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The response of the green halotolerant alga *Dunaliella* to osmotic stress: effects on pyridine nucleotide contents

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Dunaliella tertiolecta **was subjected to osmotic stress by upward (salt stress) and downward (dilution stress) shifts in NaCl concentration, and the effects on glycerol content, photosynthesis, respiration and pyridine nucleotide contents determined. The increase in glycerol content after salt stress was not dependent on photosynthesis, since it also occurred in the dark and larger stresses were inhibitory to photosynthesis, but was always associated with an increase in the contents of NADPH and $NADP^+$, a decrease in NAD^+ and an initial increase in the $NADH/NAD^+$ quotient. The possible contribution of these changes towards increasing the rate of glycerol synthesis is evaluated. When compared with published contents for dihydroxyacetone phosphate and glycerol phosphate, the results obtained here show that the glycerol phosphate dehydrogenase reaction is far from equilibrium, suggesting that the activity of this enzyme is inhibited in vivo. The decrease in glycerol content after dilution stress was associated with a partial inhibition of photosynthesis, an increase in respiratory oxygen consumption, and an increase in the $NADPH/NADP^+$ quotient in the light but not in the dark. It is considered unlikely that changes in NADP contents are directly responsible for the accelerated glycerol dissimilation after dilution stress.**

Introduction

The cell content of glycerol, the major volume-regulatory solute in the wall-less flagellate, *Dunaliella*, is determined predominantly by a two-step synthesis and dissimilation to and from DHAP [1]. The mechanism by which these reactions are regulated and by which they respond to changing external salt concentration are unknown. Inasmuch as reduction of DHAP requires NADH or NADPH, and the oxidation of glycerol to dihydroxyacetone requires $NADP^+$ [2,3], it is logical that the effects of stresses on pyridine nucleotide levels within the cell should be ascertained. The present paper describes such analyses; a preliminary account of some findings has been given elsewhere [4].

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; DHAP, dihydroxyacetone phosphate; Chl, chlorophyll.

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Materials and Methods

Growth and treatment of cultures. Dunaliella tertiolecta was maintained and cultured in a de-

fined medium [5], with NaCl concentrations as specified in the results, essentially as described by Borowitzka and Brown [6], but with the following modifications. The experimental cultures were grown at $27 \pm 2°C$ with continuous aeration and shaking. The light intensity was 200 $\mu E \cdot m^{-2} \cdot s^{-1}$, with a light/dark regime of 16/8 h. The culture was harvested in the late exponential growth phase, 7 days after inoculation. Cultures were centrifuged at $1000 \times g$ for 3–5 min and resuspended in isoosmotic growth medium (pH 7.5) in which Tris-HCl^- was replaced by 20 mM potassium-Hepes.

Extraction of pyridine nucleotides. Algal suspensions (10–25 μg chlorophyll per ml) were placed in a glass cylinder (28 mm diameter) and stirred continually with a magnetic stirrer. The glass cylinder was held in a constant temperature water bath ($27 \pm 2°C$) and illuminated from opposite sides, when required, by white light from two slide projectors with quartz-iodine lamps (photosynthetic photon flux, 800 $\mu E \cdot m^{-2} \cdot s^{-1}$). The suspension was preincubated for 15 min under these conditions before the start of the experiment.

Osmotic stress was imposed by adding appropriate amounts of either 4 M NaCl (salt stress) or water (dilution stress) to the stirred suspension. Several samples were taken before an osmotic stress was imposed, and at each subsequent sampling time, three samples were taken in rapid succession. The sampling and metabolic quenching of the cell suspension was achieved by withdrawing a 1 ml sample and ejecting it rapidly into a small glass test-tube containing the quenching agent, held adjacent to the glass cylinder. The transfer of the sample was done in a way that maintained the illumination or dark conditions until the suspension was mixed with the quenching agent in a preheated vial. The quenching agents used were HCl (0.2 M, 1 ml, preheated to 75°C) for oxidised pyridine nucleotides and NaOH (0.2 M, 1 ml, preheated to 75°C) for reduced pyridine nucleotides. Extracts were placed in a water bath (75°C) for 12 min, then cooled on ice and centrifuged before neutralisation with NaOH or HCl as appropriate to give a final pH of 6.0 (oxidised pyridine nucleotides) or 9.0 (reduced pyridine nucleotides). All extracts were stored at −85°C for up to 48 h before assay.

