Regulation of Glycerol Synthesis in Response to Osmotic Changes in *Dunaliella*

Edith Chitlaru and Uri Pick*

Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT

Changes in phosphometabolites, following osmotic shock, were analyzed by two-dimensional thin layer chromatography, in extracts of the halotolerant alga *Dunaliella salina* in order to clarify the regulation of glycerol synthesis from starch. The experiments were carried out in wild-type and in osmotically defective mutant cells. It is demonstrated that hyperosmotic shock induces a decrease in fructose 6-phosphate and an increase in fructose-1,6-bisphosphate indicating the activation of phosphofructokinase. Two mutants, which are specifically defective in their response to hyperosmotic shock, accumulate glucose 6-phosphate or phosphogluconate following shock, and have remarkably reduced activities of glucose-6-phosphate dehydrogenase and of phosphogluconate dehydrogenase, respectively. These results indicate that the pentose-phosphate oxidative pathway has a major role in glycerol synthesis. Hyperosmotic shock leads to a transient accumulation of phosphorylcholine and to a decrease of inositolbisphosphate in *D. salina* extracts. Accumulation of phosphorylcholine is not detected in osmotically defective mutants. Hypoosmotic shock induces an increase in inositolbisphosphate but not in phosphorylcholine. These results are consistent with previous indications for differential activations of phospholipases by hyper or hypoosmotic shock in *Dunaliella*. Based on these results we suggest that (a) phosphofructokinase is an important checkpoint enzyme in the regulation of glycerol production, and (b) that the pentose-phosphate pathway has a major role in keeping oxidation-reduction balance during glycerol synthesis. The possible role of lipid breakdown products as second messengers in regulating glycerol production in *Dunaliella* is discussed.

*Dunaliella* is a unicellular halotolerant green alga which adapts to a large range of NaCl concentrations, from 0.1 to 5 M, by the synthesis of intracellular glycerol. Previous studies have shown that glycerol is made primarily from starch reserves in the chloroplast (3, 4, 6, 9), but the exact metabolic pathway leading to glycerol synthesis is not known. Four enzymes which apparently are involved in glycerol metabolism have been described, three of them unique to *Dunaliella*. It has been proposed that glycerol is made from DHAP (Table 1 contains complete list of abbreviations) via a GP-DH and a GP-ase, or reconverted to DHAP via a G-DH and a DHA-K (reviewed in ref. 4). The exact enzymatic pathway leading to DHAP formation from starch is still unclear and the energetic requirements in ATP and reduced pyridine nucleotides are not known. The observation that glycerol production proceeds either in the light or in the dark suggests that there is no obligatory requirement for photosynthesis neither as an energy source nor as a carbon source. It is also unclear how is glycerol synthesis and its reconversion to starch being regulated. It has been clearly established that protein synthesis is not involved in the induction of glycerol production since inhibitors of protein synthesis do not affect glycerol production in response to hyperosmotic shocks (24). It is also unknown which enzymes in the metabolic pathway for glycerol synthesis are involved in regulating the process. Previous observations of a transient increase in the level of GP following hyperosmotic shocks in *Dunaliella tertiolecta* (1) points to GP-DH as a possible candidate. However, *in vitro* comparisons of the activity of GP-DH isolated from the alga before or after osmotic shocks gave no indications for changes in its activity and the same was observed for other suspected key enzymes in the process, suggesting that covalent modification of enzymes is not likely to be involved in the activation of glycerol synthesis (1). A further complication in the regulation of glycerol synthesis is the intracellular compartmentation of the process, between the cytoplasm and the chloroplast which calls for an integrated multicompartmental regulation (7).

Several factors which have been suggested to be involved in regulating glycerol synthesis are the intracellular pH, ATP and phosphate. Intracellular pH changes following hyperosmotic shocks in *Dunaliella* have been reported (15, 20) and suggested to be involved in the induction of starch breakdown in view of the sharp pH dependence of starch-mobilizing enzymes in this alga (15). The rapid and transient drop in ATP following hyperosmotic shock (1, 10, 22) may contribute to the activation of GP-DH and of PFK which are inhibited by ATP concentration exceeding 1 mm (19, 21). Observations of an increase in inorganic phosphate in *Dunaliella* following hyperosmotic shocks (14, 15, 20) and our observations of a correlation between the rate of glycerol synthesis or elimination with the cellular phosphate level, have led us to propose that the influx of phosphate from the cytoplasm into the chloroplast, in exchange for triose phosphates via the phosphate translocator, is an important link in the regulation of glycerol synthesis (5).

