Plant Physiol. (1972) 49, 240–243

Photosynthetic Activities of the Halophilic Alga *Dunaliella parva*

Received for publication June 18, 1971

AMI BEN-AMOTZ AND MORDHAY AVRON
Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel

ABSTRACT

***Dunaliella parva*, a unicellular halophilic alga, was found to evolve oxygen photosynthetically only in the presence of a high osmolar concentration. Cell free preparations were obtained by placing the cells in a medium of low osmolarity. The fragments obtained showed a high photoreducing and photophosphorylating activity except for their inability to catalyze all ferredoxin dependent photoreactions. Placing the cells in a medium of intermediate osmolarity produced a "chloroplast" preparation which maintained some capacity for O_2 evolution and CO_2 fixation, while possessing the ability to catalyze the photoinduced reduction of ferricyanide. Enzymic and photosynthetic reactions of cell-free preparations from *D. parva* were inhibited, rather than stimulated, by the salt concentration optimal for growth. These results were interpreted as indicating the existence of a steep NaCl gradient *in vivo* between the medium and the cell compartments which are not permeable to salt.**

Dunaliella parva, an halophilic unicellular green alga, was previously shown to lack a rigid cell wall (14). Most of the alga space is permeable to sucrose or inulin (11) and even to tris or phosphate (7). During photosynthesis it produces glycerol, which is one of the earliest labeled products (8–10, 21).

This communication will describe an investigation of the *in vivo* and the *in vitro* photosynthetic activities of *D. parva*. It will be shown that the high salt concentration required for *in vivo* photosynthesis is not manifested by *in vitro* photosynthetic and other reactions. Furthermore, advantage can be taken of the bursting of *D. parva* cells when placed in media of low osmotic strength to prepare photochemically competent cell fragments. Finally it will be shown that *D. parva* apparently lacks the ability to catalyze all ferredoxin-dependent photochemical reactions.

MATERIALS AND METHODS

Lettuce chloroplasts were prepared as previously described (3). *D. parva* was cultured as previously described (7, 15). *D. parva* "fragments" were prepared by concentrating the algae by centrifugation at 1500*g* for 10 min at room temperature, washing once in 1.5 M NaCl, 20 mM Tricine, pH 7.4, followed by dilution of a sample of the concentrated algae suspended in NaCl-Tricine in the desired reaction mixture. The resulting final NaCl concentration was 25 mM. For obtaining "chloroplasts," the reaction mixture in addition contained 0.35 M sorbitol. Chlorophyll was determined by the method of Arnon (2). NADP, cytochrome *c*, and ferricyanide reductions were measured in a Cary 14 recording spectrophotometer equipped with a scatter attachment, at 350, 550, and 420 nm, respectively. Ferredoxin reduction was measured in an Aminco-Chance dual-wavelength spectrophotometer as previously described (5).

Oxygen evolution or uptake were measured with a Yellow Springs Instrument Clark type oxygen electrode connected to a photovolt recorder. ATP formation was assayed as previously described (3).

Unless otherwise specified, light was provided by a 500 w projector, filtered through a Baird-Atomic sharp cut-off interference filter peaking at 640 nm. The intensity of the actinic light was about 4×10^4 ergs cm^{-2} sec^{-1}.

Ferredoxin of *D. parva* was isolated from algae containing 25 mg of chlorophyll (about 10 liters of algae culture suspension). After harvesting, the cells were washed twice with 1.5 M NaCl, 10 mM tris, pH 7.5, at room temperature. The resulting pellet was diluted 1/30 with 10 mM tris-HCl, pH 7.5, and allowed to stay for 20 min at 4 C. Broken cells were removed by centrifugation and NaCl was added to the supernatant up to a final concentration of 0.2 M. The supernatant was passed through a diethylaminoethyl cellulose column (1 × 15 cm), washed with 50 ml of 0.11 M NaCl, 0.15 M tris-HCl, pH 7.5, followed by 50 ml of 0.13 M NaCl, 0.15 M tris-HCl, pH 7.5. Ferredoxin was eluted with 0.56 M NaCl, 0.3 M tris-HCl, pH 7.5. This fraction showed a typical ferredoxin absorption spectrum in the visible region. The quantity isolated amounted to about 1/500 of the total chlorophyll content of the cells used for isolation.

