BBA 46116

OSMOTIC REGULATION OF PHOTOSYNTHETIC GLYCEROL PRODUCTION IN DUNALIELLA

KLAUS WEGMANN

Institut für Chemische Pflanzenphysiologie der Universität Tübingen, Tübingen (Germany)

(Received January 18th, 1971)

SUMMARY

1. The influence of osmotically active substances on photosynthetic glycerol production and on gas exchange in *Dunaliella tertiolecta* was investigated.

2. With increasing concentrations of NaCl, sucrose or 2-deoxy-D-glucose in the medium, the photosynthetic incorporation of [^{14}C]bicarbonate decreases; the percentage of ^{14}C incorporated into glycerol increases to a distinct maximum at a concentration of approx. 2.8 M NaCl, and then declines.

3. Increasing the concentration of osmotic substances first causes a decline of photosynthetic oxygen evolution, then oxygen consumption, accompanied by CO_2 evolution. Both show distinct maxima at approx. 2.8 M NaCl, in good agreement with the maximum of glycerol formation. The respiratory quotient indicates a fermentation process at higher osmotic pressures.

4. The glycerol formation is considered to be a protective mechanism for the survival of Dunaliella in its natural habitat.

INTRODUCTION

High salt concentrations in their natural habitat are tolerated (facultative halophilism) or required (obligate halophilism) by a great number of organisms. Since enzymes have ionic concentration optima (see, for instance, ref. 1), osmoregulatory mechanisms are necessary to maintain suitable osmotic pressure within organisms. Known examples for such osmoregulatory mechanisms are: (a) the active excretion of water from pulsing vacuoles, as reported for Chlamydomonas[2]; (b) the accumulation of free amino acids with increasing salinity of the medium, as in the brain tissue of the Western toad, *Bufo boreas*[3], in invertebrates[4], the marine ciliate *Miamiensis avidus*[5], and the soft-shell clam *Mya arenaria*[6,7]; (c) the reversible conversion of polysaccharides to α-galactosylglycerides in *Ochromonas malhamensis* and *Porphyra perforata*[8–11]. However, the single cell phytoplankter Dunaliella exhibits a broad salt tolerance in its natural habitat, which varies from the dryness of a salt crust to the low salinity of brackish water. This wide salt concentration range makes it of interest to physiologists and biochemists to determine how Dunaliella gets over hard osmotic stress.

Studies on glycerol production in Dunaliella by CRAIGIE AND MCLACHLAN[12] led to the suggestion that Dunaliella produces glycerol in response to the salt concentra-

tion of the medium. From the present investigations it is apparent that this glycerol formation is a response to osmotic pressure rather than ionic strength and that the whole metabolism of Dunaliella is drastically changed over the investigated osmotic range.

MATERIALS AND METHODS

Dunaliella species, No. 19-6 of the algal collection of the University of Göttingen (Germany), and considered to be identical with *Dunaliella tertiolecta Butcher*, was grown in an inorganic medium in 400 mm × 40 mm glass tubes which were placed in a light thermostate[13]. The inorganic medium (according to W. KOCH, private communication) consisted of: 6.0 g NaCl, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.15 g KCl, 0.2 g $CaSO_4 \cdot 2H_2O$, 0.02 g $NaNO_3$, 0.002 g KH_2PO_4, 10 ml soil extract, and distilled water up to 100 ml. The pH of the autoclaved medium was 6.0, identical with the pH for maximum growth. The culture was aerated with CO_2-enriched compressed air, containing 2 % of CO_2. Illumination consisted of a combination of two TL 40 W/55 and three TL 40 W/32 fluorescent lamps from Philips. The light intensity was about 1 mW/cm² on the surface of the culture tubes.

Dunaliella cultures were always grown in the above-mentioned medium. For variation of the osmotic conditions the algae were spun down in a Christ centrifuge (Junior III) at 3000 rev./min for 1 min and the sedimented cells resuspended in a tenth of the original volume of experimental medium. The experimental media contained the same ingredients as the normal culture medium except that NaCl and $MgSO_4$ contents were reduced to 0.5 and 0.2 g/100 ml, respectively. Osmotic series were obtained by the addition of NaCl or osmotically active organic substances to this basic medium, as given in the experiments. The algae were suspended in the experimental media 15 min before starting the experiments. In order to avoid osmotic shock the changes into very high osmotic solutions were carried out in two steps by suspending the cells in a solution of intermediate osmotic pressure, spinning down after 15 min, and resuspending them in the final medium.