Determination of pyridine nucleotides. Pyridine nucleotides were estimated by an enzymic cycling method [7] in which the rate of reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide coupled to a phenazine ethosulphate/pyridine nucleotide recycling redox system was measured. The reduction was driven by ethanol and alcohol dehydrogenase (NAD^+, NADH) or glucose and glucose 6-P dehydrogenase ($NADP^+$, NADPH). The reaction was monitored by recording the change in absorbance at 570 nm using a Varian-Cary 210 spectrophotometer. Recoveries of the reduced and oxidised forms of NAD and NADP were determined by separately adding 200 and 400 pmol of each pyridine nucleotide to 1 ml algal suspension and, after performing the full extraction and analysis procedures, measuring the additional pyridine nucleotide attributable to the added standard. Not less than 97% of an added internal standard of each of the four pyridine nucleotides was recovered.

Glycerol determination. Algal suspensions were preincubated for 10–15 min in light or dark before being subjected to an osmotic stress. Aliquots were centrifuged (as above) and the pellet extracted with 10 ml 80% (v:v) acetone at room temperature by vortex mixing and centrifugation. The extraction was repeated two or three times on the pellet and the pooled extracts treated with 100 mg activated charcoal, before clarifying by centrifugation, drying in a rotary evaporator (Büchi) and resuspension in water. Glycerol was determined by a spectrophotometric procedure modified [8]. The sample (0.2 ml, containing 2–50 nmol glycerol) was preincubated for 5 min at 25°C in a reaction mixture containing triethanolamine (71.4 μmol), $MgSO_4$ (71.4 μmol), P-enol pyruvate (1 μmol), NADH (100 nmol), ATP (2 μmol), pyruvate kinase (4 units), lactate dehydrogenase (5.5 units) in a total volume of 1.0 ml (pH 7.5). The change in extinction at 340 nm was then measured after the addition of glycerokinase (0.5 units).

Oxygen evolution and consumption. Photosynthetic oxygen evolution and respiratory consumption were measured polarographically [9] in 2 ml of algal suspension at 25°C. For these experiments the alga was resuspended as before but in a solution containing 0.25 mM sodium phosphate (pH 7.5), 0.2 mM $MgCl_2$ and 2.5 mM $NaHCO_3$.

Respiration was measured after preincubation of the alga for 10 min in the dark and photosynthesis was measured during illumination with red light ($300\ \mu E \cdot m^{-2} \cdot s^{-1}$).

Chlorophyll estimation. Chlorophyll was determined in acetone extracts of cell suspensions by the method of Walker [10].

Results

Effects of osmotic stress on glycerol content

When *D. tertiolecta* was grown in 0.17 M NaCl the intracellular glycerol content was about 8 μmol per mg chlorophyll. After a salt stress in the light to 0.7 M NaCl, a lag of about 2 min ensued before the glycerol content of the cells increased linearly at a rate of 50 μmol per mg chlorophyll per h during the first 30 min but decreased as glycerol approached a new steady-state level after 60 min. After a larger salt stress, from 0.17 to 1.0 M NaCl, the glycerol content increased at a lower rate (30 μmol per mg chlorophyll per h). When the alga was subjected to these salt stresses in darkness, the glycerol content also increased, but at rates of 36 and 28 μmol per mg chlorophyll per h for stresses to 0.7 and 1.0, respectively (not illustrated).

When grown in 0.7 M NaCl, *D. tertiolecta* contained about 60 μmol glycerol per mg chlorophyll. Following a dilution stress to 0.17 M NaCl, the glycerol content fell initially at a rate of about 75 μmol per mg chlorophyll per h. This response was essentially identical in light and dark. When it was grown in 1.0 M NaCl, *D. tertiolecta* contained about 90 μmol glycerol per mg chlorophyll and dilution stress to 0.17 M NaCl, resulted in the glycerol content falling intially at about 130 μmol per mg chlorophyl per h (not illustrated).