RESULTS

Properties of Whole Cells. The absorption spectrum of a suspension of cells, and of an 80% (v/v) acetone extract of the cells, is similar to that of other green algae, with chlorophyll *a*, chlorophyll *b*, and carotenoids prominent (18). The light-induced difference spectrum is again rather typical (22) with the "520 shift" and cytochrome changes prominent. Figure 1 illustrates the light-induced oxidation of cytochrome *f* in an algae suspension containing 1 μM DCMU. As previously observed for higher plant chloroplasts (5), a clear cytochrome *f* spectrum can also be seen under these conditions in *D. parva* cells. The ratio of cytochrome *f* to chlorophyll in these cells is about 1/800.

Figure 2 illustrates the absolute requirement for a high osmolarity to maintain photosynthetic oxygen evolution. NaCl, glucose, or glycine can satisfy this requirement at similar osmolar concentrations. About 3 osmolar is required for maximal activity, and no significant inhibition can be observed until a concentration of 3.0 M NaCl is exceeded. Oxygen evolution was completely inhibited below 0.3 osmolar. Oxygen evolution in *D. parva* was fully sensitive to inhibition by DCMU or *n*-butyl-3,5-diiodo-4-hydroxy-benzoate. Half maximal inhibition was observed at 0.2 μM and 30 μM, respectively.

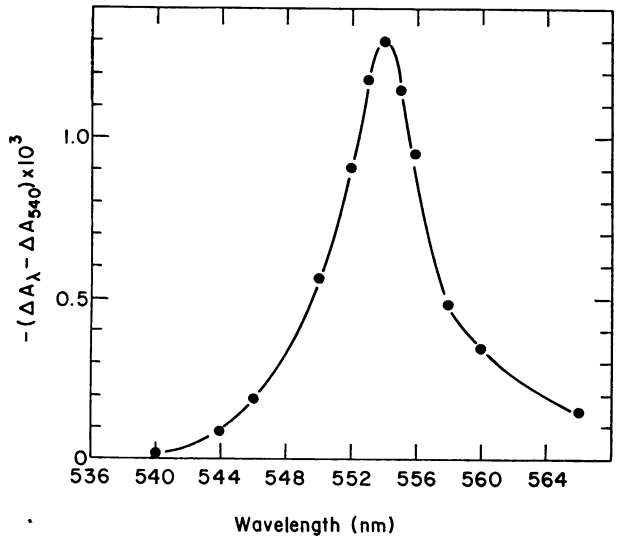

FIG. 1. Light-induced difference spectrum of cytochrome *f* of *D. parva*. The reaction mixture (3 ml) contained: 1.5 M NaCl, 20 mM Tricine, pH 7.4, 1 μM DCMU, and algae containing 162 μg of chlorophyll. Plotted is the change induced by illumination with 640 nm light (4.5×10^4 ergs cm^{-2} sec^{-1}) measured in an Aminco-Chance dual-wavelength spectrophotometer with 540 nm serving as the reference wavelength.

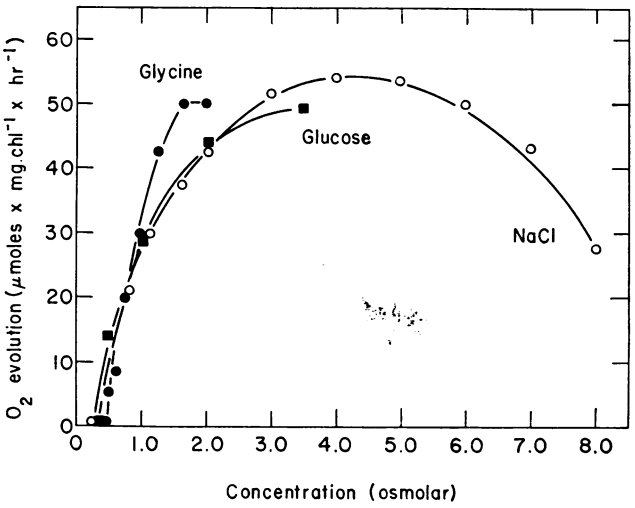

FIG. 2. Effect of the osmolar concentration of the medium on O_2 evolution in whole cells of *D. parva*. The algae were washed in NaCl, glucose, or glycine at 3.0 osmolar and then were suspended in the reaction mixture containing the indicated solution and 20 mM Tricine pH 7.4, at a final concentration of 125 μg chlorophyll/3 ml.