$^{14}CO_2$ fixation experiments were carried out in 25-ml erlenmeyer flasks with 5-ml cell suspensions, containing approx. $8 \cdot 10^8$ cells. As a rule the cell suspensions were illuminated for 10 min with white light of about 170 mW/cm² from an Attralux incandescent lamp before adding radioactive bicarbonate. A water-streamed cuvette was used to aviod strong infrared irradiation and to maintain an experimental temperature of 25° during the fixation experiments. 0.2 ml of radioactive bicarbonate solution containing 80 μC ^{14}C was added. After 3 min the cells were killed and extracted with 50 ml boiling ethanol. Further extractions were performed with 50 % ethanol and distilled water. The pooled extracts were taken to dryness with a flash evaporator at 40° and the residue was dissolved in a small known volume of distilled water. The insoluble cell residue was hydrolyzed overnight by boiling 8 M HCl and the resulting solution treated in the same way as the water-soluble extracts.

Aliquots were dried on aluminum planchettes and counted with a methane flow counter FH 407 (Frieseke and Hoepfner, Erlangen) to obtain total fixation rates. Additional aliquots of the water-soluble extracts were separated by two-dimensional thin-layer chromatography[14] or by the combination of thin-layer electrophoresis and chromatography[15]. Labelled compounds on the chromatograms were detected by

autoradiography on X-ray films (Osray DW, Agfa–Gevaert). The distribution of ^{14}C was obtained by counting the β-ray activities of the spots by the methane flow counter FH 407 placed on the surface of the thin-layer plate. In some experiments quantitative radioactivity determinations were carried out in a Beckman liquid scintillation counter after scraping off the radioactive spots and collecting the material in counting vials. Substances were identified by chromatography with authentic substances.

Gas exchange measurements were performed in a Warburg apparatus (Braun, Melsungen, Type FL 26). O_2 and CO_2 exchange as well as photosynthetic and respiratory quotients were obtained by a modified two-vessel method using Warburg vessels containing two separate compartments as described by METZNER[16]. The light intensity obtained using a built-in fluorescent lamp was about 1 mW/cm^2. 10 ml of Dunaliella suspension was used in each experiment, the total vessel volume being about 50 ml.

RESULTS AND DISCUSSION

The frequency of cell division is strongly dependent on the salt concentration in the medium. A distinct optimum concentration was obtained at about 1.2 M total salts, approximately twice the salt concentration in the oceans. Between concentrations of 2.5–3.5 M salt the growth rate drops to half the maximum rate and there is no cell division at concentrations higher than 5 M.

Labelling experiments

With increasing salt concentration the total fixation rates decrease (Fig. 1). The radioactivity in the soluble fraction tends to higher percentages with increasing

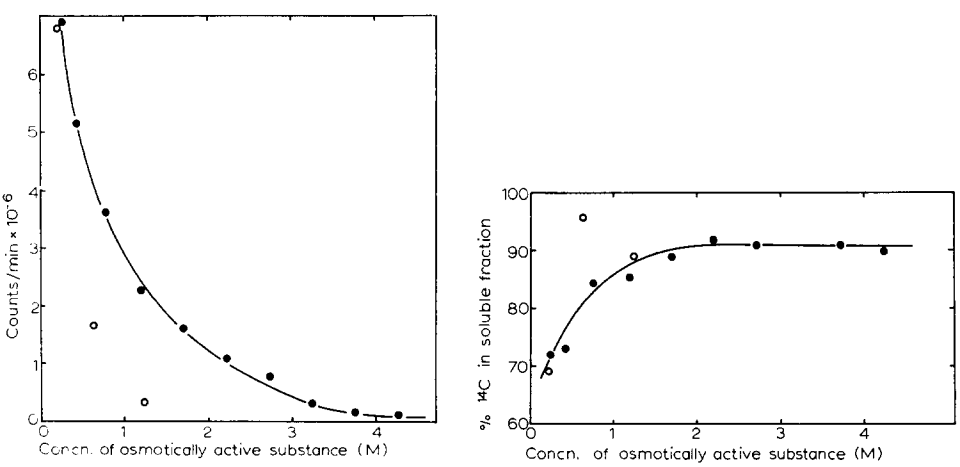

Fig. 1. Total ^{14}C incorporation into *Dunaliella tertiolecta* after 3-min photosynthesis in the presence of NaCl (●) or 2-deoxy-D-glucose (○) as osmotically active substance. For experimental conditions see text.