Effects of osmotic stress on photosynthesis and respiration

Photosynthetic oxygen evolution was initially inhibited by salt stress to 0.7 M NaCl (Fig. 1), but recovered after a few minutes to give rates slightly higher than those of the unstressed alga. The organism took longer to recover from the transfer to 1.0 M NaCl and the ultimate rate of oxygen evolution was about half that of the 'control' suspension in 0.17 M NaCl. Dilution stress caused

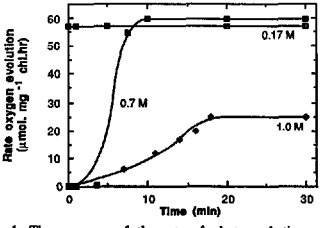

Fig. 1. The response of the rate of photosynthetic oxygen evolution by *D. tertiolecta* to salt stress. The alga was cultured in 0.17 M NaCl and the NaCl concentration in the medium increased in the light to 0.7 M (■) or 1.0 M (◆), or remained at 0.17 M (□, control).

a reduction in the rate of oxygen evolution (Table I).

The application of salt stress in darkness to *D. tertiolecta* grown in 0.17 M NaCl had little effect on respiratory oxygen uptake (Table II). When the alga was grown at higher salt concentrations and subjected to dilution stress, however, respiration increased by more than 30%.

Effects of osmotic stress on pyridine nucleotide contents

D. tertiolecta, when grown in 0.17 M NaCl, contains about 10 nmol NADPH per mg chlorophyll in darkness and twice that amount when illuminated (Figs. 2a and 3a). Smaller amounts of NADH were present. When the alga was subjected to an increase in salt concentration from

TABLE I

EFFECT OF DILUTION STRESS ON PHOTOSYNTHETIC O_2 EVOLUTION

Cultures were grown at the initial NaCl concentrations shown. Rates measured immediately after the stress are expressed as μmol O_2 per mg chlorophyll per h. Bracketed numerals denote the number of replicate preparations; where no such numbers are shown there were two preparations.

Initial NaCl concentration (M)	Rate O_2 evolution	NaCl concentration after transfer (M)	Rate O_2 evolution
0.7	75.0	0.17	38.5
1.53	86.0±2.3 (6)	0.53	34.5±2.0 (6)

TABLE II

EFFECTS OF OSMOTIC STRESS ON O_2 UPTAKE IN DARK

Rates (expressed as μmol O_2 per mg chlorophyll per h) were measured immediately after application of stress. Bracketed numerals denote the number of replicate preparations; where no such numbers are shown there were two preparations.

Conditions (M NaCl)	Rate before stress	Rate after stress
Salt stress:		
0.17–0.7	17.5 ± 2.3 (6)	18.4 ± 2.7 (6)
0.17–1.0	17.5 ± 2.3 (6)	18.2 ± 3.0 (6)
Dilution stress:		
0.7–0.17	17.5	24.0
1.0–0.17	18.8	24.5
1.53–1.06	18.9 ± 3.5 (6)	24.8 ± 2.2 (6)
1.33–0.53	18.9 ± 3.5 (6)	28.2 ± 2.7 (6)

0.17 M to 0.7 M, the levels of NADPH rose substantially during the first 5 min in both the light and dark while NADH remained essentially unchanged (Fig. 2a). After 30 min had elapsed, the level of NADPH in the light had dropped to a value close to that in darkness, but remained higher than before the salt stress was applied.

The $NADP^+$ content of *D. tertiolecta* grown in 0.17 M NaCl was similar in magnitude to the reduced coenzyme (Fig. 2b), giving reduced/oxidised quotients in the range 0.6–0.9 NAD was predominantly in the oxidised form in both light and darkness with reduced/oxidised quotients of about 0.1 (figs. 2c, 3b). After a salt stress to 0.7 M NaCl, the $NADP^+$ content increased in a manner similar to NADPH, but NAD^+ levels decreased over the first 10 min, especially in the light (Fig. 2b).