Properties of Cell Fragments. Whole cells of *D. parva* can neither photoreduce ferricyanide nor photophosphorylate (Fig. 3). However, as the osmolarity of the suspension is decreased the cells burst, and the fragments obtained are competent in catalyzing the above reactions. Maximal activity was observed below 0.15 M NaCl.

This unique feature of *D. parva* enables one to prepare cell fragments by simply diluting a concentrated sample of the algae in a reaction mixture whose total osmolarity is below 0.2. Table I illustrates the activity observed with such fragments. It is clear that these fragments show a relatively high photoreducing and photophosphorylating activity; the activity being equal or better than that previously observed with preparations from green algae (17). The one notable exception was the photoreduction of NADP in which these particles were essentially inactive. With the addition of 3 μM ferredoxin, which is sufficient to saturate this photoreduction in higher plant chloroplasts, essentially no activity could be observed. Much higher amounts of ferredoxin did elicit a poor reaction, the

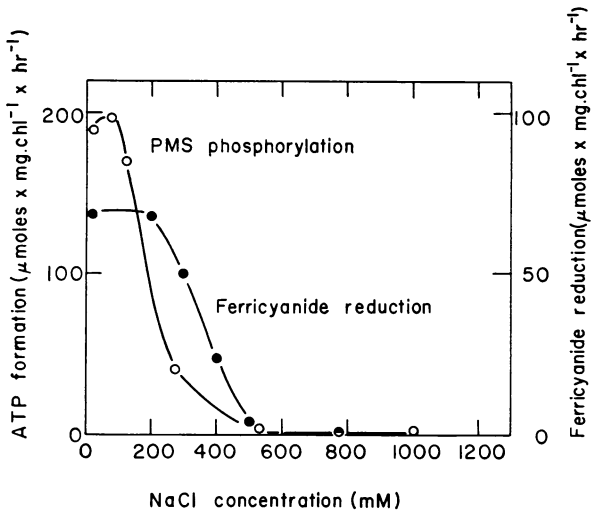

FIG. 3. Photoinduced electron transport and phosphorylation in *D. parva* as a function of the external salt concentration. Whole cells of *D. parva* were washed in 1.5 M NaCl, 20 mM Tricine, pH 7.4 and then diluted in a reaction mixture containing the indicated NaCl concentration. For ferricyanide photoreduction the reaction mixture (3 ml) contained in addition: 25 mM Tricine, pH 7.8, 0.35 mM $K_3Fe(CN)_6$, and *D. parva* cells containing 65 μg chlorophyll. The reaction mixture for PMS phosphorylation contained: 25 mM Tricine, pH 7.8, 2.5 mM sodium potassium phosphate (containing 3×10^6 cpm ^{32}P), 1.3 mM ADP, 35 μM PMS, 4 mM $MgCl_2$, and *D. parva* containing 30 μg of chlorophyll in a total volume of 3 ml.

Table I. *Photosynthetic Activities of Fragments of D. parva*

The reaction mixture (3 ml) contained 25 mM NaCl, 25 mM Tricine, pH 7.8, and *D. parva* containing 60 μg of chlorophyll and the following additions: for ferricyanide reduction, 0.5 mM $K_3Fe(CN)_6$; for reduction of NADP from H_2O, 0.5 mM NADP and ferredoxin as indicated; for Diquat reduction from H_2O, 1.2 mM azide; 50 μM Diquat; for Diquat reduction from ascorbate and DCIP, 1.2 mM azide, 50 μM Diquat, 30 μM DCIP, and 3.5 mM ascorbate; for reduction of NADP from ascorbate and DCIP, 0.5 mM NADP, 40 μM ferredoxin, 3.5 mM ascorbate, and 30 μM DCIP. Phosphorylating reaction mixtures contained, in addition, 4 mM $MgCl_2$, 1.3 mM ADP and 4 mM sodium potassium phosphate, pH 7.8, containing 3×10^6 cpm of ^{32}P. Where indicated 35 μM PMS was added.