Fig. 2. Percentage of incorporated ^{14}C in the soluble fraction after 3-min photosynthesis in the presence of NaCl (●) or 2-deoxy-D-glucose (○) as osmotically active substance. Data taken from the same experiment as in Fig. 1.

salinity (Fig. 2). The same correlation was observed when the salt concentration was only 1.5 %, but the osmotic pressure increased by the addition of sucrose or 2-deoxy-D-glucose.

The author's earlier experiments[17,18] resulted in the recognition of glycerol as a very early labelled major product of CO_2 fixation in the presence of [^{14}C]bicarbonate, and of the strict light dependence of its formation. The proposed pathway for glycerol formation is presented in Fig. 3.

The evaluation of two-dimensional chromatograms revealed large differences in the percentage of radioactivity incorporated into glycerol when the salinity was changed, which is in good agreement with the results of CRAIGIE AND MCLACHLAN[12]

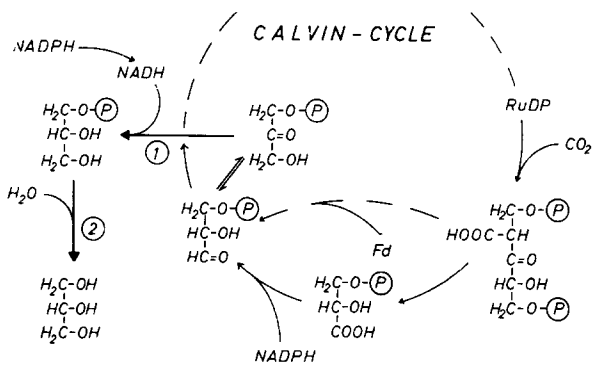

Fig. 3. Proposed pathway for the photosynthetic glycerol formation in *Dunaliella tertiolecta*[18].

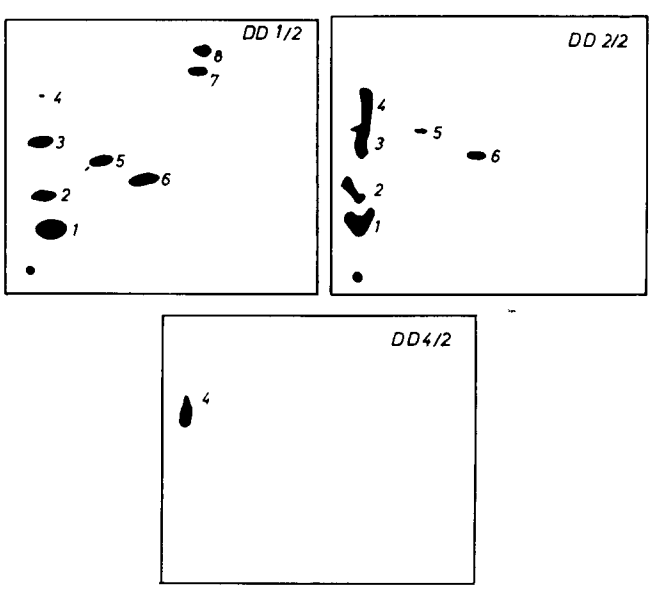

Fig. 4. Electrophero-chromatograms of soluble fractions of *Dunaliella tertiolecta* after 3-min photosynthesis in the presence of [^{14}C]bicarbonate. DD 1/2. Medium is about 0.25 M in salts; DD 2/2. Medium is about 0.25 M in salts + 0.5 M in 2-deoxy-D-glucose; DD 4/2. Medium is about 0.25 M in salts + 1.8 M in 2-deoxy-D-glucose. 1, sucrose; 2, glycine; 3, alanine; 4, glycerol; 5, glutamic acid; 6, aspartic acid; 7, malic acid; 8, glycollic acid.

for long incubation times. An increase from 13 to 65 % glycerol was observed, when the salt concentration was changed from 0.17 to about 2.7 M. With further increasing salt concentration the relative labelling rate in glycerol drops again. The percentage of labelled phosphate esters is much less affected by changes in salinity.