Another interesting set of observations in *Dunaliella*, whose connection to the regulation of glycerol synthesis is not clear, is the enhanced turnover of specific phospholipids following osmotic changes in this alga. It has been observed by the group of Thompson that hypoosmotic shocks induced increased turnover of phosphoinositol phospholipids in *Dunaliella salina*, indicating the activation of a specific PL-C in the plasma membrane of this alga (11). Conversely, hyperosmotic shocks inhibit the turnover of phosphoinositol phos-
phospholipids and stimulate the turnover of phosphatidylcholine and of phosphatidic acid indicating the activation of a different type of phospholipases (12). Since such phospholipases are involved in regulating a variety of physiological responses in animals and in plants, it may be expected to be involved also in regulating glycerol production in Dunaliella.

In an attempt to get a better understanding of the regulation of glycerol synthesis in Dunaliella, we have analyzed changes in different phosphometabolites following osmotic shocks by two-dimensional TLC. Our aims were (a) to identify checkpoint enzymes in glycerol metabolism; (b) to identify the metabolic defects in osmotically defective D. salina mutants which we have recently isolated (8); (c) to identify phospholipid breakdown products which are expected to accumulate as a result of phospholipase activation.

MATERIALS AND METHODS

Growth Conditions

Dunaliella salina was obtained from the culture collection of Dr. Thomas (Fisheries Institute, La Jolla, CA). The cells were grown in batch cultures, as previously described (8) in the presence of 0.1 or 1 mM NaCl.

Cell concentration and volume was determined using a Coulter Counter (model F) with a 100 μm orifice.

Preparation of Water-Soluble Cell Extracts of Dunaliella

The extracts were prepared essentially according to Ishii et al. (17). Cells (5 x 10^6) were prelabeled with 32P (80 μCi carrier free, 12 h). The cells were hyperosmotically or hypoosmotically shocked by dilution with 5 M NaCl, or H2O, respectively or with isoosmotic media (control samples). Cells were collected by centrifugation (5 min at 3000g at 0°C), and quenched with a cold (0°C) mixture containing chloroform/methanol/water (0.6 mL/1.5 mL/0.2 mL), mixed on vortex and incubated for 30 min at 0°C. Cell debris and denatured protein were removed by centrifugation (20 min at 1000 rpm at 0°C). Cold chloroform (0.6 mL) and H2O (0.8 mL) were added to the supernatant to achieve phase separation and after centrifugation (20 min at 1000 rpm at 0°C) the upper phase was separated, lyophilized, and resuspended in H2O (40 μL).

Separation of 32P Water-Soluble Metabolites on Two-Dimensional TLC Plates

The separation on cellulose TLC plates (20 x 20, Macherey Nagel MN 300) was done following the protocols described by Feige et al. (13). A sample of extracts containing 32P-labeled metabolites (2 μL) and nonradioactive markers (1 μL of 0.1 M solutions) were applied to the plates, dried, and developed in the following solvent mixtures for 5 to 8 h at each state with about 10 h of drying in between.

First dimension (first run): isobuteric acid, NH3, H2O, and EDTA (250 mL, 30 mL, 120 mL, and 0.125 g). Solvents for the second dimension (second run): n-butanol, n-propanol, propionic acid, and H2O (200 mL, 87.5 mL, 142.5 mL, and 186.5 mL); followed by (third run): n-butanol, acetic acid, and H2O (500 mL, 100 mL, and 40 mL). The upper phase of this biphase mixture was used. The radioactive chromatograms were exposed to Kodak films for 24 h at room temperature and the autoradiograms were scanned with a Molecular Dynamics 300A computing densitometer.

Detection of Phosphometabolite Markers on the Chromatograms

Dried chromatograms were sprayed with a molybdate reagent containing: perchloric acid, hydrochloric acid, ammonium molybdate, and H2O (5 mL of 60% w/w, 10 mL of 1 N, 25 mL of 4% w/v, and water to 100 mL) and after drying, were illuminated with a portable UV lamp. Phosphate-containing metabolites appear within seconds as blue spots.

