Arch. Microbiol. 113, 131–138 (1977)

Archives of
Microbiology

The Salt Relations of *Dunaliella*

Further Observations on Glycerol Production and Its Regulation

LESLEY JOYCE BOROWITZKA*, DAVID STUART KESSLY, and AUSTIN DUNCAN BROWN**

School of Microbiology, University of New South Wales, Kensington, N.S.W. 2033,
and Department of Biology, University of Wollongong, N.S.W. 2500, Australia

Abstract. *Dunaliella tertiolecta* (marine) and *D. viridis* (halophilic) were each trained by serial transfer to grow at salt concentrations previously regarded as the other's domain. *D. viridis* then had a salt optimum at 1.0–1.5 M sodium chloride whereas that for *D. tertiolecta* was less than 0–2 M. Nevertheless *D. tertiolecta* grew faster than the halophil at all salt concentrations up to 3.5 M, the highest at which they were compared.

Both species accumulate glycerol, which is necessary for growth at elevated salinities and which responds in its content to water activity (a_w) rather than specifically to salt concentration. Variation in glycerol content is a metabolic process which occurs in the dark from accumulated starch as well as photosynthetically. Regulation of glycerol content by a_w does not require protein synthesis. The NADP-specific glycerol dehydrogenase of each of the algae is likely to be directly involved in the regulation of glycerol content. Kinetic studies, together with those described in an earlier publication, show that the enzyme has regulatory properties and that both glycerol and dihydroxyacetone act as effectors as well as reactants. A mechanism of the reaction is tentatively proposed.

Key words: Alga – Compatible solute – *Dunaliella* – Glycerol – Halophil – Marine – Osmoregulation – Salt relations.

The algal genus, *Dunaliella*, contains species with a marked tolerance of and even an apparent requirement for high salt concentrations. Indeed a recent report by Brock (1975) suggests that some species of *Dunaliella* are the most halotolerant eucaryotic microorganisms known. The salt requirements of these algae are closely related to their ability to accumulate glycerol, the intracellular concentration of which responds positively to changes in environmental salinity (Craigie and McLachlan, 1964; Wegman, 1971; Ben-Amotz and Avron, 1973a; Borowitzka and Brown, 1974; Ben-Amotz, 1975).

The halophilic physiology of a species such as *D. viridis*, in contrast with the moderate salt requirements of the marine species, *D. tertiolecta*, cannot be explained, however, simply by its ability to produce glycerol. Firstly compatible solutes characteristically confer a tolerance rather than a requirement although they might contribute indirectly to a xerophilic physiology (by which is meant a requirement for a reduced water activity) under some circumstances (Brown, 1976). Secondly, the marine species, *D. tertiolecta*, also accumulates glycerol in direct response to salinity and, furthermore, two of its enzymes, glucose-6-phosphate dehydrogenase and glycerol dehydrogenase, are functionally indistinguishable from the corresponding enzymes of *D. viridis* (Borowitzka and Brown, 1974).

On the other hand, the glycerol contents of the two species are normally quite different, in rough proportion to their respective environmental salinities (Borowitzka and Brown, 1974). Glycerol content is thus related to the salt requirements of the two species but the details of cause and effect in this relation are obscure. Nevertheless it would seem that the actual regulation of glycerol accumulation is intimately associated with the salt requirements of the two species. The regulation of glycerol accumulation appears a priori to be interesting and complex, the more so because it presupposes highly specific metabolic regulation by variations in the concentration of an extracellular solute. We can reasonably assume that the NADP-specific glycerol dehydrogenase previously

* *Present address:* Roche Research Institute of Marine Pharmacology, Dee Why, N.S.W. 2099, Australia
** *Address for offprint requests:* Department of Biology, University of Wollongong, Wollongong, N.S.W. 2500, Australia

described by Ben-Amotz and Avron (1973 b) and Borowitzka and Brown (1974) is involved in the process.

The present paper describes additional aspects of *Dunaliella* physiology and of the kinetics of the NADP-specific glycerol dehydrogenase.

MATERIALS AND METHODS

Organisms

The two species *Dunaliella tertiolecta* (marine) and *D. viridis* (halophilic) were maintained and cultured as described by Borowitzka and Brown (1974). For all purposes, except the intermediate steps in training experiments, inocula of the two species were grown respectively in 0.17 M and 3.4 M sodium chloride. In the "training" experiments, *D. tertiolecta* was first transferred from a standard inoculum to a medium containing 1.6 M sodium chloride and thereafter transferred successively to media of higher salt concentration as described in the "Results" section. Conversely, *D. viridis* was transferred initially to a medium containing 1.5 M sodium chloride and thereafter successively to media of lower salt concentration. An inoculum of 1% was used for each serial transfer. In each case the transfer was made soon after the algae had commenced growing exponentially.

The sizes of the steps (positive or negative) in salt concentration were as shown in the results. Algae which had been so trained were plated onto media of several salt concentrations in order to ensure that the training had not been merely a process of selection.

The nitrogen-free medium used in adaptation experiments was the normal growth medium (of Johnson et al., 1968) in which Tris was replaced by phosphate of equivalent concentration and the same pH, nitrate was omitted, ammonium molybdate replaced by sodium molybdate and EDTA by sodium citrate at equivalent concentration.