In order to ascertain whether the changes in glycerol are caused by changes in the ionic strength in the medium, further experiments were undertaken using organic compounds to increase the osmolarity without changing the ionic strength. Low-molecular-weight polyethylene glycols proved poisonous for Dunaliella cells. Several polyethylene glycols from different manufacturers caused clumping of the culture and inhibition of photosynthetic oxygen evolution, even at low concentrations. Further investigations were undertaken with sucrose or 2-deoxy-D-glucose, the latter compound being regarded as non-metabolizable by the cells. The resulting glycerol formation was found to be quite similar to that in the presence of NaCl. The distribution of radioactivity over the metabolic intermediates was obtained by electrophoresis–chromatography. At high osmotic values the incorporation of ^{14}C into other substances than glycerol becomes less important (Fig. 4).

An optimum in the dependence of ^{14}C incorporation into glycerol on osmotic values suggests that at least two different metabolic processes exist which are influenced by osmotic pressure in different ways.

Gas exchange measurements

Strong support for this view was obtained by gas exchange measurements with the Warburg micromanometer technique. Fig. 5 presents the results of a larger number of experiments; it shows the O_2 and CO_2 exchange as well as the photosynthetic and respiratory quotients. The most surprising results are that with increasing salt concentration photosynthesis inverts to an oxygen consumption while large amounts of carbon dioxide are evolved, and that there is again a distinct maximum at 2.7 M salt, as was obtained for the incorporation of ^{14}C into glycerol. Similar results

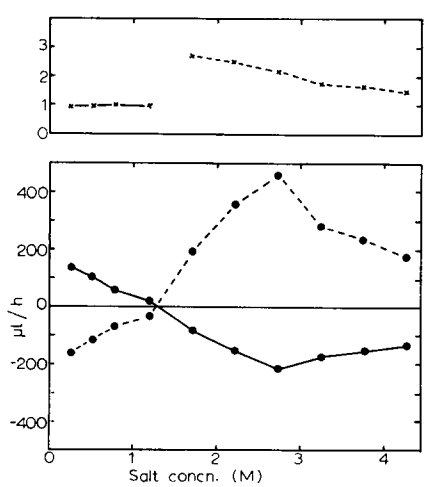

Fig. 5. Gas exchange of illuminated *Dunaliella tertiolecta* in dependence on the salinity. ●——●, oxygen evolution or consumption; ●- - - -●, carbon dioxide evolution or uptake; ×——×, photosynthetic quotient $O_2/-CO_2$; ×- - - -×, respiratory quotient $CO_2/-O_2$.

were obtained in sucrose solutions. The respiratory quotients were found to be greater than 1.5, indicating decarboxylation without oxygen consumption. Such results are consistent with fermentation processes.

As Fig. 3 shows, glycerol is derived from dihydroxyacetone phosphate of the Calvin cycle by reduction. The pathway functions only if glycerol 1-phosphate is hydrolyzed by an active phosphatase, as the reaction is limited by product inhibition. JOHNSON *et al.*[19] demonstrated that Calvin cycle enzymes (phosphoribose isomerase, ribulose diphosphate carboxylase) of *Dunaliella viridis* are sensitive to increased salt concentration. If this is also true for *Dunaliella tertiolecta*, increasing salt concentration would cause inhibition of photosynthesis so that ^{14}C incorporation into glycerol drops due to limited availability of triose phosphates. From the gas exchange data Dunaliella cells seem to switch over to a fermentation metabolism (probably after break-down of storage substances, such as starch) when put under severe osmotic conditions. It appears possible that the production of glycerol is continued in a similar way as in the halophilic yeast *Pichia miso*[20]; however, this glycerol remains unlabelled. The decreased specific activity of glycerol at the highest salt concentration found by CRAIGIE and MCLACHLAN[12] would support this opinion.

Part of the photosynthetic products is lost into the medium[17]. Electron micrographic findings[21,22] indicate a rapid exchange of water (and salts) between cell organelles and the medium without being forced to overcome a cell wall resistance. Thus the enzymes should be directly affected by osmotic pressure. It is apparent that the influence is neither a change in electrostatic charge of the protein by ions, nor an allosteric effect because it lacks the expected specifity of an allosteric effector. In the author's opinion it may be a conformational change of the protein due to the extraction of structural water by osmosis. Different enzymes may exhibit different responses or sensitivities. This kind of osmoregulation is probably more universal than is recognized. HINTON *et al.*[23] reported in connection with enzyme assays after sucrose density gradient centrifugation, that for several enzymes the activity drops by increasing (relatively low) sucrose concentrations.