Enzymatic Treatments of Extracted Metabolites

Fru-1,6-bisP

The reaction mixture contained 10 mM Tris buffer (pH 9.6), 2 mM MgCl2, 0.6 mM EDTA, 20 μL extract, and H2O to final volume of 200 μL. The reaction was started by addition of 2 μL Fru-1,6-bisP phosphatase (6 units/mg protein, Sigma),

<table>
<thead>
<tr>
<th>Table I. Abbreviations Used in This Paper</th>
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<tr>
<td>DHAP</td>
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<tr>
<td>Fru-6P</td>
</tr>
<tr>
<td>Fru-1,6-bisP</td>
</tr>
<tr>
<td>Glc-6P</td>
</tr>
<tr>
<td>GP</td>
</tr>
<tr>
<td>IP3, IP6</td>
</tr>
<tr>
<td>PyrC</td>
</tr>
<tr>
<td>PGA</td>
</tr>
<tr>
<td>6PGlc</td>
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<tr>
<td>Rib-1,5-bisP</td>
</tr>
<tr>
<td>DHA-K</td>
</tr>
<tr>
<td>Glc-6P-DH</td>
</tr>
<tr>
<td>GP-ase</td>
</tr>
<tr>
<td>GP-DH</td>
</tr>
<tr>
<td>6PGlc-DH</td>
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<tr>
<td>PL-C</td>
</tr>
</tbody>
</table>
Figure 1. Changes in Dunaliella phosphometabolites following hypertonic shock. Water-soluble phosphometabolites extracted from 32P-labeled cells grown at 100 mM NaCl (A) or transferred for 15 min to 300 mM (B) were separated by two-dimensional TLC on cellulose plates. After 24 h exposure the autoradiographs were scanned by a densitometer (C before and D after hypertonic shock).

Table II. Changes in Phosphometabolites in Dunaliella Induced by Hypertonic Shock

TLC autoradiographs of the experiment shown in Figure 1 were scanned and analyzed by a densitometer connected to a programmed computer. Analysis of components 2, 3, and 5 was performed with chromatograms developed twice in the second dimension (including a third run) for better resolution of spots 3/4 and 2/5. The content of identified metabolites was calculated with reference to ATP (spot No. 10, 3.5 nmol/10^7 cells, measured by the luciferase assay), and expressed as percentage of the overall phosphometabolites on the TLC plate (relative intensity) and in nmole per 10^7 cells. Averages of two experiments are presented.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Metabolite</th>
<th>Relative Intensity</th>
<th>Calculated Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before hyperosmotic shock</td>
<td>After hyperosmotic shock</td>
</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>nmol/10^7 cells</td>
</tr>
<tr>
<td>2</td>
<td>Glo-6P</td>
<td>2.5</td>
<td>3.4</td>
</tr>
<tr>
<td>3</td>
<td>Fru-1,6-bisP</td>
<td>3.9</td>
<td>12.8</td>
</tr>
<tr>
<td>5</td>
<td>Rib-1,5-bisP</td>
<td>3.9</td>
<td>6.6</td>
</tr>
<tr>
<td>8</td>
<td>PyrC</td>
<td>0.0</td>
<td>0.7</td>
</tr>
<tr>
<td>9</td>
<td>Fru-6P</td>
<td>4.5</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>ATP + ADP</td>
<td>9.1</td>
<td>8.6</td>
</tr>
<tr>
<td>13</td>
<td>IP_2</td>
<td>3.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>
incubated for 15 min at 25°C, supplemented with 3.6 mM Fru-1,6-bisP and incubated for an additional 15 min. The extracts were leophylized and resuspended in 20 μL H2O.

**Glc-6P**

The reaction mixture contained 10 mM Tris-Mops (pH 7), 2 mM MgCl₂, 3.9 mM NADP, and H₂O to final volume 200 μL. The reaction was started by the addition of 0.6 μL Glu-6P-DH (470 units/mg protein, Sigma), incubated for 35 min at 25°C, supplemented with 3 mM Glu-6-DH and incubated for an additional 15 min. The extracts were leophylized and resuspended in 10 μL of H₂O.

**Phosphogluconate**

The reaction mixture contained 10 mM Tris-Mops (pH 7), 2 mM MgCl₂, 0.4 mM NADP, H₂O to final volume 200 μL. The reaction was started by the addition of 1 μL of 6P-Glu-DH (33 units/mg protein, Sigma), incubated for 15 min at 37°C, supplemented with 3 mM GP-Glu for an additional 15 min. The extracts were leophylized and resuspended in 10 μL of H₂O.