Addition	Photoreduction	Photophosphorylation
	μmoles electrons/mg chl·hr	μmoles ATP/mg chl·hr
None		25
Ferricyanide	60	65
PMS		200
Diquat	80	95
Ascorbate + DCIP + Diquat	280	35
NADP (40 μM ferredoxin)	14	30
NADP (3 μM ferredoxin)	1	
Ascorbate + DCIP + NADP	2	

Table II. *Effect of Ferredoxin from Swiss Chard Leaves or from D. parva Cells, on NADP Photoreduction in Lettuce Chloroplasts, or D. parva Fragments*

The reaction mixture (3 ml) contained: 25 mM Tricine, pH 7.8, 0.5 mM NADP, 25 mM NaCl, 3 μM Swiss chard ferredoxin, or 0.53 μM *D. parva* ferredoxin (prepared as described in "Materials and Methods"). Lettuce chloroplasts or *D. parva* fragments contained 30 μg of chlorophyll.

Source of Ferredoxin	Lettuce Chloroplasts	*D. parva* Chloroplasts
	μmoles NADP reduced/mg chl·hr	
B. vulgaris var. cicla (Swiss chard)	90	0
D. parva	22	0

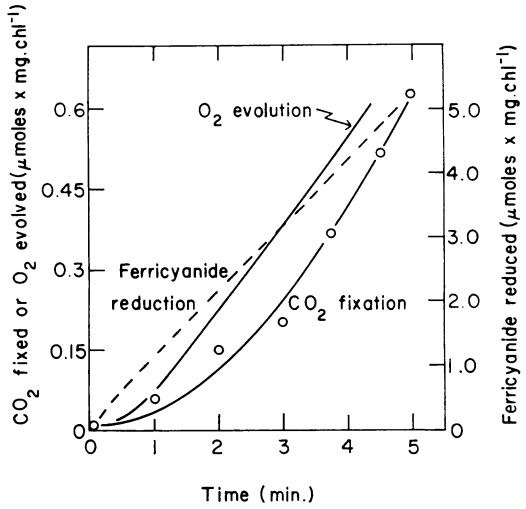

FIG. 4. O_2 evolution, $^{14}CO_2$ fixation and ferricyanide photoreduction in "chloroplasts" of *D. parva*. The reaction mixture (3 ml) for O_2 evolution contained: 0.35 M sorbitol, 50 mM HEPES pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM $NaHCO_3$, 240 mM NaCl, and *D. parva* containing 83 μg of chlorophyll. The reaction mixture for $^{14}CO_2$ fixation contained in addition 1.0 μc ^{14}C-bicarbonate. Assay for $^{14}CO_2$ fixed was as described (6). The reaction mixture for ferricyanide reduction contained in addition 0.5 mM $K_3Fe(CN)_6$ and no $NaHCO_3$.

rate of which increased with the amount of ferredoxin added up to 40 μM. No significant NADPH oxidation was observed in the dark, and no inhibition of NADP reduction was observed on mixing *D. parva* fragments with lettuce chloroplasts. To check whether this inability to photoreduce NADP may be related to specificity for an endogenous ferredoxin, we isolated ferredoxin from *D. parva* cells and tested its ability to substitute for the Swiss chard ferredoxin which was routinely used. As can be seen (Table II), either ferredoxin was effective in promoting the photoreduction of NADP in lettuce chloroplasts, but neither was effective in promoting the reaction in *D. parva* fragments.

