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Glycerol production by *Dunaliella*

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Summary. Species of the unicellular alga *Dunaliella* possess outstanding tolerance of a wide range of salinities. They can adapt to grow in salt media which range from less than 0.5 M to saturated salt solutions and withstand enormous osmotic shocks through a unique osmotic adaptation. The osmoregulating mechanism depends on photosynthetic production of glycerol, whose intracellular concentration varies in direct proportion to the extracellular salt concentration and reaches values in excess of 50% of the total dry weight of the cells. *Dunaliella,* and another halotolerant glycerol producing alga, *Asteromonas gracilis,* osmoregulate biochemically by controlling glycerol biosynthesis and degradation. 3 new enzymes, NADPH-dihydroxyacetone-reductase, dihydroxyacetone kinase and glycerol-1-phosphatase seem to be involved in the osmoregulatory response via glycerol in *Dunaliella* and *Asteromonas.* A hypothetical scheme of glycerol metabolism in these algae utilizing these enzymes is presented. Growth studies of *Dunaliella* indoors and outdoors showed that salt concentrations favoring maximal glycerol productivity are not identical with those required for maximal algal productivity. Maximal yield of glycerol occurred around 2 M NaCl while maximal algal productivity occurred below 0.5 M NaCl. Observed yields of glycerol in *Dunaliella* culture outdoors are compared with theoretically calculated maximal yield.

1. *Introduction*

Utilization of the photosynthetic machinery for the production of energy, chemicals and food has a particular appeal because it is the most abundant energy storing and life-supporting process on earth. Starting with the photosynthetic reaction converting carbon dioxide and water into organic carbon and oxygen with solar irradiation as the energy source, photosynthetic plants and algae utilize intricate biochemical pathways to produce a variety of organic metabolites. Serveral potential crops have been suggested in recent years as possible candidates for converting solar energy via photosynthesis into biofuels and/or valuable organic compounds[1]. These include fast-growing tree species grown at high densities, conventional crops such as corn, sugar beet, sugarcane, and plants native to arid environments. Though each has its own specific advantages and disadvantages, there are several drawbacks common to almost all terrestrial plants, such as low solar conversion efficiency due to reflection and partial absorption, storage capacity limitations, investment of much of the photosynthate in nonrecoverable parts of the plant, competition with high economic value agricultural land and a high consumptive use of fresh water. The halotolerant unialga *Dunaliella*[2] has only a few of the drawbacks and in addition offers advantages not found in the other systems. It can be grown in a population density resulting in the presentation of an optimum absorbing surface area-to-unit land area ratio throughout the year. It can grow in salt water on arid land where there is maximum availability of solar energy and where the land is not utilizable for any other kind of potential crop. Lacking a typical polysaccharide cell wall, *Dunaliella* invests a much smaller fraction of the photosynthetic products in difficult to utilize structural constituents than do other algae and plants. Most significantly, the major photosynthetic end product in *Dunaliella* is glycerol, the concentration of which varies in direct proportion to the extracellular salt concentration reaching a maximum of around 80% of the algal dry weight[3,4]. The use of *Dunaliella* for direct conversion of solar energy into a useful chemical product is therefore of particular interest. Also of interest are the rather unique metabolic pathways which exist in this alga, permitting the synthesis and regulation of the massive intracellular concentrations of glycerol. The purpose of this manuscript is to examine the basic biochemistry of glycerol production and regulation in *Dunaliella* and to describe indoor and outdoor ex-

periments in which glycerol and algal material yield have been measured and compared.

2. *Growth of Dunaliella*

The genus *Dunaliella* contains species whose normal habitats range from seawater of around 0.4 M NaCl to salt lakes containing NaCl at concentrations up to saturation (>5 M). Moreover, algae originated from seawater can be adapted to high salt concentration and vice versa[3,5]. Figure 1 illustrates the effect of the NaCl concentration of the medium on the growth of *D. salina* as measured by chlorophyll content. The remarkable adaptation to a wide range of salt concentrations is evident. Algae grown at 4 M NaCl multiply at a rate which approximates only about one third that of algae grown under optimal conditions. A lack of requirement for high salt concentration for growth coupled with the halotolerance adaptability to a wide range of salinities differentiate these eukaryotic algae from the obligate halophilic bacteria[6].