Analytical Methods

(i) Chemical Analyses. Chemical analyses were essentially as described by Borowitzka and Brown (1974) except that acetone (70% w/v) was used instead of ethanol for extracting the algae and glycerol was estimated by gas chromatography as well as by periodate oxidation. For the chromatographic estimation a column (1 m) of Parapak Q was used isothermally at 170° C. The injection temperature was 250° C and the carrier gas (N_2) used at a flow rate of 56.6 ml/min.

(ii) Enzyme Assays. Glycerol dehydrogenase was assayed in the "forward" direction (glycerol dehydrogenation) as described by Borowitzka and Brown (1974). In the "reverse" direction (reduction of dihydroxyacetone) it was assayed under essentially the same conditions in a reaction mixture (1.0 ml) of the following composition: Tris-HCl (pH 7.5, 81 µmole); enzyme, 0.034 ml (60–200 µg protein); magnesium sulphate (3.4 µmole); dihydroxyacetone and NADPH (sodium salt) as specified. The mixture without NADPH was preincubated for 3 min in a cuvette in a water bath at 30° C and then transferred to the water-jacketed housing of the spectrophotometer, also at 30° C. The reaction was started by adding NADPH and assayed by measuring spectrophotometrically the rate of disappearance of that substance.

(iii) Gel Electrophoresis. Enzyme preparations were subjected to electrophoresis in polyacrylamide gels with a discontinuous buffer system. The method used was that of Aitken and Brown (1972) with the omission of solution 4 (1.0 M sodium chloride). After electrophoresis separate gels were stained specifically for glycerol dehydrogenation or dihydroxyacetone reduction. For the former the reaction mixture contained glycerol (4.0 ml), $NADP^+$ (sodium salt, 7.65 mg), phenazine methosulphate (0.8 mg), nitroblue tetrazolium (8.0 mg), magnesium chloride (100 µmole), potassium cyanide (40 µmole), all in Tris-HCl buffer (pH 8.0, 0.1 M), final volume 20 ml. The gels were incubated in this reagent for 1–2 h at room temperature in the dark and then stored in a solution containing methanol + acetic acid + water (5 : 1 : 5 by vol).

For the reduction of dihydroxyacetone the procedure was as follows. The gels were first incubated in the dark in a solution containing dihydroxyacetone (835 mg), NADPH (10 mg), magnesium chloride (100 µmole) all in Tris-HCl buffer (pH 8.0, 0.1 M), final volume 20 ml. After 10 min the solution was decanted off and the gels washed once with water. They were then incubated for a further 0.5–1.0 h at room temperature in the dark in a solution containing phenazine methosulphate (1.2 mg), nitroblue tetrazolium (12 mg), magnesium chloride (100 µmole), all in Tris buffer as above, final volume, 20 ml. After incubation the gels were stored in methanolic acetic acid as above.

RESULTS

Growth Characteristics

We previously reported that, from standard inocula, *Dunaliella tertiolecta* will grow at sodium chloride concentrations from 0.17 M to about 1.6 M and *D. viridis* from about 1.6 M to at least 4.8 M (Borowitzka and Brown, 1974). Both algae grew fastest at the bottom of their respective salinity ranges. Figure 1 shows, however, that these ranges can be greatly extended by training. In fact, *D. tertiolecta* could be trained to grow in 3.5 M sodium chloride, the highest concentration tested, and *D. viridis* could be trained to grow in 0.3 M sodium chloride, the lowest concentration tested against that species. When they were thus growing in each other's domain, there was still a major physiological difference between the two species; *D. tertiolecta* grew faster than the halophil over the entire concentration range (i.e., "normal" plus trained range). Adaptation to the new range resulted in readjustment of glycerol content to levels normally appropriate (in the other species) to that range (Fig. 2).

Moreover, *D. viridis*, on a single transfer from a "standard" inoculum, grew in a medium containing 0.05 M sodium chloride + sucrose in the range 1.0–2.0 M. Sucrose concentration had little effect on growth rate (Fig. 1) but glycerol content of the alga responded positively to sucrose concentration (Fig. 2).

Glycerol Production in the Dark

The foregoing evidence, together with that already reported, shows glycerol to be an important, probably essential factor in the growth of either species of *Dunaliella* in the presence of salt or in a medium of reduced water activity. In the light, glycerol is an early product of photosynthesis (Wegmann, 1971). It remained to

