Plant Physiol. (1986) 82, 594–596
0032-0889/86/82/0594/03/$01.00/0

Communication

Effect of Osmotic Stress on Carbon Metabolism in *Chlamydomonas reinhardtii*[1]

ACCUMULATION OF GLYCEROL AS AN OSMOREGULATORY SOLUTE

Received for publication May 22, 1986

H. DAVID HUSIC[2] AND N. E. TOLBERT*
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

NaCl, KCl, and sucrose at equiosmolar concentrations had similar inhibitory effects on photosynthetic carbon metabolism by the freshwater green alga, *Chlamydomonas reinhardtii*. Inhibitory concentrations of these solutes altered the products of photosynthetic $^{14}CO_2$ incorporation, resulting in reduced incorporation into starch, sugar phosphates, lactate, and glycolate, but caused an accumulation of glycerol both intracellularly and in the medium.

Some species of green algae thrive in saline environments because of the intracellular accumulation of metabolites which provide an osmotic balance with the environment. Glycerol is an important osmoregulatory solute in some halophilic green algae including *Dunaliella*, *Zooxanthellae*, *Asteromonas*, and also in several halotolerant species of *Chlamydomonas* (see Ref. 3 for a review). The accumulation of polyalcohols, sucrose, mannitol, and glycosides in response to osmotic stress have been reported in other species of green algae (3). Although this accumulation of osmoregulatory metabolites is believed to be responsible for halotolerance, it has not been determined whether species of green algae which are intolerant of high salt conditions synthesize these compounds in response to osmotic stress. In this communication, we report the synthesis of glycerol in response to osmotic stress in the freshwater green alga *Chlamydomonas reinhardtii*. The effect of this stress on the distribution of $^{14}CO_2$ into intracellular and excreted photosynthetic and photorespiratory products has also been examined.

RESULTS AND DISCUSSION

Chlamydomonas reinhardtii (UTEX 90 from the University of Texas at Austin) was grown while being aerated with 5% CO_2, harvested, and photosynthetically labeled with $H^{14}CO_3^-$ in 6 mM K-phosphate, pH 7.5, as described previously (13). Salts or sucrose were added immediately prior to the labeling where indicated. Under these conditions, CO_2 levels were less than saturating for photosynthesis, which resulted in significant labeling and excretion of glycolate. Maximal rates of photosynthesis were determined in 6 mM K-phosphate, pH 7.5, by measuring the rate of photosynthetic O_2 evolution in an O_2 electrode after the addition of 3.3 mM $NaHCO_3$. Sample preparation, glycolate determination, chromatographic separations, and quantitation of ^{14}C-labeled metabolites were carried out as previously outlined (13). Glycerol was determined enzymically with a coupled enzyme assay utilizing glycerol kinase (5). Crude extracts of algae were prepared as described previously (8). Published procedures were used to measure the activities of glycerol-1-P phosphatase (12), dihydroxyacetone-P phosphatase (12), glycerol-1-P dehydrogenase (9), and dihydroxyacetone reductase (2). All substrates and coupling enzymes were from Sigma Chemical Co.

RESULTS

Effects of Osmotic Stress on Growth and Rates of Photosynthesis. When the cells were incubated in growth medium containing 500 mM sucrose or 250 mM KCl or NaCl during the logarithmic phase of growth, increase in total cell mass after 5 d was less than 5% of that of a control culture which had no additional salts or sucrose added (data not shown). This intolerance of *C. reinhardtii* growth to osmotic stress is similar to results reported previously with KCl and NaCl (6, 11).