The reduced/oxidised quotients for NAD and NADP increased after salt stress in both the light and dark. The elevated quotients for NADP in the light and NAD and NADP in darkness persisted throughout the 30 min after the salt stress was applied, except for NAD in the light (Fig. 2c). The total amount of NADP (i.e., the sum of the oxidised and reduced forms) increased about two-fold during the first 5 min in the light, but thereafter there was a sharp decline in the light but not in the dark. 30 min after the stress was applied both light and dark values were similar (Fig. 2d). The total amounts of NAD, however, decreased in both light and dark after the salt stress.

When *D. tertiolecta* was subjected to a larger salt stress (from 0.17 to 1.0 M), qualitatively similar changes occurred in the individual pyridine nucleotide contents (Fig. 3a, b), except that NADPH levels increased more slowly than at the lower salt concentration and there was slightly more NADH present in the light than in darkness. The reduced/oxidised quotient for NADP increased after the stress in the light, but in the dark it initially decreased and then recovered slowly; 30 min after the stress it had still not regained the starting level (Fig. 3c). Total NADP increased whereas total NAD decreased (Fig. 3d).

When *D. tertiolecta* was grown in 0.7 M NaCl, the content and proportions of the individual pyridine nucleotides were similar to those of algae cultured at the lower salt concentration (Fig. 4). The application of a dilution stress to 0.17 M NaCl caused a small transient increase in the NADPH content in the light in the first 2 min, followed by a decline to a value close to the original level; there was little or no change in the dark (Fig. 4a). The $NADP^+$ level increased in the dark but decreased on illumination (Figs. 4b). A transient elevation of the reduced/oxidised quotients for NAD and NADP in the light was the most noticeable effect of the dilution stress. The quotients declined again after 2 min to a value about 30% above the original quotient (Fig. 4c). There was relatively little change in the total contents of NAD and NADP in either light or dark (Fig. 4d). An increase in the quotient for NAD and decrease in that for NADP in the dark was also evident.

Discussion

Two points should be noted before considering the biological significance of these results. The first is that the procedures used in this work can be assumed to extract the total amount, that is free and bound pyridine nucleotides [11]. Since substantial changes in the levels of NAD^+, $NADP^+$ and NADPH were observed after salt stress, it is likely that the bulk of these nucleotides is thermodynamically active. The NADH content was consistently much lower and displayed little