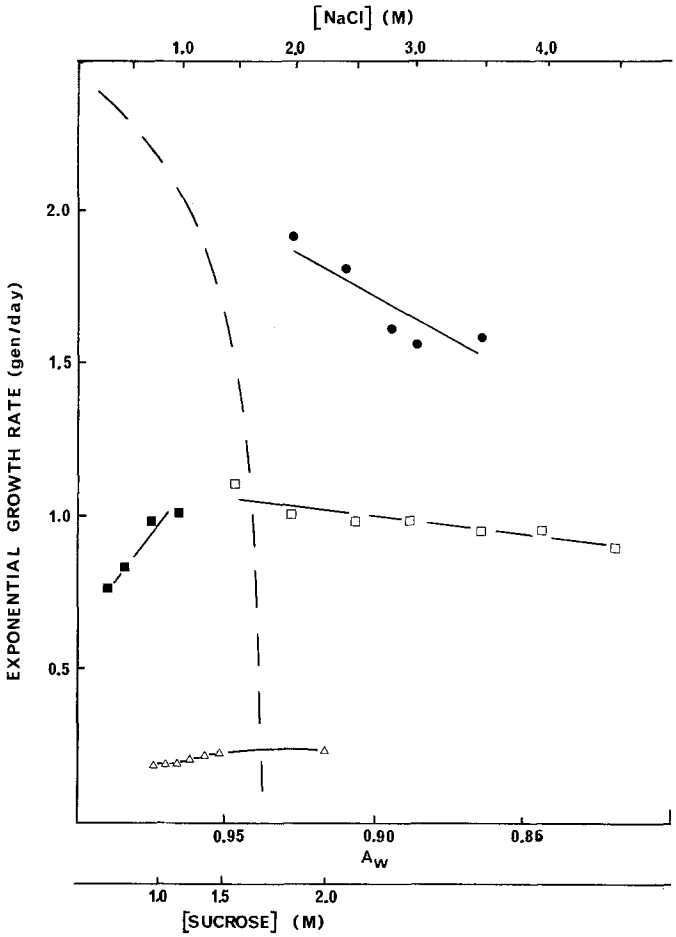

Fig. 1. Effect of water activity (A_w), adjusted with sodium chloride or with sucrose, on the exponential growth rate of "trained" and "untrained" *Dunaliella*. ● *D. tertiolecta* trained by serial transfer to higher salt concentrations; ■ *D. viridis* trained by serial transfer to lower salt concentrations; □ *D. viridis* on direct transfer from a "standard" inoculum in 3.4 M sodium chloride; △ *D. viridis* on direct transfer from a "standard" inoculum to a medium containing 0.05 M sodium chloride + sucrose as indicated. The broken line without points represents results of Borowitzka and Brown (1974) for *D. tertiolecta* on direct transfer from a "standard" inoculum in 0.17 M sodium chloride

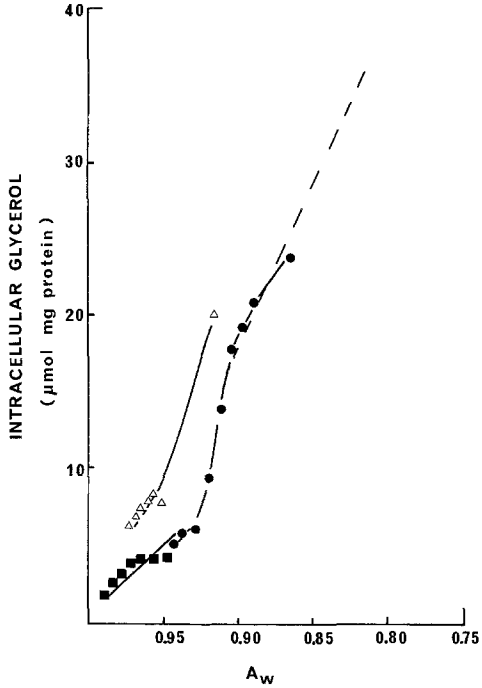

Fig. 2. Effect of water activity (A_w) adjusted with sodium chloride or with sucrose on intracellular glycerol content of "trained" and "untrained" *Dunaliella*. ■ *D. viridis* trained by serial transfer to lower salt concentrations; △ *D. viridis* transferred directly from a "standard" inoculum in 3.4 M sodium chloride to a medium containing 0.05 M sodium chloride + sucrose as in Figure 1. The broken line without points represents the results of Borowitzka and Brown (1974) for *D. viridis* on direct transfer from a standard inoculum in 3.4 M sodium chloride. ● *D. tertiolecta* trained by serial transfer to higher salt concentrations; the unbroken line without points in the lower left hand corner represents results of Borowitzka and Brown (1974) for *D. tertiolecta* grown on direct transfer from a "standard" inoculum in 0.17 M sodium chloride

determine whether the algae were metabolically or energetically able to adapt in the dark to a higher salt concentration. Accordingly *D. viridis* was grown under standard conditions in 1.5 M sodium chloride, checked microscopically for starch granules, harvested and transferred to fresh media containing 2.5, 3.5 and 4.5 M sodium chloride and incubated in the dark at 25° C. Samples were withdrawn at intervals and assayed for intracellular glycerol content. The results are shown in Figure 3 which demonstrates the ability of the algae to resort to heterotrophic metabolism to produce the additional glycerol needed for adaptation to the higher salt concentration. In 2.5 M sodium chloride, algae incubated in the dark produced as much glycerol as when illuminated, almost as much in 3.5 M but much less in 4.5 M sodium chloride. The difference in the last comparison was presumably the result of a starch limitation. At all three concentrations, extended incubation led to a diminution of accumulated glycerol in the dark but not in the light.