D. parva fragments isolated by osmotic bursting, as described, were incapable of fixing CO_2. We, therefore, tried to develop a milder treatment, which would permit the entry of electron acceptors into the cells without destroying its ability to fix CO_2. Figure 4 illustrates that such a situation was approached when the cells were placed in a solution containing 0.35 M sorbitol. To differentiate these treated cells from the fragments used previously, they will be termed *D. parva* "chloroplasts." Such chloroplasts maintained about 10% of the cells' ability to evolve O_2 and fix CO_2, while possessing a capacity for photoreduction of ferricyanide equal to that of *D. parva* fragments. Nevertheless, such chloroplasts were still totally unable to photoreduce NADP. This unusual inability of *D. parva* chloroplasts or fragments will be further described in a separate communication (6).

Effect of Salt Concentration on *in Vitro* Activity. As was earlier noted (Fig. 2), the ability of *D. parva* cells to perform photosynthesis was absolutely dependent upon the maintenance of a high osmolarity in the medium. It was of interest,

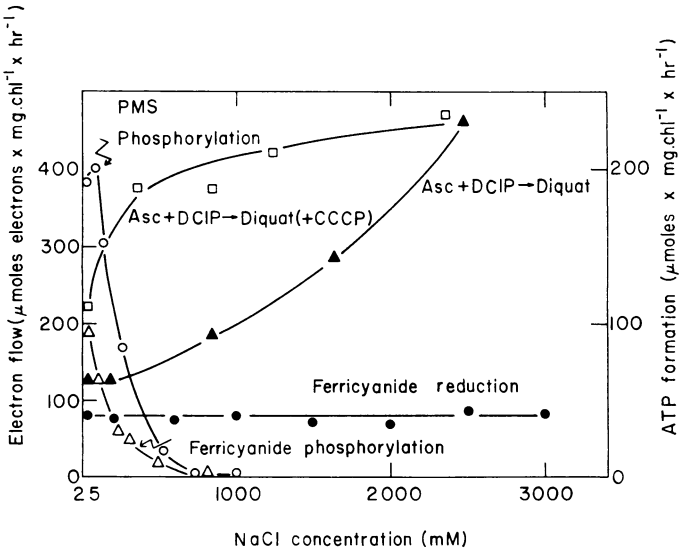

FIG. 5. Effect of salt on photoinduced electron flow and phosphorylation in fragments of *D. parva*. Algae suspended in 1.5 M NaCl, 20 mM Tricine, pH 7.4, were diluted 50-fold in the reaction mixture (final NaCl concentration 25 mM), and then NaCl was added up to the indicated concentration. Other conditions as described under Table I. Where indicated CCCP was added at 0.8 μM.

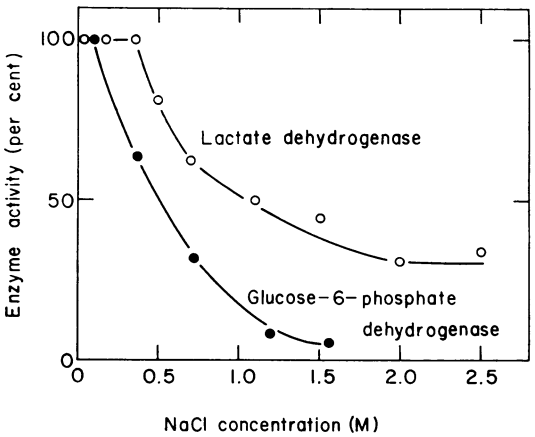

FIG. 6. Effect of salt on the activity of lactate dehydrogenase and glucose-6-phosphate dehydrogenase in fragments of *D. parva*. The reaction mixture for lactate dehydrogenase contained: 25 mM Tricine, pH 7.4, 4 mM $MgCl_2$, 0.2 mM pyruvate, 40 μM NADH, *D. parva* containing 40 μg of chlorophyll, and NaCl at the indicated concentration. The reaction mixture for glucose-6-phosphate dehydrogenase contained: 25 mM Tricine, pH 7.4, 4 mM $MgCl_2$, 1.0 mM glucose-6-phosphate, 50 μM NADP, *D. parva* containing 40 μg of chlorophyll, and NaCl at the indicated concentration. Activity was continuously monitored in a Cary 14 spectrophotometer at 340 nm. One hundred percent activity refers to: 15 μmoles NADP reduced per mg chl per hr or to 10 μmoles of NADH oxidized per mg chl per hr.

therefore, to test whether some of its *in vitro* activities would also show a dependence upon the osmolarity of the medium.