**Crude Enzyme Extracts**

Enzyme preparations from *Dunaliella* were made essentially according to Belmans and Van Laere (1). Cells (10⁶ cells/sample, cultured at 1 mM NaCl) were washed with extraction buffer (50 mM Tris buffer [pH 7.5], 1.6 mM glycerol, 2 mM DTT, and 2 mM EDTA) by centrifugation (2,000g, 0°C, 10 min) and resuspended in 1 mL buffer. The cells were broken by vigorous shaking (2 min) with 3 g glass beads (0.5 mm diameter). The extracts were filtered by centrifugation through miracloth (1,000g, 5 min, 0°C), and the insoluble material was removed by centrifugation (10,000g, 4 min). The supernatant was quickly desalted by centrifugation (1,000g, 10 min, 0°C) through a 10 mL Sephadex G-50 column equilibrated with extraction buffer. The enzyme activities were measured at 25°C within 15 min after the preparation.

**Enzymatic Assays**

Reactions were carried out in a Cary 18 spectrophotometer at 340 nm. Initial rates of NADPH formation were monitored at 340 nm following addition of 0.15 mg NADP to 1 mL.

**Glc-6P-DH**

The reaction mixture contained 80 mM Tris buffer (pH 7.5), 6.6 mM MgCl₂, phosphogluconate 0.33 mg/mL, 50 μL extract, and H₂O to 1 mL final volume.

**DHA-Reductase**

The reaction mixture contained 20 mM Tris-glycine (pH 9.2), 2.5 mM glycerol, 50 μL extract, and H₂O to 1 mL final volume.

**Glycerol Content**

Glycerol was determined as previously described (8).

**RESULTS**

**Changes in Phosphometabolites following Hyperosmotic Shocks**

Identification of cellular metabolites depends on an effective extraction procedure which does not activate hydrolytic enzymes, and on a sensitive analytical separation technique. In *Dunaliella*, water-soluble metabolites can be extracted by a cold mixture of chloroform/methanol (adapted by E Chiariu and M Bental from Ishii et al. [17]). The ATP yield obtained by this extraction procedure is essentially identical to the values estimated by the Luciferase assay in intact cells (3–4 nmol/10⁷ cells) indicating that the extraction is complete and does not trigger hydrolytic activities.

Separation of ³²P-labeled phosphometabolites was obtained...
by TLC on cellulose sheets following a procedure developed especially for analysis of plant extracts (8). Because salt was found to interfere with the TLC separation, some of the experiments were performed with cells which have been adapted to relatively low salinities (0.1 or 0.3 mM NaCl), and the extracellular NaCl has been removed by a brief centrifugation of the cells before the extraction.

It may be noted that the physiological and biochemical responses of Dunaliella salina to a hyperosmotic shock from 0.1 → 0.3 mM NaCl resembles that from 1 → 2.5 mM NaCl with respect to changes in volume, glycerol, and starch contents and effects on photosynthesis, ATP, and internal Na⁺ except that in the former the recovery of the cells is faster.

Figure 1 demonstrates autoradiograms of phosphometabolites from cells cultured at 0.1 mM NaCl before (A) or 15 min after a hypertonic shock (B). Since two developments did not always lead to a complete separation of components, a third development (in the second dimension) was often employed for better resolution. The densitometric traces including the indicated spot numbers are shown in Figure 1, C and D, and the calculated contents of corresponding metabolites are summarized in Table II. Not all the metabolites are well resolved by this technique—for example the major spot, designated No. 1, contains inorganic phosphate, PGA, DHAP, and GP. Therefore, these metabolites were not analyzed in this work.

Nevertheless, several phosphometabolites which are resolved by the TLC undergo considerable changes following hyperosmotic shock. Spot No. 3, which comigrates with Fru-1,6-bisP, is increased by 3- to 4-fold, whereas spot No. 9, comigrating with Fru-6P decreases by about 10-fold. A direct demonstration that spots 3 and 9 are indeed Fru-1,6-bisP and Fru-6P, respectively, is shown in the experiment summarized in Table III. Incubation of the extract of hypertonically shocked cells with Fru-1,6-bisP phosphatase significantly reduces spot No. 3 and also converts externally added Fru-1,6-bisP (identified with the molybdate spray reagent) to components which comigrate with Fru-6P and Pi (spots 9 and 1, respectively, not shown). These results suggest that hyperosmotic shock stimulates the conversion of Fru-6P to Fru-1,6-bisP.

Figure 3. Enzymatic identification of accumulated phosphometabolites in Dunaliella osmotic mutants, following hyperosmotic shock. Water-soluble extracts of 32P-labeled Dunaliella mutant cells UV22 and NG8 were separated by TLC (in one dimension), before (A, B) or 180 min after (A, B, E, F) hyperosmotic shock (100–300 mM NaCl). The extracts were treated with Glc-6P-DH (B) or P-Glc-DH (D and F). Two different concentrations of each extract (parallel lanes except for E) were separated. Arrows indicate the location of Glc-6P (solid) or P-Glc (open).