Glycerol Production and Protein Synthesis

Although the time scale of response suggested that the adaptive change in glycerol content was a process mediated by preexisting enzymes, the possibility remained that the regulatory mechanism included induction or repression of enzyme synthesis. Accordingly *Dunaliella viridis* was grown in 1.5 M sodium chloride and transferred to a suspension in 3.5 M sodium chloride in the following solutions: (a) complete growth medium; (b) "N-free" medium (see "Materials

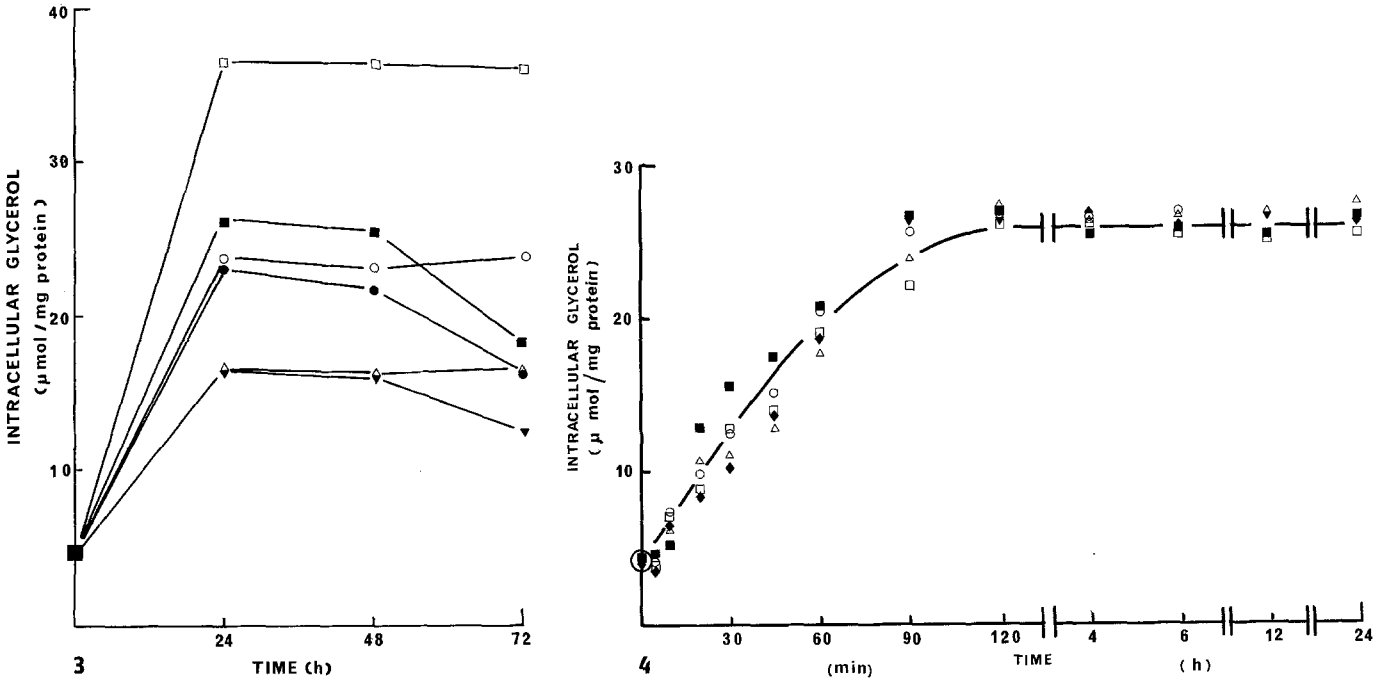

Fig. 3. Glycerol production by *D. viridis* in the light and in the dark. *Open symbols:* illuminated; *closed symbols:* dark; *squares:* incubated in 4.5 M sodium chloride; *circles:* incubated in 3.5 M sodium chloride; *triangles:* incubated in 2.5 M sodium chloride

Fig. 4. Effect of inhibitors of protein synthesis on glycerol accumulation in *D. viridis* when transferred from 1.5–3.5 M sodium chloride. ○ complete growth medium; △ N-free medium; □ N-free medium + rifampicin (25 μg/ml); ◆ N-free medium + chloramphenicol (12.5 μg/ml); ■ N-free medium + cycloheximide (2 μM)

and Methods" section); (c) "N-free" medium + cycloheximide (2 μM); (d) "N-free" medium + chloramphenicol (12.5 μg/ml); (e) "N-free" medium + rifampicin (25 μg/ml). The suspensions were incubated at 26° C with illumination. Samples were collected at intervals and intracellular glycerol content determined. The conditions of inoculation and incubation were as follows. The alga was grown under standard conditions in 1.5 M sodium chloride and harvested from exponential growth phase by centrifugation. The cells were washed once in 1.5 M sodium chloride and resuspended in N-free medium (containing 1.5 M sodium chloride). The cell density of this suspension was adjusted to a level about three times that of the growth medium from which the alga was harvested. This suspension was dispensed into volumes of 2.5 ml and incubated in the light for 24 h to starve the cells of nitrogen. (Suspensions to which rifampicin had been added were pre-incubated in the dark for 24 h to facilitate release of RNA polymerases from chloroplast DNA.) A "zero time" sample was then taken after which the remaining suspensions were mixed with equal volumes of N-free medium containing 5.5 M sodium chloride and inhibitors as indicated. The method of collecting samples was to add acetone (10 ml, 4–6° C) shake vigorously, centrifuge and wash as previously described by Borowitzka and Brown (1974). The "zero time" sample was treated a little differently from the others inasmuch as, after addition of the acetone, 2.5 ml N-free medium in 5.5 M sodium chloride was added. The effect of this was simply to bring acetone concentration down to the same level as in the other samples.