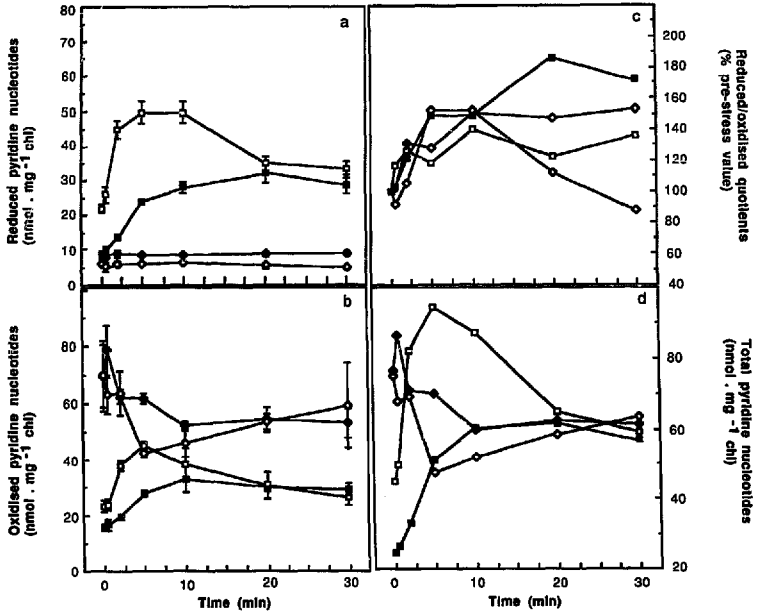

Fig. 2. The response of the pyridine nucleotide content of *D. tertiolecta* to salt stress (means of three determinations). The alga was cultured in 0.17 M NaCl and the NaCl concentration in the medium increased to 0.7 M in the light (open symbols) or dark (closed symbols). (a) NADH (◇, ◆) and NADPH (□, ■). The vertical bars represent the standard deviation where this is larger than the symbol. (b) NAD^+ (◇, ◆) and $NADP^+$ (□, ■). The vertical bars represent the standard deviation where this is larger than the symbol. (c) Reduced/oxidised quotients (% pre-stress quotient, pre-stress value follows symbol); NAD (◇, 0.092; ◆, 0.110) and NADP (□, 0.939; ■, 0.580). (d) Sum of reduced and oxidised forms of NAD (◇, ◆) and NADP (□, ■).

change; it may be that much of the NADH in the alga is bound and of restricted metabolic availability.

The second point is that our results differ fundamentally from the findings of Belmans and Van Laere [12], who reported that "the amount of nicotinamide coenzymes and their degree of reduction did not change significantly" in *D. tertiolecta* subjected to salt stress from 0.1 M to 0.6 M NaCl. This transition was very close to that used here (0.17–0.7 M), and caused similar changes in glycerol content. The reason for the discrepancy between their results and ours in unclear: Belmans and Van Laere used an analytical technique based on polarographic recycling, and presented data only for the reduced/oxidised quotients. Their quotients for NAD are comparable to those reported here, but their NADP quotients are lower, in the range of 0.3–0.5. A recent assessment of methods for extracting pyridine nucleotides from

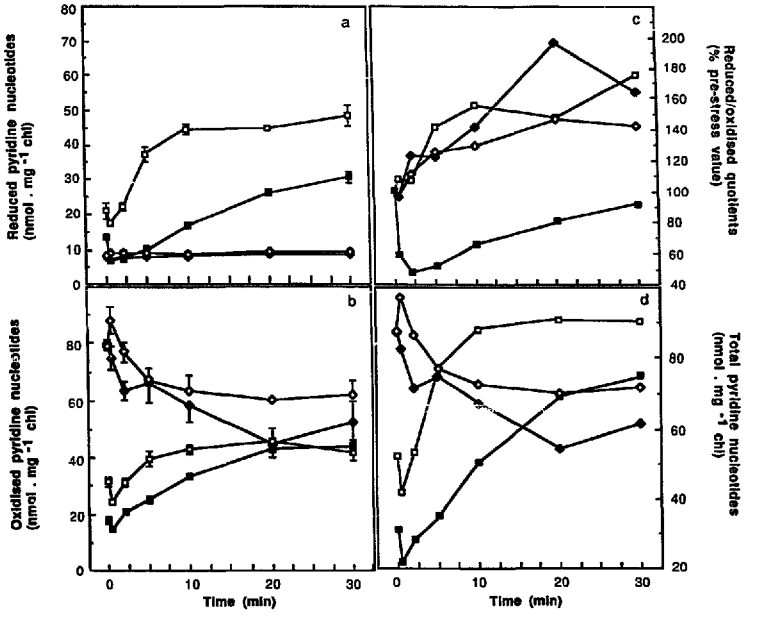

Fig. 3. The response of the pyridine nucleotide content of *D. tertiolecta* to salt stress. The alga was cultured in 0.17 M NaCl and the NaCl concentration in the medium increased to 1.0 M in the light (open symbols) or dark (closed symbols). (a) NADH (◇, ◆) and NADPH (□, ■). The vertical bars represent the standard deviation where this is larger than the symbol. (b) NAD^+ (◇, ◆) and $NADP^+$ (□, ■). The vertical bars represent the standard deviation where this is larger than the symbol. (c) Reduced/oxidised quotients (% pre-stress quotient, pre-stress value follows symbol): NAD (◇, 0.110; ◆, 0.101) and NADP (□, 0.665; ■, 0.759). (d) Sum of reduced and oxidised forms of NAD (◇, ◆) and NADP (□, ■).

plant tissue [13] reported that extraction and analysis procedures essentially similar to those used here gave satisfactory determinations.