When cells grown in normal medium were exposed to salts or sucrose, equiosmolar concentrations of KCl, NaCl, or sucrose had similar inhibitory effects on the rate of photosynthesis as determined by the rate of photosynthetic O_2 evolution at saturating CO_2 levels (Fig. 1). Inhibition was observed at greater than 100 mM sucrose or 50 mM KCl or NaCl. The similarity in inhibition of photosynthesis by these three solutes indicates that the inhibition was due primarily to the osmotic potential of the solution. A similar result was obtained by measuring the rates of $^{14}CO_2$ fixation (data not shown). Inhibition of photosynthesis by *Chlamydomonas* has also been observed earlier by mannitol (4). The extent of inhibition of photosynthesis by varying concentrations of KCl and NaCl are also similar in magnitude to the concentration dependence for the inhibition of cell growth by these salts (6, 11).

Effect of Osmotic Stress on the Photosynthetic Incorporation of $^{14}CO_2$ into Intracellular Metabolites. $^{14}CO_2$ fixation was carried out for 30 min in the presence or absence of 100 mM KCl or 200 mM sucrose. These concentrations of solutes resulted in about 65% inhibition of the maximal rate of photosynthesis measured as O_2 evolution (Fig. 1). Chromatographic analysis of the ^{14}C-labeled products revealed an accumulation of glycerol in the osmotically stressed cells relative to the other labeled meta-

[1] Supported in part by National Science Foundation grant PCM 8003917 and by the McKnight Foundation. Journal article No. 11093 of the Michigan Agricultural Experiment Station.

[2] Present address: Chemistry Department, Lafayette College, Easton, PA 18042.

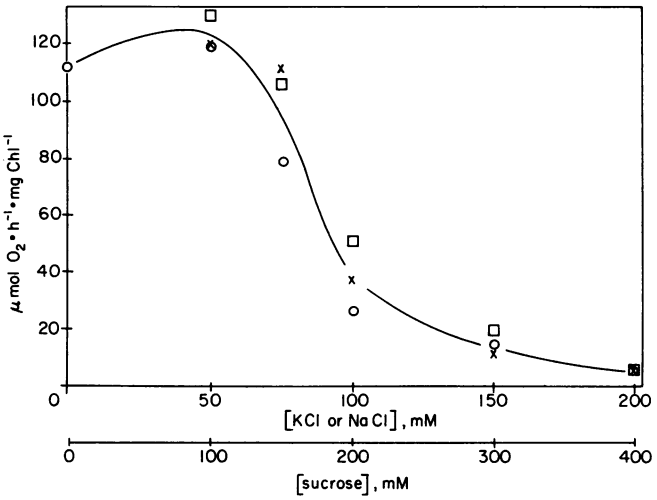

FIG. 1. Effect of varying concentrations of solutes on photosynthetic O_2 evolution by *C. reinhardtii*. Maximal rates of O_2 evoluation were measured at varying concentrations of NaCl (×), KCl (□), or sucrose (○), as described in "Materials and Methods."

Table I. *Distribution of ^{14}C-Labeled Metabolites in the Cellular and Medium Fractions of C. reinhardtii after 30 Min $^{14}CO_2$ Fixation*
$H^{14}CO_3^-$ was added at 0 and 15 min to a concentration of 1 mM.

Metabolite	Treatment		
	Control	100 mM KCl	200 mM Sucrose
		% total ^{14}C	
Cellular Products			
Insoluble	20.1	2.6	2.1
Glycerol	0.2	29.0	54.3
Phosphate esters	32.8	18.1	8.4
Lactate	14.9	5.2	2.8
Glycolate	0.3	0.2	0.4
Glycine + serine	2.2	7.1	6.7
Glutamate	6.0	20.8	9.8
Malate	7.2	6.6	3.5
Alanine	2.2	6.0	1.6
Aspartate	0	0	1.7
Excreted Products			
Glycerol	0.2	1.7	1.7
Glycolate	13.6	2.9	6.5

Table II. *Specific Activity of Glycolate and Glycerol Excreted from C. reinhardtii after 60 Min $^{14}CO_2$ Fixation*