The results are shown in Figure 4 from which it is evident that conditions selected to inhibit protein synthesis did not significantly change the rate of glycerol production in response to increased salinity.

Glycerol Dehydrogenase

(a) Effects of pH. Figure 5 shows the response to changes in pH of the dehydrogenation of glycerol and the hydrogenation of dihydroxyacetone catalyzed by glycerol dehydrogenase preparations from both algae. The two reactions responded differently to pH, the optimum for the "forward" reaction being in the region of pH 8.5–9.0 and for the "reverse" reaction, pH 7.0–7.5. Activity fell sharply at pH values away from the optimum, especially for dihydroxyacetone reduction; at pH 8.8, about the optimum for the forward reaction, the reverse reaction had 16% or less of its maximal activity. No species differences were observed.

In contrast to its effect on reaction velocity, pH had little or no effect on apparent Michaelis constants

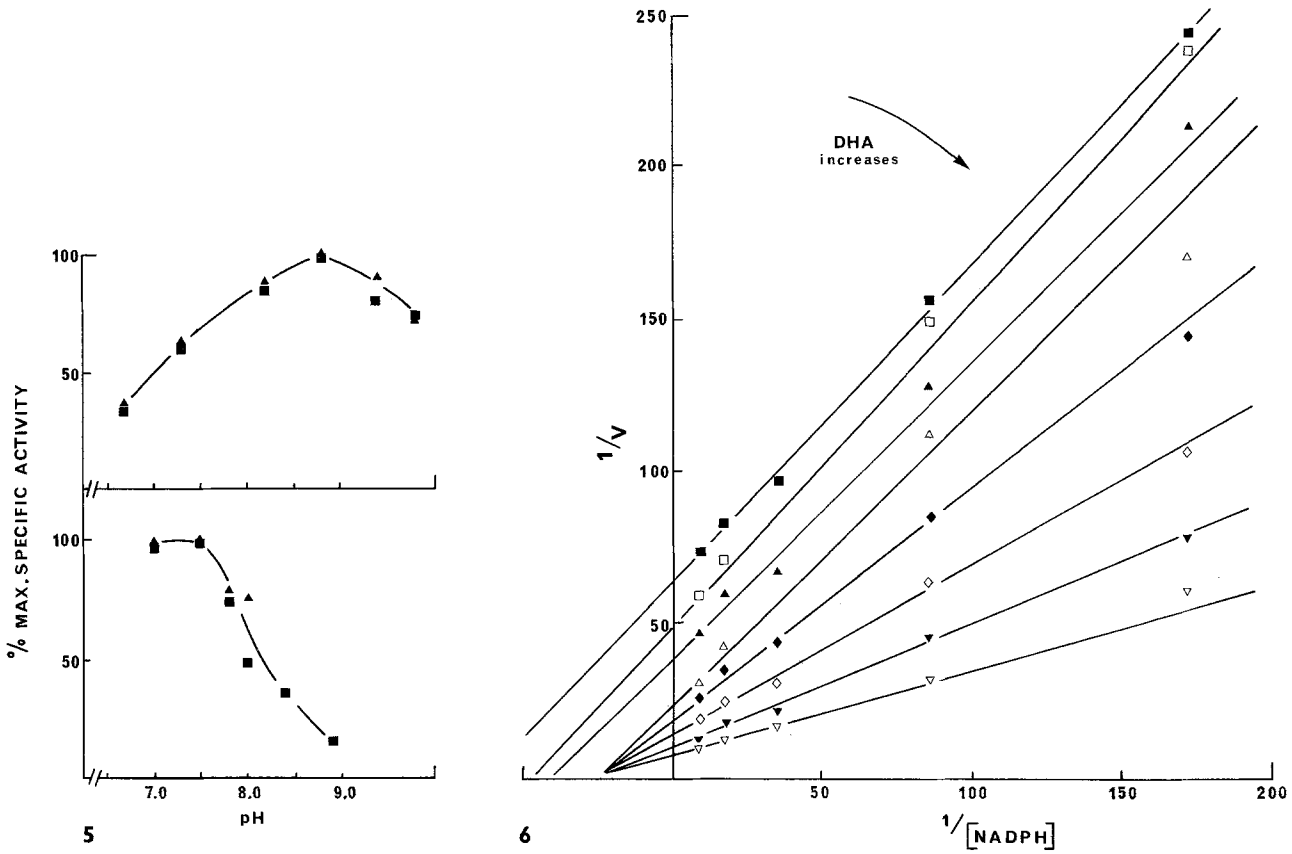

Fig. 5. Upper panel: The effect of pH on the dehydrogenation of glycerol by $NADP^+$ with the glycerol dehydrogenase from *D. tertiolecta* (■) and *D. viridis* (▲). Maximum specific activities were respectively 0.225 and 0.192 μmole $NADP^+$ reduced/min × mg protein. Lower panel: The effect of pH on the reduction of dihydroxyacetone by NADPH with the glycerol dehydrogenase from the two species (symbols as above). Maximum specific activities were 0.012 and 0.023 μmole NADPH oxidized/min × mg protein for *D. tertiolecta* and *D. viridis* respectively. Substrates were used at the following concentrations: glycerol, 2.95 M; $NADP^+$, 0.29 mM; dihydroxyacetone, 0.74 mM; NADPH, 0.029 mM