The presence of a much higher reduced/oxidised quotient for NADP than NAD is typical for plant [14,15] and animal liver [16] tissue. The ability of the alga to increase its glycerol content on salt stress is not linked obligatorily to photosynthesis, since it can increase glycerol in the dark, which occurs at the expense of starch [17].

Salt stress, which stimulated glycerol accumulation, reduced the NAD^+ content, did not appreciably change NADH and thus raised the reduced/oxidised quotient for NAD, although some decay occurred after 10 min. This happened at both levels of salt stress in both light and dark. NADPH and $NADP^+$ increased overall and the reduced/oxidised quotient for NADP increased under all conditions except one, namely stress to 1.0 M NaCl in the dark where it initially de-

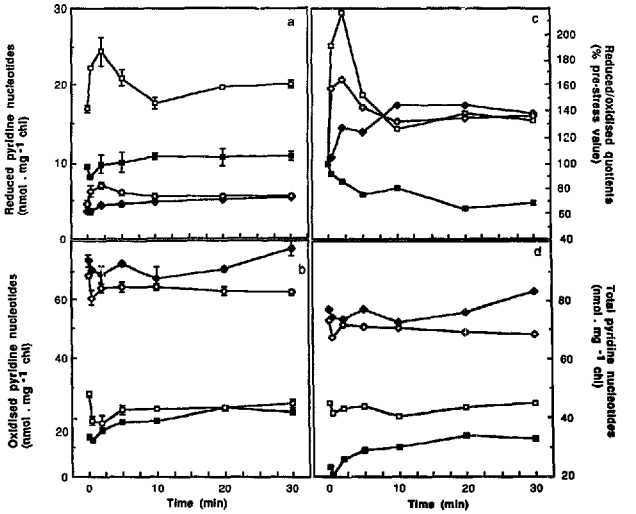

Fig. 4. The response of the pyridine nucleotide content of *D. tertiolecta* to dilution stress. The alga was cultured in 0.7 M NaCl and the NaCl concentration in the medium decreased to 0.17 M in the light (open symbols) or dark (closed symbols). (a) NADH (◇, ◆) and NADPH (□, ■). The vertical bars represent the standard deviation where this is larger than the symbol. (b) NAD^+ (◇, ◆) and $NADP^+$ (□, ■). The vertical bars represent the standard deviation where this is larger than the symbol. (c) Reduced/oxidised quotients (% pre-stress quotient, pre-stress values follows symbol): NAD (◇, 0.067; ◆, 0.050) and NADP (□, 0.602; ■, 0.709). (d) Sum of reduced and oxidised forms of NAD (◇, ◆) and NADP (□, ■).

creased (Fig. 3b). This exception is important, since glycerol did accumulate under these conditions.

The NADPH content of 20 nmol per mg chlorophyll for the alga in 0.17 M NaCl represents an intracellular concentration of 147 μM if the cellular volume is 136 μl per mg chlorophyll (Marengo, T., personal communication), or 222 μM if the cellular volume is 90 μl per mg chlorophyll [18]. The NADH concentration on the same bases would be about one quarter of these values. In crude cell-free extracts, the true K_m(NADH) for glycerol phosphate dehydrogenase was 41–64 μM, depending on the buffer. The true K_m(NADPH) was indeterminate but values for the apparent constants were similar for the two coenzymes, although more variable for NADPH [2]. Thus, intracellular NADH concentrations were of similar magnitude to the K_m(NADH) for glycerol phosphate dehydrogenase, while the intracellular concentration of NADPH is from 3 to 10 times higher than the K_m for the enzyme. Such calculations, it should be noted, take no account of intracellular binding or compartmentation.