Other sugar phosphates are affected to a smaller extent. The slight accumulation of Rib-1,5-bisP may be a reflection of the partial inhibition of photosynthesis under these conditions (by about 30%, not shown). The two other prominent changes following an osmotic shock are the appearance of a new component (spot 8), and the disappearance of another component (spot 13) which will be described below.

Identification of Metabolic Defects in Two Osmotically Defective Mutants

In a previous publication we have described the selection and characterization of D. salina mutants which are specifically defective in their capacity to respond to hyperosmotic shock (8). These mutants, which grow like wild-type cells at 1 mM NaCl, require a prolonged period of time to adjust to hyperosmotic shocks (days instead of hours) indicating that an essential step in glycerol formation is defective.

When two of these mutants are exposed to a hyperosmotic shock, different metabolites accumulate and become the major soluble phosphometabolites (Fig. 2). In the mutant UV22 (term U22 in ref. 8) a component which comigrates with Glc-6P appears as the major metabolite (B), whereas in the mutant NG8 (term D8 in ref. 8) a different spot appears which is not apparent in control cells and comigrates with 6P.
Table V. Changes in Phosphometabolites Induced by Hypertonic Shocks in Osmotically Defective Mutants of Dunaliella

32P-labeled phosphometabolites were extracted from 1 M NaCl adapted wild-type, UV22, and NG8 cell lines before (0 time), 40 min, or 180 min after transfer to 2.5 M NaCl. Separation and analysis of phosphometabolites was performed as in Figure 1 and Table I.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Metabolite</th>
<th>Wild type</th>
<th>UV22</th>
<th>NG8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>40</td>
<td>180</td>
</tr>
<tr>
<td>1</td>
<td>Pi, PGA, DHAP</td>
<td>56.6</td>
<td>33.5</td>
<td>42.2</td>
</tr>
<tr>
<td>2</td>
<td>Glc-6P</td>
<td>5.4</td>
<td>3.5</td>
<td>8.1</td>
</tr>
<tr>
<td>3</td>
<td>Fru 1,6 bis P</td>
<td>1.7</td>
<td>6.4</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>nib</td>
<td>1.8</td>
<td>6.0</td>
<td>2.6</td>
</tr>
<tr>
<td>5</td>
<td>Rib 1,5 bis P</td>
<td>4.8</td>
<td>16.4</td>
<td>6.8</td>
</tr>
<tr>
<td>6</td>
<td>ni</td>
<td>5.2</td>
<td>5.4</td>
<td>9.9</td>
</tr>
<tr>
<td>7</td>
<td>ni</td>
<td>0.06</td>
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<td>1.1</td>
</tr>
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<td>8</td>
<td>PyrIC</td>
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<td>0.3</td>
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<td>Fru-6 P</td>
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<td>0.08</td>
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<td>16.8</td>
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</tr>
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<td>11</td>
<td>ni</td>
<td>10.4</td>
<td>7.9</td>
<td>10.2</td>
</tr>
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<td>12</td>
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<tr>
<td>14</td>
<td>6P-Glc</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
</tbody>
</table>

* Not identified.

Figure 4. Relative changes in phosphometabolites in Dunaliella osmotic mutants, UV22 and NG8, following hypertonic shock. Water-soluble phosphometabolites extracted from 32P-labeled Dunaliella wild-type (center) and osmotic mutants, UV22 (bottom) and NG8 (top) were separated by two-dimensional TLC, 0 (open bars), 40 (striated bars), and 180 (solid bars) min after hypertonic shock from 1 to 2 M NaCl.
Glu (C). In order to ascertain the identification of these metabolites, the extracts from the osmotically shocked mutants have been exposed to specific enzymatic tests. As is demonstrated in Figure 3 treatment of the UV22 extract with Gls-6P-DH converts the accumulated metabolite to 6P-Glc (Fig. 3, A and B). Treatment of the NG8 extract with 6P-Glc-DH converts the accumulated metabolite to a substrate which comigrates with Gls-6P (Fig. 3, E and F). These results may indicate that enzymes which metabolize Gls-6P and 6P-Glc are defective in the mutants. In order to check this possibility, soluble extracts of the mutants and wild-type cells were tested for Gls-6P-DH, 6P-Glc-DH, and dihydroxyacetone reductase activities. The latter enzyme served as a control. As is demonstrated in Table IV the UV22 mutant has a greatly reduced activity of Gls-6P-DH, whereas the NG8 mutant has a reduced activity of both 6P-Glc-DH and Gls-6P-DH. These results are consistent with the accumulation of Gls-6P and 6P-Glc in the mutants (Fig. 2). Since these dehydrogenases catalyze the first steps in the oxidative pentose-phosphate pathway, the results suggest that this pathway is essential for glycerol production in Dunaliella.