Fig. 6. Double reciprocal plots of the reduction of dihydroxyacetone with NADPH as variable substrate using the glycerol dehydrogenase from *D. tertiolecta*. Similar results were given by preparations from *D. viridis*. Dihydroxyacetone was used at the following fixed concentrations (mM): from top to bottom, 0.222, 0.296, 0.370, 0.556, 0.740, 1.11, 2.22, 4.44. The unit of V is μmol/min × mg protein. The unit of NADPH concentration is mM

of glycerol and $NADP^+$, the only two substrates for which the constants were determined at two levels of pH. Table 1 lists apparent Michaelis constants for all four substrates.

(b) Initial Velocity Patterns of the "Reverse" Reaction. Borowitzka and Brown (1974) reported results of initial velocity studies of glycerol dehydrogenation. The following kinetic studies of dihydroxyacetone reduction are complementary to the earlier report. Figures 6 and 7 show results of initial velocity measurements, Figure 6 with NADPH as variable substrate, Figure 7 with dihydroxyacetone as variable substrate. Both situations gave complex families of double reciprocal plots. When NADPH was the variable substrate (Fig. 6) there was little or no effect on slope of concentration of the fixed substrate (dihydroxyacetone) at low dihydroxyacetone concentrations. On the other hand, when dihydroxyacetone was the variable substrate, double reciprocals plots were non-linear (concave down) at low fixed NADPH concentrations; they were linear at high fixed $NADP^+$ concentrations (Fig. 7). Identical results were obtained with preparations from both species.

Secondary plots of slope and intercept emphasized these effects. Intercept replots were linear for both forward and reverse reactions whereas slope replots were concave up for the forward and concave down for the reverse reactions (L. J. Borowitzka, Ph.D Thesis, University of New South Wales, Sydney, 1974).

(c) Gel Electrophoresis. Throughout this work no difference, other than in specific activity, could be detected between glycerol dehydrogenase preparations from the two species. Gel electrophoresis, revealed one enzymically active band with the same mobility

Table 1. Apparent Michaelis constants for the forward and reverse reactions of glycerol dehydrogenase preparations from the two algae

	K_m (glycerol) (M)		K_m ($NADP^+$) (mM)		K_m[a] (dihydroxy-acetone (mM)	K_m[a] (NADPH) (mM)
	pH 7.5	pH 9.0	pH 7.5	pH 9.0		
Dunaliella tertiolecta	1.6–3.5	2.2–3.4	0.04–0.10	0.04–0.05	1.8–2.1	0.02–0.05
Dunaliella viridis	2.5–2.7	2.0–2.3	0.04–0.08	0.03–0.06	1.6–2.3	0.02–0.04

Note that the unit of K_m (glycerol) is 1000 times that of the other constants

[a] Measured at pH 7.5

"Fixed" substrates were used at the following concentrations: Glycerol, 0.30–5.87 M; $NaDP^+$ (pH 7.5) 0.23–0.58 mM; (pH 9.0) 0.058–0.23 mM; dihydroxyacetone. 0.22–4.44 mM; NADPH, 0.029–0.12 mM