Figure 5 illustrates that most of the photoreactions catalyzed by *D. parva* fragments were either unaffected or inhibited rather than stimulated by high osmolarity in a manner similar to that described for higher plant chloroplasts (16). The only exception was the reaction from ascorbate + DCIP to Diquat,[1] and even in that case most of the effect seemed to be due to an uncoupling effect since it disappeared on addition of the uncoupler CCCP. This reaction was previously shown to be highly stimulated by treatments such as sonication or detergent treatment (13, 19).

Figure 6 illustrates that the activity of soluble enzymes not bound to the cell membrane, such as lactate dehydrogenase or glucose-6-P dehydrogenase, was also severely inhibited rather than stimulated by the salt concentrations required for cell growth. Thus, it seems that the inside of these cells most probably maintains a considerably lower salt concentration than that present in the surrounding medium.

DISCUSSION

D. parva was previously shown to require NaCl for growth and for photosynthetic glycerol production (8). These results, together with the observation that most of the cell volume contains a high NaCl concentration (11, 12), implies some essential role for NaCl in these algae. Our observations that glucose and glycine can replace NaCl for O_2 evolution indicates that at least for this function the major requirement is an osmotic one. The interpretation that NaCl freely permeates the cell membrane (12) is in contrast to our observations on the inhibition by NaCl of enzymic and photosynthetic reactions. These results point to the existence of compartments within the cell which are not permeable to salt. This should result in the formation of steep NaCl gradients between the medium and such compartments.

The photochemical activities of *D. parva* cells, the absorption spectrum, the light-induced difference spectrum, and the sensitivity to electron transport inhibitors are typical of green algae and of higher plant chloroplasts. However, the special characteristics of *D. parva* make it possible to utilize the effect of osmolarity changes for preparing subcellular particles capable of catalyzing photosynthetic reactions. A decrease in the osmotic concentration of the medium made the algae permeable to electron mediators and acceptors while maintaining some of its ability to evolve O_2 while fixing CO_2. This intermediate state produces an algal preparation which is very similar to the so-called "whole chloroplasts" isolated from higher plants (20). A further decrease in the osmolarity resulted in bursting of the cells and inhibition of O_2 evolution coupled with CO_2 fixation.

The observation that *D. parva* fragments can catalyze the photoreduction of viologens of a low redox potential such as Diquat with water at the electron donor indicates that both photosystem II and photosystem I are active in this preparation. Other results show the activity of each photosystem separately. However, they lack the ability to photoreduce NADP. This might be due to one of the following reasons: (*a*) on breakage of the cell an essential unknown component is released, or destroyed. This component is most probably located between photosystem I and ferredoxin (see also [6], and may be identical with "ferredoxin-reducing substance" or similar active species [23]). (*b*) CO_2 assimilation in *D. parva* utilizes directly the reducing power of the unknown reduced component produced by photosystem I (4).

[1] Abbreviations: CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; Diquat: 1,1′-ethylene-2,2′-dipyridylium dibromide; PMS: phenazine methosulfate.

