 1990). Freezing tolerance also can be induced at room temperature in response to exogenous application of abscisic acid (ABA; Chen and Gusta 1983, Lång et al. 1989). Recently, sequencing of complementary DNA clones from messenger RNA transcripts produced during ABA-induced freezing tolerance in bromegrass has indicated that genes expressed in response to ABA were likely to be involved in sugar metabolism and osmotic stress (Lee and Chen 1993). Presently, we are conducting experiments to assess whether or not ABA has a role in osmotic stress-induced freezing tolerance in *F. distichus*. Recently, a cold-acclimation-specific transcript has been isolated from alfalfa (*Medicago falcata*) with homology to the dehydrin/LEA/RAB proteins. This protein appears to be regulated by low temperatures and not by ABA (Wolfraim et al. 1993). Such proteins may be involved in readjustment of endogenous rhythms during the cold-acclimation process as well as directly protecting the cell during freeze-induced dehydration by solvating intracellular structures due to the presence of many hydroxyl groups (Baker et al. 1988). It would be interesting to know whether or not homologous proteins are present in desiccation and freezing-tolerant versus -susceptible brown algae and whether their regulation is controlled by temperature, osmotic stress, or both.

The results presented in this article support the

hypothesis that osmotic dehydration and freezing stress in *F. distichus* exert similar physiological effects. However, evidence also suggests that osmotic dehydration and freezing may have different effects on plasma membrane integrity, on respiration, and possibly on some aspects of photosynthesis (e.g. fluorescence quenching). Acclimation to hyperosmotic conditions induces an increased ability to recover from a freezing event although an initial inhibition of P_{max} and P_{subsat} was still observed. The ability to increase freezing tolerance by osmoacclimation may confer an adaptive advantage for a rock pool alga such as *F. distichus* and also provides a useful model system in which to study the physiological and molecular mechanisms of water stresses in algae.

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STORAGE OF PHOSPHORUS IN NITROGEN-FIXING *ANABAENA FLOS-AQUAE* (CYANOPHYCEAE)[1]

Patsy-Ann Thompson,[2] Hee-Mock Oh, and G-Yull Rhee[3]

Wadsworth Center for Laboratories and Research and School of Public Health, State University of New York at Albany, New York State Department of Health, Empire State Plaza, Albany, New York 12201–0509

ABSTRACT

P accumulation and metabolic pathway in N_2-fixing Anabaena flos-aquae *(Lyngb.) Bréb were investigated in P-sufficient (20 μM P) and P-limited (2 μM P) turbidostats in combined N-free medium. The cyanobacterium grew at its maximum rate (μ_{max}, 1.13 d^{-1}) at the high P concentration and at 65% of μ_{max} under P limitation, with total cell P concentrations (Q_P) at steady states of 12.0 and 5.2 fmol·cell^{-1}, respectively. At steady state, polyphosphates (PP_i) accounted for only 3% of Q_P (0.4 fmol·cell^{-1}) in P-rich cells. Its concentration in P-limited cells was 5.8% (0.3 fmol·cell^{-1}). On the other hand, sugar P was very high at 22% of Q_P in P-rich cells and was undetectable in P-limited cells. Pulse chase experiments with ^{32}P showed that P-rich cells initially incorporated the labeled P into the acid-soluble PP_i fraction within the first few minutes and to a lesser extent into nucleotide P. Radioactivity in the PP_i then declined rapidly with concomitant increases in sugar P and nucleotide P fractions. In contrast, in P-limited cells, no radiolabel was detected in acid-soluble PP_i, and ^{32}P was initially incorporated into nucleotide P, sugar P, and ortho P fractions. The latter two fractions then subsequently declined. Therefore, under N_2-fixing conditions the cyanobacteria appeared to store P as sugar P and also utilize P through different pathways under P-rich and -limited conditions. When nitrate was supplied as the N source under P-sufficient conditions, PP_i accounted for about 15% of steady-state Q_P, but no sugar P was detected. Therefore, the same organism stored P in different cell P fractions depending on its N sources.*

Key index words: Anabaena flos-aquae; *cyanobacteria; N_2 fixation; P limitation polyphosphate, sugar phosphate*

In most freshwaters, phytoplankton growth is regulated by the availability of phosphorus (P). Under P limitation, growth is related to intracellular P, or cell quota (Q_P), due mostly to a large storage capacity for P by these organisms (Rhee 1980). Phosphorus is stored as polyphosphates (PP_i) (see Kulaev and Vagabov 1983, Cembella et al. 1984), and growth as well as P uptake is directly related to the size of P pools (Rhee 1973, 1974). In *Scenedesmus* sp., PP_i at a relative growth rate (μ/μ_{max}) of 0.8 was greater than the minimum cell quota (Rhee 1973). Such a large storage capacity for P uncouples phytoplankton growth from external P concentration.

Phosphorus storage in cyanobacteria appears to be much larger than in other species, and this capacity was reported to give them a competitive advantage over diatoms and chlorophytes when P was supplied in pulses (Sommer 1985, Reinertsen et al. 1986, Sakshaug and Olsen 1986). In eutrophic Green Lake, the colonies of a benthic population of *Gleo-*

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[2] Present address: Atomic Energy Control Board, Radiation and Environmental Protection Division, P.O. Box 1046, Station B, Ottawa, Ontario, Canada K1P 5S9.
[3] Author for reprint requests.