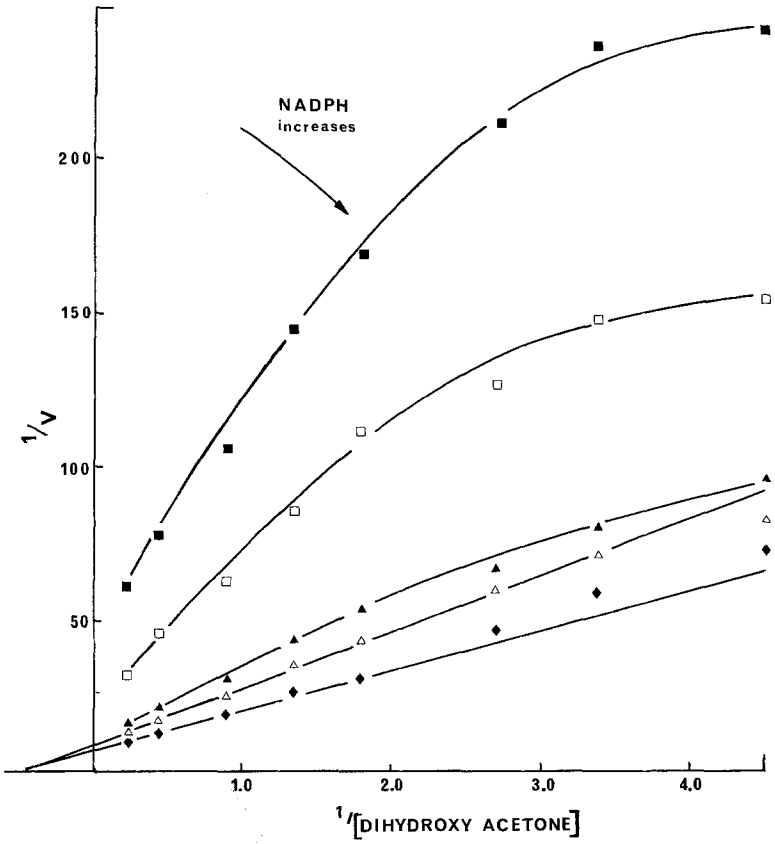

Fig. 7. Double reciprocal plots of the reduction of dihydroxyacetone with NADPH using the glycerol dehydrogenase from *D. tertiolecta*. Dihydroxyacetone was the variable substrate and NADPH was used at the following fixed concentrations (mM): from top to bottom, 0.0058, 0.0116, 0.029, 0.058, 0.116. The two bottom lines were computed, the others were fitted by eye. Enzyme preparations from *D. viridis* gave similar results. The unit of V is μmol/min × mg protein. The unit of dihydroxyacetone concentration is mM

when located either by the forward or reverse reaction and failed to show any difference between the two species.

DISCUSSION

When trained and untrained algae are considered together, *Dunaliella viridis* has a salt optimum for growth rate in the region of 1.0–1.5 M sodium chloride whereas that for *D. tertiolecta* is very much lower (Fig. 1). Indeed this is the only evidence so far available which seems to justify the label "halophilic" for *D. viridis* but even this optimum does not give it any competitive advantage over *D. tertiolecta* under our experimental conditions. The different natural habitats of the two species imply, however, that *D. viridis* does have a selective advantage over *D. tertiolecta* at high salt concentrations in the field. The physiological reasons for this have not been identified but might well involve response to nutrient concentration.

The slow growth rate of *D. viridis* is reminiscent of the xerotolerant ("osmophilic") yeasts which, as

a group, have lower growth rates than those of non-tolerant but otherwise similar species (Anand and Brown, 1968). In that case the lower growth rates correlate with an enhanced contribution by the pentose phosphate pathway to overall metabolic budgets (Brown, 1977). No such correlation is demonstrable with the algae. It is possible that photosynthetic energy conversion occurs at a slower rate in the halophilic species but we have no information on this point.

For all practical purposes, the glycerol contents of the two algae respond identically to any set of experimental conditions which we have used. There is virtually no leakage of glycerol from either species except at very low salt concentrations (D. S. Kessly, unpublished results). Similarly Ben-Amotz (1975) could detect no glycerol leakage from *D. parva* at salt concentrations above 0.6 M. The response of glycerol content to water activity is therefore a metabolic event. Since glycerol is essential for growth at high salinities or low a_w (see Brown, 1976, 1977), the means by which the glycerol content is regulated is central to the salt relations of these algae. The essential features of the control mechanism are that an intracellular metabolic process is regulated across the plasma membrane by the concentration of an extracellular solute which is apparently non-specific and largely impermeant. A similar mechanism operates in yeast (Brown, 1977).

Since the response to a_w occurs in the light or the dark, the regulatory site(s) should be located on that segment of the pathway common to glycolysis and photosynthetic production of glycerol, in other words close to glycerol itself. The mechanism evidently does not involve protein synthesis and thus does not require the induction or repression of an enzyme. The NADP-specific glycerol dehydrogenase is well placed for a central role in the regulation of glycerol content. This enzyme can be assumed to be freely reversible in vivo and is probably immediately responsible for the appearance or disappearance of glycerol in response to salinity changes. Under such circumstances its pH relations are significant. It would thus be susceptible, for example, to the proton flux from the cytoplasm into the chloroplast which occurs during photophosphorylation.

The kinetics of glycerol dehydrogenation by this enzyme are complex (Borowitzka and Brown, 1974). The present work adds to the earlier results with the demonstration that the kinetics of the "reverse" reaction are no less complex. In this direction dihydroxyacetone has a cooperative action but it is negative in contrast to the positive cooperativity of glycerol. There was no evidence of any cooperative action of either $NADP^+$ or NADPH.

$$\begin{array}{ccccccccccc} \text{G.E} & \rightleftharpoons & \text{G.E.N} & \rightleftharpoons & \text{G.E.N.G} & \rightleftharpoons & \text{G.E.N'.D} & \rightleftharpoons & \text{G.E.N'} & \rightleftharpoons & \text{G.E} \quad (1) \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ \text{E} & \rightleftharpoons & \text{E.N} & \rightleftharpoons & \text{E.N.G} & \rightleftharpoons & \text{E.N'.D} & \rightleftharpoons & \text{E.N'} & \rightleftharpoons & \text{E} \quad (2) \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ \text{D.E} & \rightleftharpoons & \text{D.E.N} & \rightleftharpoons & \text{D.E.N.G} & \rightleftharpoons & \text{D.E.N'.D} & \rightleftharpoons & \text{D.E.N'} & \rightleftharpoons & \text{D.E} \quad (3) \end{array}$$

Scheme 1. *D*, dihydroxyacetone; *E*, enzyme; *G*, glycerol; *N*, $NADP^+$; *N'*, NADPH. Effector molecules are shown before the enzyme, reactants after the enzyme

Double reciprocal plots which are concave down can mean either a negative cooperativity, as proposed, or alternatively, a mixture of (iso)enzymes with different Michaelis constants for the variable substrate. Our reasons for rejecting the second explanation are: (i) gel electrophoresis revealed only one enzymically active band able to catalyze either the forward or reverse reaction; (ii) components of an enzyme mixture are unlikely to have substantially different Michaelis constants for one substrate but identical Michaelis constants for the other three; (iii) the negative cooperativity of dihydroxyacetone in one direction and the positive cooperativity of glycerol in the other correlate too well for any explanation of their action which invoked two widely different mechanisms.