3. *Intracellular composition*

The unique ability of *Dunaliella* to survive in highly saline water bodies was found to depend on the photosynthetic production and accumulation of high intracellular concentrations of glycerol[3,7,8]. A linear relation between the concentrations of intracellular glycerol and extracellular salt is maintained over a broad range of salt concentrations from 0.5 M to 4.5 M (fig.2). This has been observed in various species of *Dunaliella* and in 1 species of *Asteromonas*[9]. All produce and accumulate glycerol, the intracellular content of which depends on the salt concentrations of the medium and the algal volume. Thus all available data suggest that glycerol is the major intracellular solute which serves to osmotically balance the medium salt concentration.

4. *Osmoregulatory mechanism*

Several lines of evidence provide information regarding the underlying mechanism which enables *Dunaliella* to display its unique halotolerance and adaptability.

Microscopic observations show that *Dunaliella* cells behave like perfect osmometers rapidly shrinking or swelling under hypertonic or hypotonic conditions, respectively (fig.3). The absence of a rigid polysac-

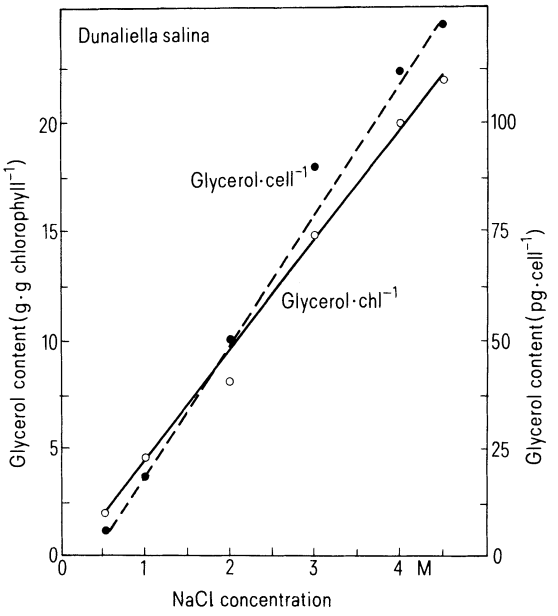

Figure 2. Effect of extracellular salt concentration on the intracellular glycerol content in *Dunaliella salina*. Assay conditions were as described previously[3].

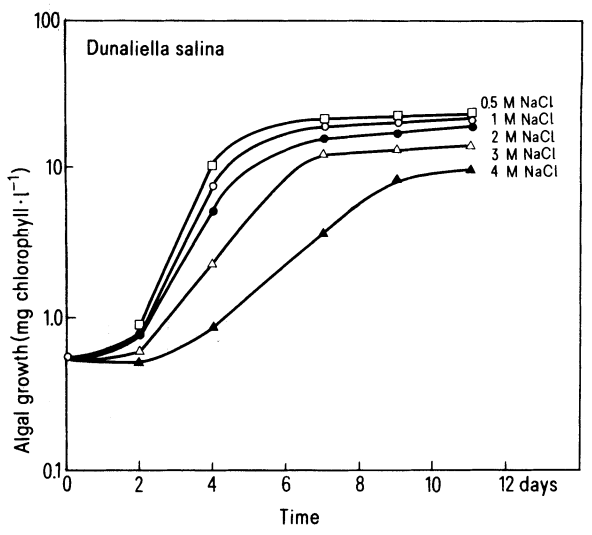

Figure 1. Growth of *Dunaliella salina* at several concentrations of salt. Growth conditions and medium were as described previously[11].

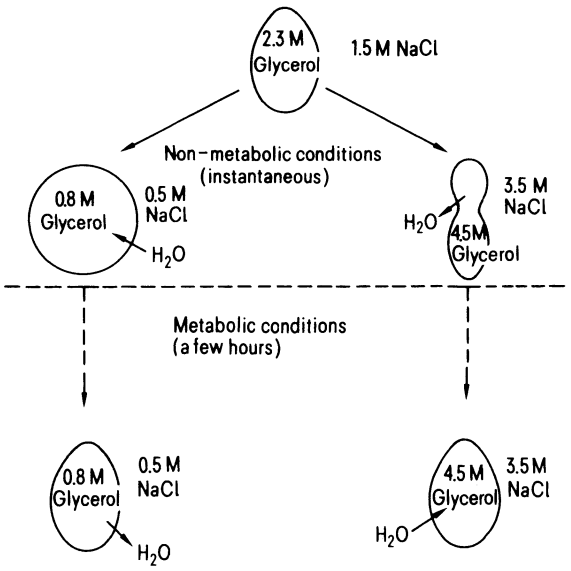

Figure 3. Osmoregulation in halotolerant wall-less algae. Schematic representation of the adjustment of *Dunaliella* to hypertonic and hypotonic conditions.

charide cell wall permits a rapid adjustment of the intracellular osmotic pressure by fluxes of water through the cytoplasmic membrane. Thereafter the cells slowly return to their original ellipsoid-like shape through a phase of metabolic adjustment. During this metabolic adjustment period under hypertonic conditions the algae produce and accumulate glycerol above the original level, while under hypotonic conditions the algae reduce the glycerol content below the original level. In either case water flows through the cytoplasmic membrane in response to the new level of intracellular glycerol so that at the steady state the original cell volume is regained[7,10]. Cellular osmoregulation in *Dunaliella* can be defined, therefore, as the ability of the cell to maintain approximately constant volume in the face of changing water potential.