The increases in NADPH, NADH and the reduced/oxidised quotient for NAD on salt stress could conceivably stimulate glycerol synthesis from DHAP thermodynamically, through a reduction in redox potential, or kinetically from their involvement in the glycerol phosphate dehydrogenase

reaction. The value of the equilibrium constant for this reaction can be calculated from the equation:

$$K = \frac{[\text{glycerol 3-P}] \cdot [\text{NAD(P)}^+]}{[\text{DHAP}] \cdot [\text{NAD(P)H}] \cdot [\text{H}^+]}$$

The glycerol phosphate/triose phosphate quotient in *D. tertiolecta* has been reported to be about 1 in 0.1 M NaCl and about 3 in 0.6 M NaCl [12]. Assuming the relevant pH to be 7, and taking typical (this work) reduced/oxidised quotients of 0.1 for NAD and 0.6 for NADP, the values of K are $1 \cdot 10^8$–$3 \cdot 10^8$ (NAD) and $1.7 \cdot 10^7$–$5 \cdot 10^7$ (NADP). These values are far below the K_{eq} for the NAD glycerol phosphate dehydrogenase reaction of 10^{12} [19]. For the enzyme to be this far from equilibrium it seems likely either that its activity is strongly inhibited in vivo, or that a product is rapidly sequestered by another reaction. The marked inhibitory effect of ATP (95% inhibition by 0.5 mM ATP) [2] may have relevance to the source of this inhibition.

The higher NADPH content of *D. tertiolecta* in the light can be attributed to additional reduction of the stromal $NADP^+$ pool when photosynthesis was operating [20]. The increase in NADPH content that accompanied salt stress in the dark may result from activation of the oxidative pentose phosphate cycle, since there was no increase in mitochondrial respiration after salt stress. Salt stress also resulted in an increase in total NADP and a decrease, of somewhat smaller magnitude, in total NAD. Thus, while some of the increase in total NADP may be accounted for by phosphorylation of NAD, as occurs in illuminated algal chloroplasts [14], the balance of the increase can be attributed to de novo synthesis.

The increase in glycerol content that occurs after salt stress is likely to be primarily a result of accelerated synthesis rather than reduced breakdown because the turnover time for the intracellular glycerol pool is of the order of 1 h for cells in 0.17 M NaCl [21]. The net synthesis of glycerol that occurs after salt stress requires DHAP as substrate. The source of DHAP for increased glycerol synthesis in darkness is from starch breakdown which is preferentially phosphorolytic in *Dunaliella* [17]. This presumably also applies when photosynthesis is inhibited in the light.

Dilution stress inhibited photosynthesis but resulted in increases of between 30 and 50% in oxygen consumption in the dark, attributed to increased mitochondrial respiration. Dilution stress also resulted in an elevated content of $NADP^+$ (the substrate for the oxidation of glycerol to dihydroxyacetone by glycerol dehydrogenase) in the dark but not in the light. The $NADP^+$ content of about 20 n mol per mg chlorophyll during dilution stress represents an intracellular concentration of 147–222 μM (as before). This is above the $K_m(NADP^+)$ of glycerol dehydrogenase which is in the range 40–100 μM [6]. However, this enzyme is located in the cytosol [22,23] where, in higher plants, the reduced/oxidised quotient for NADP is high irrespective of light-induced changes in this quotient in the chloroplast stroma [20]. It is thus likely that the $NADP^+$ concentration in the cytosol is lower than the whole cell content.

The dissimilation of glycerol on dilution stress should be expected, a priori, to respond directly to changes in $NADP^+$ concentration rather than NAD^+, since glycerol dehydrogenase in NADP-specific [3,6]. Nevertheless, there were sufficiently large differences in the response of NADPH and the reduced/oxidised quotient for NADP between light and dark conditions to discourage further enquiries in this direction as a direct explanation of the stress-induced dissimilation of glycerol.

When *Dunaliella* is subjected to a dilution stress there is little leakage of glycerol to the medium [24,25], except under extreme conditions [26]. Findings contrary to this view have been presented recently [27]. The reason for the extensive leakage of glycerol from *D. tertiolecta* during dilution stress reported by Zidan et al. [27] is unclear, although one possibility is their technique of applying dilution stress to centrifuged pellets of cells, while Kessley and Brown [25] applied it to cell cultures that were preincubated for 24 h after harvesting and resuspension.

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