A more complete analysis of the changes of different metabolites is given in Table V, and the major changes in the mutants are summarized in Figure 4. In the UV22 mutant there is a significant increase in the steady-state level of Gls-6P (2-fold) and of Fru-6P (30-fold!) before the shock (spots 2 and 9 in Table V, respectively) as well as a massive accumulation of Gls-6P following upshock (Fig. 4, bottom). These results may indicate that the low activity of Gls-6P-DH limits the degradation of carbohydrates in this mutant also under steady-state conditions. In the NG8 mutant, the massive accumulation of 6P-Glc is accompanied by a minor accumulation of Gls-6P (cf. Fig. 4, top to center). Interestingly, in this mutant the steady-state level of Fru-6P is 5-fold higher than in control cells but drops following osmotic upshocks, similar to wild-type cells, indicating activation of PFK. An interesting difference between the 1 m NaCl adapted cells (Table V) and the 0.1 m NaCl cells (Table II) is the level of Fru-6P. The steady-state concentration of Fru-6P is 7- to 8-fold lower than in low-salt adapted cells, and the Fru-1,6-bis P/Fru-6P ratio is about 3-fold higher (2.7 compared to 0.87 in 1 m and 0.1 m NaCl, respectively). These results may indicate that in high NaCl the activity of PFK is higher than in low NaCl also under steady-state conditions, as might be expected from the higher glycerol synthesis in the former. Nevertheless, the effect of hyperosmotic shocks on fructosephosphates in 1 m NaCl cell extracts are generally similar to the changes in 0.1 m NaCl cell extracts. The shock induces a 30-fold increase in Fru-1,6 bisP/Fru-6P in both high and low NaCl-adapted cells indicating a similar activation of PFK. These results suggest that the activation of PFK is a response to the osmotic shock and not to the increased NaCl in the medium.

### Changes in Phospholipid Metabolic Products following Hyperosmotic Shocks

As mentioned above, two major changes following hyperosmotic shocks are the appearance of a new component (spot 8, Fig. 1), which comigrates with PrylC, and the disappearance of another component (spot 13, Fig. 1) which comigrates with IP3. In order to find out if the accumulation of the putative PrylC is correlated with the stimulated turnover of phosphatidylcholine (12) we tried to estimate the time course of PrylC accumulation. As shown in Figure 5B the accumulation of PrylC is transient, starting within minutes after the shock and decreasing toward the completion of glycerol synthesis (Fig. 5; see also Table VA, spot 8). This transient accumulation resembles the transient stimulation of phosphatidylcholine turnover reported earlier (12).

The transient accumulation of PrylC following hyperosmotic shocks is observed also in 1 m NaCl cells (Table V, spot 8) indicating that it is a response to the osmotic shock and

**Figure 5.** Kinetics of glycerol synthesis and phosphorylcholine accumulation during hypertonic shock. Samples of osmotically shocked cells (100-300 mm NaCl) were taken at different times after the shock for glycerol (A) and phosphorylcholine (B) measurements. Averages of two experiments are presented. The phosphorylcholine content was calculated in Table I.
not to the increased salt in the medium. Therefore, it might be expected that in osmotically defective *Dunaliella* mutants, PC accumulation may be affected. As is demonstrated in Table V, PrylC does not appear following hyperosmotic shocks in either of the mutants, substantiating the correlation between appearance of this metabolite and the capacity of the cells to respond to hyperosmotic shocks.

The appearance of the putative IP$_2$ in control cells is not reproducible (cf. Figs. 1 and 6) and therefore has not been further explored.

**Changes in Phosphometabolites following Hypoosmotic Shocks**

In view of the changes in metabolites following hyperosmotic shock, it seemed of interest to check the effect of hypoosmotic shock on *Dunaliella* cells, in order to get an indication as to whether the same or different metabolites are involved in the regulation of glycerol conversion to starch.