The kinetics of synthesis and elimination of glycerol upon transition from low to high salt concentration or vice versa have been studied in detail[3,5,7,8] and indicate that: a) the process is very rapid; glycerol synthesis or elimination can be detected within minutes after the transition, and b) such synthesis is independent of protein synthesis or of illumination; thus, the mechanism of response is ever present and rapid in responding.

Three unique enzymes have been described for *Dunaliella* which are likely to be involved in its osmoregulatory response (table 1). The 1st is an $NADP^+$-dependent dihydroxyacetone reductase which catalyzes the interconversion of dihydroxyacetone and glycerol[8,11]. The classical NAD^+-dependent glycerol-3-phosphate dehydrogenase has also been described in *Dunaliella*[12]. The 2nd unique enzyme is dihydroxyacetone kinase which is highly specific toward dihydroxyacetone[13], and the 3rd is glycerol-1-phosphatase which specifically dephosphorylates α-glycerol phosphate.

Taking into consideration the presence of these enzymes in *Dunaliella*, a reasonable hypothetical scheme of the osmoregulatory metabolism of *Dunaliella* may be as shown in figure 4. Glycerol may accumulate by production of triose phosphate via photosynthesis or from polysaccharide degradation followed by reduction to α-glycerol phosphate and dephosphorylation. Conversion of glycerol back to polysaccharides may proceed via oxidation to dihydroxyacetone and phosphorylation to dihydroxyacetone phosphate.

Finally, it is of interest to note that these same unique enzymes were found in another halotolerant alga which osmoregulates with glycerol, *Asteromonas gracilis* (table 1)[9].

Table 1. The activity of several enzymes involved in glycerol metabolism in halotolerant algae

Algal species	Dihydroxyacetone reductase	Dihydroxyacetone kinase	Glycerol-1-phosphatase
	(μmoles substrate consumed · min^{-1} · mg chl^{-1})		
Dunaliella salina	0.89	0.54	4.27
Dunaliella bardawil	0.25	0.35	1.75
Asteromonas gracilis	2.22	1.01	5.18

Algae were grown in growth medium containing 3 M NaCl in an illuminated room as previously described[10,11]. Enzymes in the crude algal extract were assayed: dihydroxyacetone reductase[11]; dihydroxyacetone kinase[13], as previously described, and glycerol-1-phosphatase by following the release of inorganic phosphate in the presence of 5mM $MgCl_2$.

5. *Glycerol yield optimization*

Much has been learned about photosynthesis in terrestrial plants, aquatic plants and algae. All share the same basic biochemical machinery for converting carbon dioxide and water into organic carbon and oxygen, and so their energy conversion efficiency is governed by the same basic principles.

Solar energy strikes the earth at a low flux of 2000 kcal m^{-2} day^{-1}, hence requiring very large collection systems for capturing the light. Moreover, photosynthetic conversion efficiencies are rather low. Therefore, under the most ideal conditions the most efficient plant can convert at best about 8% of solar irradiation into stored energy in the form organic matter[14,15]. In reality, photosynthetic conversion efficiencies of natural terrestrial systems are considerably lower and seldom exceed 1-2%, primarily because other factors such as light availability, nutrients, water, etc., are limiting. Aquatic plants including microalgae are among the most efficient converters of

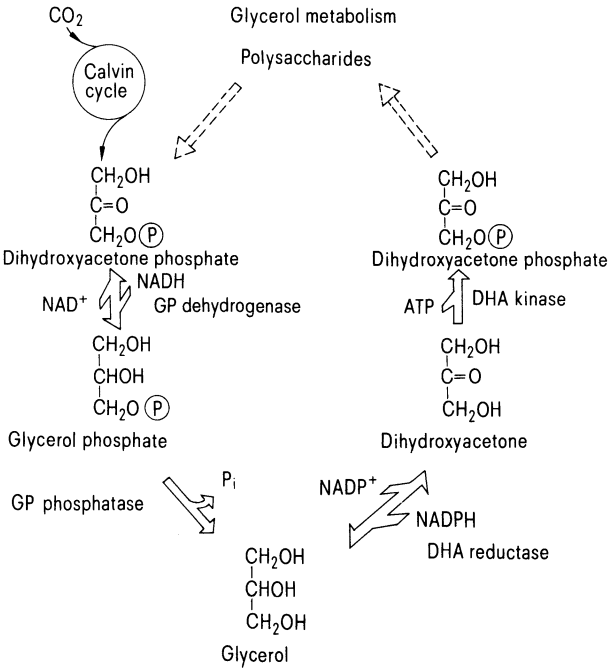

Figure 4. A hypothetical metabolic pathway of glycerol biosynthesis and degradation in *Dunaliella*.