Conditions	Specific Radioactivity	
	Glycolate	Glycerol
	cpm/nmol	
No additions	1040	—[a]
100 mM KCl	1330	284
200 mM KCl	789	110
200 mM Sucrose	ND[b]	313

[a] None detected. [b] Not determined because sucrose interfered with the colorimetric determination of glycolate.

bolic products (Table I). No glycerol accumulation was observed with no added KCl or sucrose, or with 50 mM KCl or 100 mM sucrose (not shown). The percentage of the fixed ^{14}C in the insoluble fraction (starch) was reduced by about 90% and there was a lower percentage of ^{14}C in sugar phosphates and lactate in osmotically stressed cells compared to controls. The lactate arose from the photosynthetic sugar phosphate pools during the brief period of anaerobiosis encountered when the cells were centrifuged into a tightly packed pellet to separate the cells from the medium (7). These findings indicate that the osmotic stress caused a diversion of fixed carbon from sugar phosphates and starch accumulation to enhance the synthesis of glycerol.

Effect of Osmotic Stress on the Excretion of Glycolate and Glycerol. There was some excretion of ^{14}C-labeled glycerol and glycolate by osmotically stressed *C. reinhardtii* cells (Table I). No other ^{14}C-labeled compounds were observed in the medium. The levels of excreted glycolate were markedly reduced in osmotically stressed Cells (Table I). The decreased level of excreted glycolate in stressed cells was in part balanced by increased relative intracellular levels of labeled glycine and serine, products of intracellular glycolate metabolism. We have suggested that glycolate is excreted when its rate of synthesis exceeds the capacity of glycolate dehydrogenase to metabolize it intracellularly (14).

Because glycolate is derived from ribulose 1,5-bisP, its specific radioactivity is indicative of the specific radioactivity of the sugar phosphate pools of the reductive photosynthetic carbon cycle from which it was derived. The specific radioactivity of the excreted glycolate was considerably higher than the glycerol that was excreted (Table II). This indicates that there was a significant contribution of unlabeled metabolite pools to the synthesis of glycerol.

Only 8% of the total glycerol that was synthesized was excreted from the cells after 30 min of photosynthesis in the presence of 100 mM KCl, although this level increased to 26% after 60 min (data not shown). Based on previous measurements of internal cell volume (10), the concentration of glycerol in the cells after 60 min CO_2 fixation in 100 mM KCl was about 16 mM, while the concentration in the medium was 0.064 mM. Thus the concentration of glycerol in the cells was 250 times that in the medium. Nevertheless, after 1 h the intracellular glycerol level is considerably less than is necessary to provide an osmotic balance to 100 mM KCl extracellularly. When cells were grown with 100 mM KCl for 20 h, an intracellular glycerol concentration of 26 mM was attained.

DISCUSSION

Chlamydomonas reinhardtii growth and photosynthetic rates are inhibited by moderate concentrations of osmotic substances in the medium. In response to osmotic stress, an alteration in carbon metabolism results in a significant accumulation of glycerol, yet these algae are rather intolerant to these conditions of osmotic stress. By comparison, the halophilic green alga *Dunaliella* thrives in media containing 5.5 M NaCl and accumulates glycerol intracellularly to levels which are nearly isoosmotic with the surrounding medium (1). The inhibition of growth and photosynthesis by *C. reinhardtii* may be because intracellular glycerol levels did not become equiosmolar with the environment. At 50 mM KCl or 100 mM sucrose no glycerol was observed, nor was any inhibition of photosynthesis observed, indicating that under conditions of mild osmotic stress the cells are able to compensate in ways other than by glycerol accumulation.

The mechanism by which a change in the osmotic potential of the medium signals the induction of glycerol biosynthesis is unclear. The induction of glycerol synthesis after the breakage of dormancy in yeast spores is due to the cAMP-dependent regulation of glycerol-P phosphatase (9). In *Dunaliella* glycerol-P phosphatase was found, but little dihydroxyacetone-P phosphatase (12). In crude extracts of *C. reinhardtii* we detected little glycerol-l-P phosphatase activity (0.3 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl); however, a dihydroxyacetone-P phosphatase activity of 2.7 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl was found. Similar levels of activity were observed in extracts prepared from control or osmotically stressed cells.