Finally the question of the biological significance of the glycerol formation remains. As glycerol is partly excreted or lost into the medium it cannot be expected to act as an osmotic regulatory substance as do free amino acids in previously mentioned organisms and tissues. In its habitat, however, Dunaliella might have another advantage. Dunaliella is found near the oceans' shorelines where the cells happen to dry out in puddles, thus being exhibited to high salt concentrations. Under these circumstances glycerol might protect the proteins against severe structural changes so that the cells survive until being washed back into the ocean. BAXTER[24] has reported lactate dehydrogenase from *Halobacterium salinarum* to be protected by glycerol even against denaturation by urea, although no explanation could be given.

The behaviour of Dunaliella enzymes under different osmotic conditions and possible regulation mechanisms are being further investigated.

ACKNOWLEDGEMENTS

Thanks are due to Prof. Dr. H. Metzner for his kind interest and support during the investigations and to the Deutsche Forschungsgemeinschaft for financial support. The skillful technical assistance of Miss M. Kraljic is gratefully appreciated.

REFERENCES

1 H. LARSEN in I. C. GUNSALUS AND R. Y. STANIER, *The Bacteria, A Treatise on Structure and Function*, Vol. 4, Academic Press, New York, 1962, p. 297.
2 R. R. L. GUILLARD, *J. Protozool.*, 7 (1960) 262.
3 C. F. BAXTER AND L. L. ORTIZ, *Life Sci.*, 5 (1966) 2321.
4 W. WILBRANDT, *Ann. Rev. Physiol.*, 25 (1963) 601.
5 E. S. KANESHIRO, G. G. HOLZ, JR. AND P. B. DUNHAM, *Biol. Bull.*, 137 (1969) 161.
6 R. A. VIRKAR AND K. L. WEBB, *Comp. Biochem. Physiol.*, 32 (1970) 775.
7 W. D. DUPAUL AND K. L. WEBB, *Comp. Biochem. Physiol.*, 32 (1970) 785.
8 H. KAUSS, *Z. Pflanzenphysiol.*, 56 (1967) 453.
9 H. KAUSS, *Nature*, 214 (1967) 1129.
10 H. KAUSS, *Z. Pflanzenphysiol.*, 58 (1968) 428.
11 H. KAUSS, *Z. Naturforsch.*, 24b (1969) 363.
12 J. S. CRAIGIE AND J. MCLACHLAN, *Can. J. Botany*, 42 (1964) 777.
13 H. SENGER, *Arch. Mikrobiol.*, 40 (1961) 47.
14 E. RIES, *Beziehungen zwischen Gaswechsel und ^{14}C – Einbau monochromatisch bestrahlter Algenzellen*, Thesis, Tübingen, 1970.
15 P. SCHÜRMANN, *J. Chromatogr.*, 39 (1969) 507.
16 H. METZNER, *Photosynthetica*, 1 (1967) 249.
17 K. WEGMANN, *Der Weg des Kohlenstoffs bei der Photosynthese und Dunkelfixierung in Dunaliella spec.*, Thesis, Tübingen, 1968.
18 K. WEGMANN, in H. METZNER, *Progress in Photosynthesis Research*, Vol. 3, Institut für Chemische Pflanzenphysiologie, Tübingen, 1969, p. 1559.
19 M. K. JOHNSON, E. J. JOHNSON, R. D. MACELROY, H. L. SPEER AND B. S. BRUFF, *J. Bacteriol.*, 95 (1968) 1461.
20 H. ONISHI, *Agric. Biol. Chem.*, 27 (1963) 543.
21 F. TREZZI, M. G. GALLI AND E. BELLINI, *Giorn. Botan. Ital.*, 71 (1964) 127.
22 F. TREZZI, M. G. GALLI AND E. BELLINI, *Giorn. Botan. Ital.*, 72 (1965) 255.
23 R. H. HINTON, M. L. E. BURGE AND G. C. HARTMAN, *Anal. Biochem.*, 29 (1969) 248.
24 R. M. BAXTER, *Can. J. Microbiol.*, 5 (1959) 47.