Figure 6 suggests that, at low fixed concentrations of dihydroxyacetone the slope effect disappeared, that is the double reciprocal plots were parallel. If that is so, it might imply a change in reaction mechanism under those conditions. Product inhibition studies were of limited value in clarifying the reaction mechanism since it was necessary to use $NADP^+$ at a fixed concentration which was effectively saturating. They did show, however, that, in the direction of dehydrogenation, $NADP^+$ was the first substrate added and NADPH the last product released (L. J. Borowitzka, Ph.D. Thesis, University of New South Wales, Sydney, 1974).

The crucial evidence to emerge from the kinetics is that both glycerol and dihydroxyacetone, in addition to their role as reactants, act as effector molecules, glycerol positively in the forward direction and dihydroxyacetone negatively in the reverse direction. That is to say, they add more than once in the reaction sequence. If the assumption is made that, at physiological concentrations of $NADP^+$, the reaction is sequential, as is common for the pyridine nucleotide-linked dehydrogenases, then the mechanism probably has a form such as shown in Scheme 1. This shows three possible reaction channels, each distinguished by the presence or absence of an effector. In initial velocity measurements channels 1 and 2 would operate in the forward direction with glycerol as effector, channels 2 and 3 in the reverse direction with dihydroxyacetone as effector. In the cell under conditions approximating equilibrium, all channels should be

functional. The scheme is not advanced as a firm conclusion. It is entirely consistent, however, with the kinetics described in this paper, by Borowitzka and Brown (1974) and by L. J. Borowitzka (Ph.D. Thesis, University of New South Wales, Sydney, 1974). It provides a useful working hypothesis for interpreting the physiological role of the glycerol dehydrogenase.

The immediately relevant physiological conclusion is that the glycerol dehydrogenase has the characteristics of a regulatory enzyme which can be controlled by the compatible solute, glycerol, and by its oxidation product, dihydroxyacetone. Furthermore, control by either of these substrates is itself subject to modification by the concentrations of $NADP^+$ and NADPH. In addition the preferred direction of the reaction is quite sensitive to pH.

Thus, at the immediate point of glycerol formation/oxidation, there is a complex regulatory mechanism able to respond to environmental stresses and to stimuli which change the concentration of any of the substrates or of protons. The way in which these variables respond to environmental salinity is under investigation.

REFERENCES

Aitken, D. M., Brown, A. D.: Properties of halophil nicotinamide-adenine dinucleotide phosphate-specific isocitrate dehydrogenase. True Michaelis constants, reaction mechanisms and molecular weights. Biochem. J. **130**, 645–662 (1972)

Anand, J. C., Brown, A. D.: Growth rate patterns of the so-called osmophilic and non-osmophilic yeasts in solutions of polyethylene glycol. J. gen. Microbiol. **52**, 205–212 (1968)

Ben-Amotz, A.: Adaptation of the unicellular alga *Dunaliella parva* to a saline environment. J. Phycol. **11**, 50–54 (1975)

Ben-Amotz, A., Avron, M.: The role of glycerol in the osmotic regulation of the halophilic alga *Dunaliella parva*. Plant Physiol. **51**, 875–878 (1973a)

Ben-Amotz, A., Avron, M.: NADP specific dihydroxyacetone reductase from *Dunaliella parva*. FEBS Letters **29**, 153–155 (1973b)

Borowitzka, L. J., Brown, A. D.: The salt relations of marine and halophilic species of the unicellular green alga, *Dunaliella*. The role of glycerol as a compatible solute. Arch. Microbiol. **96**, 37–52 (1974)

Brock, T. D.: Salinity and the ecology of *Dunaliella* from Great Salt Lake. J. gen. Microbiol. **89**, 285–292 (1975)

Brown, A. D.: Microbial water stress. Bact. Rev. **40**, 803–846 (1976)

Brown, A. D.: Compatible solutes and extreme water stress in eucaryotic microorganisms. Advanc. Microbial Physiol. **17**, (in press, 1977)

Craigie, J. S., McLachlan, J.: Glycerol as a photosynthetic product in *Dunaliella tertiolecta* Butcher. Canad. J. Bot. **42**, 777–778 (1964)

Johnson, M. K., Johnson, E. J., MacElroy, R. D., Speer, H. L., Bruff, B. S.: Effects of salts on the halophilic alga *Dunaliella viridis*. J. Bact. **95**, 1461–1468 (1968)

Wegmann, K.: Osmotic regulation of photosynthetic glycerol production in *Dunaliella*. Biochim. biophys. Acta (Amst.) **234**, 317–323 (1971)

Received December 8, 1976