As is demonstrated in Figure 6, hypoosmotic shock induces different changes in phosphometabolites as compared to hyperosmotic shock. The most prominent change is a clear accumulation of two fast-moving components (indicated by arrows), the larger of which comigrates with IP$_2$. It should be stressed that the accumulation of this metabolite is reproducible irrespective of its presence or absence before the shock. Another difference with respect to hyperosmotic shocks is that hypoosmotic shocks induce only small changes in Fru-6P and Fru-1,6-bisP (a slight increase in both, not shown). These results suggest that the metabolic changes involved in regulating glycerol conversion to starch differ from those involved in regulating glycerol formation.

**DISCUSSION**

The exact metabolic pathway leading to glycerol synthesis from starch in *Dunaliella* is not known yet, particularly the initial steps, which take place inside the chloroplast (15). We have chosen to approach this subject by the isolation and characterization of mutants which are impaired in their osmoregulation. The isolation and preliminary characterization of the mutants was described in a previous paper (8). The selection strategy of the osmotically defective mutants has been directed to isolate mutants which are capable of growing like wild-type cells in salt-containing media but are limited in their capacity to respond quickly to a hyperosmotic stress, which requires massive glycerol production from starch (8). Such mutants are expected to be unimpaired in their central metabolic pathways as well as in the capacity to produce glycerol but may be defective in a stage which becomes rate limiting when massive glycerol synthesis is required. The observation that mutants UV22, NG8, and several other mutants which are not described in this work are defective in enzymes of the oxidative pentose-phosphate pathway suggests that this pathway plays a major role in glycerol production in *Dunaliella*, but is not operating at full capacity during normal metabolic activities in this alga. This conclusion seems logical in view of the requirement for reduced pyridine nucleotide for glycerol synthesis from starch in the glycolytic pathway (see scheme in Fig. 7):

\[
\text{[glucose] + ATP + 2 NADH} \rightarrow 2 \text{glycerol} \\
+ \text{ADP + Pi + 2 NAD}^+ 
\]

Because the major function of the pentose-phosphate pathway is to provide reduced pyridine nucleotides for biosynthetic purposes, and since it is active in higher plant systems, it may be expected also to play a role in glycerol production in *Dunaliella*. Previous attempts to detect changes in the level of reduced pyridine nucleotides following osmotic shocks in *Dunaliella* have failed (1, 16), perhaps because of a very efficient production of NADPH by the pentose-phosphate pathway. The high activity of Glc-6P-DH and PGlc-DH in *Dunaliella* lysates (Table IV) is consistent with this interpretation. It is interesting to note that glycerol may be produced by the pentose phosphate pathway from glyceraldehyde phosphate, an intermediate in this pathway, at half of the efficiency of the glycolytic pathway. The overall stoichiometry of glycerol production by the oxidative pentose-phosphate pathway is:

\[
\text{[glucose] + 5 NAD (P)}^+ \rightarrow \text{glycerol} \\
+ 3 \text{CO}_2 + 5 \text{NAD(P)}^- 
\]

Theoretically, it is possible to maintain oxidation/reduction balance in the absence of photosynthesis or respiration by a combined synthesis of glycerol via glycolysis, and the pentose-phosphate pathways according to the following stoichiometry:

![Figure 6. Changes in Dunaliella phosphometabolites following hypotonic shock. Water-soluble phosphometabolites extracted from $^{32}$P-labeled cells grown at 100 mm NaCl (A) or diluted for 15 min to 50 mm (B) were separated by two-dimensional TLC on cellulose plates.](image)
GLYCEROL SYNTHESIS IN DUNALIELLA

Glycolysis:
5[glucose] + 10 NADH + 5 ATP → 10 glycerol
+ 10 NADH^+ + 5 ADP + 5 P_i

Pentose phosphate:
2[glucose] + 10 NAD(P)^+ → 2 glycerol
+ 6 CO_2 + 10 NAD(P)H

Overall:
7[glucose] + 5 ATP → 12 glycerol
+ 6 CO_2 + 5 ADP + 5 Pi

In this combination the only energy source required for glycerol production in addition to glycerol, is ATP. This prediction is consistent with the reported drop in ATP following hyperosmotic shocks (1, 10) and with the dependence of glycerol synthesis on cellular ATP level in Dunaliella (2).