LITERATURE CITED

1. ANTIA, N. J. AND A. WATT. 1965. Phosphatase activity in some species of marine Phytoplankters. J. Fish. Res. Board Can. 22: 793–799.
2. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
3. AVRON, M. 1961. Photophosphorylation as a tool for the synthesis of specially labeled nucleotides. Anal. Biochem. 2: 535–543.
4. AVRON, M. 1967. Mechanism of photoinduced electron transport in isolated chloroplasts. *In:* D. R. Sanadi, ed., Current Topics in Bioenergetics, Vol. 2. Academic Press, New York. pp. 1–22.
5. AVRON, M. AND B. CHANCE. 1966. Relation of phosphorylation to electron transport in isolated chloroplasts. Brookhaven Symp. Biol. 19: 149–160.
6. BEN-AMOTZ, A. AND M. AVRON. 1972. Is NADP an obligatory intermediate in photosynthesis? Plant Physiol. 49: 244–248.
7. BEN-AMOTZ, A. AND B. Z. GINZBURG. 1969. Light-induced proton uptake in whole cells of *Dunaliella parva*. Biochim. Biophys. Acta 183: 144–154.
8. CRAIGIE, J. S. AND J. MCLACHLAN. 1964. Glycerol as a photosynthetic product in *Dunaliella tertiolecta*. Can. J. Bot. 42: 777–778.
9. CRAIGIE, J. S., J. MCLACHLAN, W. MAJAK, R. G. ACKMAN, AND C. S. TOCHER. 1966. Photosynthesis in algae. II. Green algae with special reference to *Dunaliella* spp. and *Tetraselmis* spp. Can. J. Bot. 44: 1247–1254.
10. CRAIGIE, J. S., J. MCLACHLAN, R. G. ACKMAN, AND C. S. TOCHER. 1967. Photosynthesis in algae. III. Distribution of soluble carbohydrates and dimethyl-β-propiothetin in marine unicellular Chlorophyceae and Prasinophyceae. Can. J. Bot. 45: 1327–1334.
11. GINZBURG, M. 1969. The unusual membrane permeability of two halophilic unicellular organisms. Biochim. Biophys. Acta 173: 370–376.
12. GUILLARD, R. R. L. 1962. Salt and osmotic balance. *In:* R. A. Lewin, ed., Physiology and Biochemistry of Algae. Academic Press, New York, pp. 529–540.
13. KATOH, S. AND A. SAN PIETRO. 1966. Activities of chloroplast fragments. I. Hill reaction and ascorbate-indophenol photoreactions. J. Biol. Chem. 241: 3575–3581.
14. MARRÉ, E. AND O. SERVANTTAZ. 1959. Sul meccanismo di adattamento condizioni osmotiche extreme in *Dunaliella salina*. II. Rapporto fra concentrazioni del mezzo externo e composizione del succo cellulare. Atti. Accad. Naz. Lincei. Rend. Cl. Sci. Fis. Mat. Natur. 26: 272–277.
15. MCLACHLAN, J. 1960. The culture of *Dunaliella tertiolecta* Butcher—a euryhaline organism. Can. J. Microbiol. 6: 367–379.
16. SHAVIT, N. AND M. AVRON. 1967. The relation of electron transport and photophosphorylation to conformational changes in chloroplasts. Biochim. Biophys. Acta 131: 516–525.
17. SHNEYOUR, A. AND M. AVRON. 1970. High biological activity in chloroplasts from *Euglena gracilis* prepared with a new gas pressure device. FEBS Lett. 8: 164–166.
18. STRAIN, H. H. AND W. A. SVEC. 1966. Extraction, separation, estimation and isolation of the chlorophylls. *In:* L. P. Vernon and G. R. Seely, eds., The Chlorophylls. Academic Press, N. Y. pp. 21–66.
19. VERNON, L. P. AND E. SHAW. 1965. Photochemical activities of spinach chloroplasts following treatment with the detergent Triton X-100. Plant Physiol. 40: 1269–1277.
20. WALKER, D. A. AND A. R. CROFTS. 1970. Photosynthesis. Annu. Rev. Biochem. 39: 389–428.
21. WEGMANN, K. 1969. On the pathways of CO_2 fixation in *Dunaliella*. Progress in Photosynthetic Research. Vol. III: 1559–1564.
22. WITT, H. T., B. RUMBERG, P. SCHMIDT-MENDE, U. SIGGEL, B. SHERRA, J. VATER, AND J. WEIKAND. 1965. On the analysis of photosynthesis by flashlight techniques. Angew. Chem. Int. Ed. Engl. 4: 799–819.
23. YOCUM, C. F. AND A. SAN PIETRO. 1970. Ferredoxin-reducing substance (FRS) from spinach. II. Separation and assay. Arch. Biochem. Biophys. 140: 152–157.