The relative levels of the phosphatases in the extracts indicate that the primary route of glycerol synthesis from dihydroxyacetone-P may be by the action of dihydroxyacetone-P phosphatase and dihydroxyacetone reductase. Similar activities of NADP-dependent glycerol-P dehydrogenase and dihydroxyacetone reductase were observed (5 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl) in crude extracts; however, the glycerol-P dehydrogenase activity may have been in part due to the combined action of dihydroxyacetone-P phosphatase and dihydroxyacetone reductase.

Acknowledgment—We thank Tamra Reed for technical assistance in some of these experiments.

LITERATURE CITED

1. AVRON M 1986 The osmotic components of halotolerant algae. Trends Biochem Sci 11: 5–6
2. BEN-AMOTZ A, M AVRON 1974 Isolation, characterization, and partial purification of a reduced nicotinamide adenine dinucleotide phosphate-dependent dihydroxyacetone reductase from the halophilic alga *Dunaliella parva*. Plant Physiol 53: 628–631
3. BEN-AMOTZ A, M AVRON 1983 Accumulation of metabolites by halotolerant algae and its industrial potential. Annu Rev Microbiol 37: 95–119
4. BERKOWITZ GA, C CHEN, M GIBBS 1983 Stromal acidification mediates *in vivo* water stress inhibition of nonstomatal-controlled photosynthesis. Plant Physiol 72: 1123–1126
5. EGGSTEIN M, E KUHLMANN 1974 Triglycerides and glycerol. Determination after alkaline hydrolysis. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, Academic Press, New York, pp 1825–1831
6. GAMBOA A, S ALFARO, T REYNOSO 1985 Taurine induction of cation tolerance in *Chlamydomonas reinhardtii*. Comp Biochem Physiol 81: 491–493
7. HUSIC DW, NE TOLBERT 1985 Anaerobic formation of D-lactate and partial purification of a pyruvate reductase from *Chlamydomonas reinhardtii*. Plant Physiol 78: 277–284
8. HUSIC HD, NE TOLBERT 1985 Properties of phosphoglycolate phosphatase from *Chlamydomonas reinhardtii* and *Anacystis nidulans*. Plant Physiol 79: 394–399
9. MARENGO T, RMcC LILLEY, AD BROWN 1985 Osmoregulation in *Dunaliella*. Catalysis of the glycerol-3-phosphate dehydrogenase reaction in a chloroplast-enriched fraction of *Dunaliella tertiolecta*. Arch Microbiol 142: 262–268
10. MORONEY JV, NE TOLBERT 1985 Inorganic carbon uptake by *Chlamydomonas reinhardtii*. Plant Physiol 77: 253–258
11. REYNOSO GT, BA DEGAMBOA 1982 Salt tolerance in the fresh water algae *Chlamydomonas reinhardtii*: effect of proline and taurine. Comp Biochem Physiol 73: 95–99
12. SUSSMAN I, M AVRON 1981 Characterization and partial purification of DL-glycerol-1-phosphatase from *Dunaliella salina*. Biochim Biophys Acta 661: 199–204
13. TOLBERT NE, M HARRISON, N SELPH 1983 Aminooxyacetate stimulation of glycolate formation and excretion in *Chlamydomonas*. Plant Physiol 72: 1075–1983
14. TOLBERT NE, HD HUSIC, DW HUSIC, JV MORONEY, BJ WILSON 1985 Glycolate excretion by microalgae. *In* JA Berry, WJ Lucas, eds, Bicarbonate Utilization by Photosynthetic Organisms. American Society of Plant Physiologists, Rockville, MD, pp 211–227