The pronounced drop in Fru-6P and increase in Fru-1,6-bisP following hypertonic shocks strongly indicate that PFK plays a major role in regulating glycerol synthesis from starch. This possibility is not surprising since PFK is a classical checkpoint enzyme in regulation of glycolysis. Moreover, PFK has been isolated from chloroplasts of Dunaliella and on the basis of its kinetic properties it has been proposed as a likely candidate for regulation of glycerol production (19). Three ligands which influence PFK activity and may be involved in glycerol production are noteworthy: (a) ATP, which is a substrate for the enzyme, also inhibits its activity above 1 mm. The rapid drop in ATP content following hyperosmotic shock is expected to partially relieve the inhi-
bition. (b) Inorganic phosphate is an activator of PFK. Previous reports of increase in Pi in Dunaliella following hypertonic shock (14, 15, 20) and our recent observation of the correlation between cellular phosphate in Dunaliella and the rate of glycerol synthesis (5) are consistent with the idea that phosphate activates glycerol production by stimulating PFK as well as starch phosphorylase activities. (c) Several photosynthesis products such as PGA drastically inhibit PFK activity (19). The inhibition of photosynthesis following hyperosmotic shock (by about 30%, E Chitlaru, unpublished observation and ref. 16) which is accompanied by an increase in Rib-1,5-bisP is expected to result in a parallel decrease in PGA and derepression of PFK activity.

It has been reported recently that in D. tertiolecta hyperosmotic shocks at low salinities, but not at higher salinities induce a decrease, rather than an increase in Fru-1,6-bisP (1). The reason for the difference with our results is not clear. It may result from the different extraction or analytical (enzymatic assay) procedures, or from differences between the two Dunaliella strains.

In the same study it has also been reported that hyperosmotic shock in D. tertiolecta induces an increase in glycerol phosphate level and it was suggested that GP-DH may be the regulatory enzyme in glycerol production (7). However, the location of this enzyme toward the end of the glycerol synthetic pathway, its high activity in Dunaliella, and the observation that it is inhibited rather than stimulated by inorganic phosphate suggest that it is not a likely candidate to be involved in regulation of glycerol synthesis. The transient accumulation of glycerol phosphate following hyperosmotic shocks may be a secondary consequence of the accumulation of Fru-1,6-bisP as a result of PFK activation. This should be expected if the rate of glycerol phosphate formation is limited by the level of Fru-1,6-bisP, which is reasonable in view of the high activity of GPDH in Dunaliella. It is unfortunate that our TLC resolution does not enable us to detect GP and DHAP following hyperosmotic shocks due to their comigration with other major metabolites (PGA, Pi).

The physiological significance of the changes in the soluble lipid metabolites following osmotic shocks is not clear. The correlation between the transient increase of IP$_2$ (Fig. 6) and the reported transient increase in the turnover of phosphoinositol phospholipids following downshock is consistent with activation of a phosphatidylinositolphosphate PL-C by hyperosmotic shocks (11). The origin of phosphorylcholine is more obscure, since this metabolite may be both a precursor for synthesis of phosphatidylcholine or its breakdown product, due to the action of PLC (23). The transient increase of lysophosphatidylcholine following upshocks (12) is kinetically correlated with the accumulation of phosphorylcholine. This may indicate that activation of a phospholipase A$_2$, which hydrolyses phosphatidylcholine, induces a secondary activation of the biosynthesis of this lipid via phosphorylcholine.

The transient accumulation of IP$_2$ or PPI/C following osmotic shocks may be, therefore, trivial consequences of the activation of specific phospholipases. Nevertheless, the increasing evidence for the role of phosphoinositides as second messengers in plants and the recent indications that in mammalian cells metabolites of phosphatidylcholine may also act as second messengers, could suggest a similar role in osmotic regulation in Dunaliella (23). The observation that in the osmotically defective mutants there is no detectable accumulation of phosphorylcholine following upshocks substantiates the correlation between glycerol production and this metabolite. However, since Fru-6P drops following hyperosmotic shocks also in the mutants, indicating activation of PFK, it appears that the accumulation of phosphorylcholine is not directly involved in triggering glycerol production, but may be a secondary response to the volume changes.

A summary of the proposed metabolic pathway leading to glycerol production in Dunaliella is presented schematically in Fig. 7. It is proposed that: (a) glycerol production from starch is activated by stimulation of phosphofructokinase and possibly also of starch phosphorylase. (b) The carbon flow from glucose to glycerol involves operation of two metabolic pathways inside the chloroplast: the glycolytic pathway, which produces glycerol phosphate with consumption of NADH and ATP, and the pentose phosphate pathway which supplements the loss of reducing equivalents. The loss of ATP is probably partially replenished by photosynthesis, respiration and by conversion of glyceraldehydephosphate to pyruvate. (c) Glycerol phosphate is transported out of the chloroplast via the phosphate translocator in exchange for Pi and is hydrolyzed in the cytoplasm by glycerol phosphatase.

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**LITERATURE CITED**

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