radiant energy; photosynthetic efficiencies under laboratory conditions with low light intensity have been reported to approach the theoretical limit. Goldman[14,15] has recently summarized the theoretical and practically observed light conversion efficiency of large-scale algal culture grown under natural and light limiting conditions. Maximal yield of around 30 g m^{-2} day^{-1} have been calculated and in practice, a similar high yield data has been reported in various locations in the world for short periods[15].

Assuming solar conversion efficiency of 8% for calculating the potential for production of glycerol by *Dunaliella,* algae containing 40% glycerol on a dry weight basis can yield 16 g glycerol m^{-2} day^{-1} [4]. Table 2 illustrates the effect of salt concentration on the actual production of glycerol in open culture of *Dunaliella.* Maximal long term productivity of about 4.5 g glycerol m^{-2} day^{-1} has been observed at a salt concentration of 3.5 M. However, since the conditions for optimal growth are not necessarily those which maximize glycerol production, laboratory experiments have been undertaken to check the effect of salt on glycerol and algal yield production (fig. 5). Indeed, optimization for high yield of glycerol occurred around 2 M NaCl while the conditions favoring maximal algal productivity were in the low range of salt concentration.

Table 2. The effect of salt concentration on the productivity of glycerol in outdoor cultures of *Dunaliella bardawil*

NaCl concentration (M)	Average level of glycerol at harvest ($g \cdot l^{-1}$)	Productivity of glycerol ($g \cdot m^{-2} \cdot day^{-1}$)
3.5	0.18	4.4
4.0	0.14	3.4
4.5	0.16	3.0
5.0	0.16	2.4

Dunaliella bardawil were grown in 10-cm-depth miniponds outdoors for about 60 days between May and June. When the algae content of the pond reached the indicated level of glycerol half of the culture volume was harvested by centrifugation and the remaining algae diluted with fresh medium to the original volume.

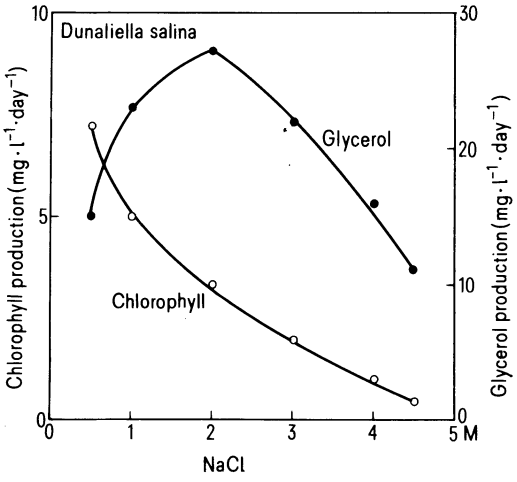

Figure 5. Effect of extracellular salt concentrations on the chlorophyll and glycerol production by *Dunaliella.* Algae were grown in a constant temperature growth room as described previously[10,11].

A variety of considerations come into play when we wish to optimize growth conditions for the production of a particular product, such as glycerol. For example, the higher the salt concentration, the lower is the interference by other organisms and predators. Since these conditions are not necessarily optimal for glycerol production, maximum yield potential may need to be sacrificed. In evaluating optimal production capability for a chemical derivative of *Dunaliella* or other microorganisms, a quantitative determination of the yield potential as a function of a variety of conditions in addition to maximal growth rate needs to be carried out. Thereafter we can assess the economic value of the algal product.

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Biological photoproduction of hydrogen and ammonia

Ammonia and hydrogen are energy-rich products which can be used as fuels. They are produced by photosynthetic microorganisms under certain limited conditions, especially stress situations. The organisms and the environmental conditions leading to a high ammonia production from nitrate or nitrogen gas are discussed by M.G. Guerrero et al., while the systems leading to gaseous hydrogen from different electron donors are presented separately for algae (H. Bothe) and photosynthetic bacteria (H. Zürrer).