Osmoregulation in *Dunaliella*. Intracellular Distribution of Enzymes of Glycerol Metabolism

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Dunaliella, Osmoregulation, Glycerol Metabolism, Glycerol Cycle, Enzyme Localization

Dunaliella tertiolecta was disrupted mechanically and resolved by centrifugation into chloroplast- and cytosol-enriched fractions. Intact chloroplasts could not be isolated because peripheral extensions of the single large chloroplast reached almost to the flagellar pole of the cell. The chloroplast envelope was closely appressed to the plasmalemma and, because of this and its dimensions, was vulnerable to mechanical damage to the cell.

Distribution of enzymes of the glycerol cycle between the two fractions was compared with that of two marker enzymes, phosphoenolpyruvate carboxylase (cytosol) and ribulose bisphosphate carboxylase (chloroplast). The two reversible steps of the cycle were found to be spatially separated; glycerol-3-phosphate dehydrogenase (NAD-specific) was located in the chloroplast whereas glycerol dehydrogenase (NADP-specific) was located in the cytosol. The distribution of the two irreversible enzymes, glycerol phosphate phosphatase and dihydroxyacetone kinase is uncertain. These enzymes might occur about equally in both major compartments (cytoplasm and chloroplast) or be mitochondrial or they might be loosely associated with a membrane system. Implications of these results for regulation of the glycerol cycle are discussed.

Introduction

The green flagellates of the genus *Dunaliella*, which are remarkable for their adaptability to change in salinity and for their tolerance of very high absolute concentrations of sodium chloride, osmoregulate with glycerol. The osmoregulatory mechanism is purely biochemical since it involves the synthesis and dissimilation of glycerol rather than synthesis complemented in one way or another by a glycerol efflux [1, 2]. Because there is glycerol dissimilation as well as synthesis, the metabolic sequence to glycerol must be either reversible or cyclic.

Avron, in association with Ben-Amotz, and we had separately proposed that glycerol is produced in *Dunaliella* by a reversible sequence dependent on an NADP-specific glycerol dehydrogenase (see [1]). Reversibility of the sequence from dihydroxyacetone phosphate was apparently established with Lerner and Avron's report [3] of dihydroxyacetone kinase in *Dunaliella*. On the other hand, Wegmann and his associates had argued that glycerol is produced essentially by a glycolytic type of sequence involving an NAD-specific glycerol phosphate dehydrogenase followed by a phosphatase [4]. The phosphatase reaction is not reversible. Our reasons for assigning no more that a minor role to this sequence were based partly on the low apparent activity (relative to glycerol dehydrogenase) of glycerol phosphate dehydrogenase in our early attempts to assay this enzyme.

More recently, however, Wegmann [5] has proposed a "glycerol cycle" involving synthesis of glycerol via glycerol phosphate dehydrogenase (NAD-specific) and dissimilation via glycerol dehydrogenase (NADP-specific). The other enzymes of the cycle are glycerol phosphate phosphatase and dihydroxyacetone kinase (Scheme I). This cycle is formally identical to the "mannitol cycle" of fungi [6]. A cyclic pathway is also involved in osmoregulation with galactosyl glycerol in *Ochromonas* [7].

We wish to acknowledge our agreement with Wegmann's proposal and report on the intracellular distribution of the enzymes of the cycle. Our agreement with the proposed cycle is based on the following observations: (i) the dihydroxyacetone kinase is readily detectable whereas we have been unable to detect any glycerol kinase activity, (ii) glycerol-3-phosphate phosphatase is readily detectable whereas we have been unable to detect significant levels of phosphatase activity with dihydroxy-

Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; RuBP, ribulose-1,5-bisphosphate; Tris, tris(hydroxymethyl) aminomethane.

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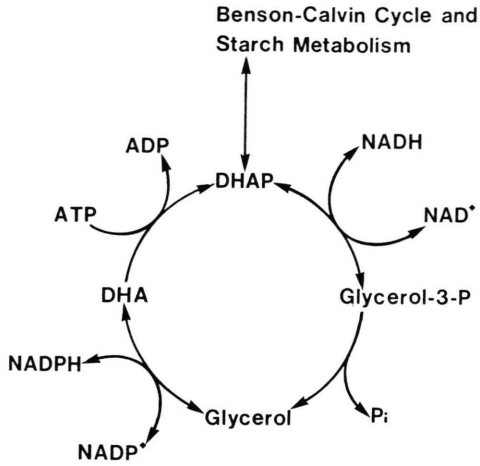

Scheme 1. The "glycerol cycle".

acetone phosphate, (iii) at the pH assumed to prevail within the cell, glycerol phosphate dehydrogenase is more active than glycerol dehydrogenase in the direction of reduction and less active in the direction of oxidation. Avron (personal communication) also acknowledges the role of this cycle. Kinetics and other properties of the glycerol cycle enzymes will be reported in due course.

Our immediate purpose is to describe the distribution of these enzymes within the cell of *Dunaliella tertiolecta*, since identification of possible compartments is essential if the site and mechanism of osmoregulation are to be identified. Determining the location of enzymes within the cell poses special problems in *Dunaliella*. Because the chloroplast envelope is closely appressed to the plasmalemma around almost the entire circumference of the cell, any treatment which ruptures or removes the plasmalemma is likely to affect the chloroplast envelope membranes as well, hence it is difficult to separate intact chloroplasts from these cells by conventional procedures.

Materials and Methods

Organism

Dunaliella tertiolecta was maintained and cultured in the defined medium of Johnson *et al.* [8], containing 0.17 M NaCl, essentially as described by Borowitzka and Brown [9] but with the following minor differences in detail. The experimental culture (one l) was inoculated to an initial density equivalent to an extinction at 700 nm of 0.04 and grown at 26° with stirring and aeration. Light intensity was equivalent to 4000 lux with a light/dark regime of 19 h and 5 h respectively. (A period of darkness was used primarily to diminish the content of starch granules which were potentially damaging to the chloroplast during subsequent fractionation.) The culture was harvested after seven days when the turbidity had reached an extinction value (at 700 nm) of 0.5.

Electron microscopy

A solution of glutaraldehyde (4% w/v, 1 vol.) in culture medium was added to *D. tertiolecta* culture (1 vol.). After 20 sec, osmium tetroxide (5% w/v in water, 2 vols) was added to the suspension which was allowed to stand for 15 min at ambient temperature. The suspension was then centrifuged briefly, dehydrated, embedded, sectioned and the sections stained conventionally with uranyl acetate and lead citrate.

Fractionation procedure

The algal suspension was centrifuged at $4000 \times g$ for 5 min at 2 °C. The pellet was gently resuspended in buffer (20 ml) of the following composition: Tris 50 mM, pH 7.5; $MgSO_4$, 5 mM; NaCl, 20 mM; dithiothreitol, 5 mM. The suspension was disrupted in a homogenizer (Polytron, Kinematica, Switzerland) at 30% maximal speed for 5 sec at 0 °C. A portion (5 ml) of the homogenate was withdrawn and designated Fraction 1. The remaining homogenate (15 ml) was centrifuged at $900 \times g$ for 60 sec at 2 °C. The supernatant fraction was designated Fraction 2 and this volume was measured. The centrifuged pellet was resuspended in a 1/10 dilution of the above homogenizing buffer (10 ml) by ten strokes of a glass/teflon homogeniser and designated Fraction 3.

The detergent, Aminoxide WS35 (Theo Goldschmidt & Co. Munich, FRG) was added to each fraction to a final concentration of 0.2% (v/v); we had previously ascertained that, except for fumarase, none of the enzymes to be assayed was inhibited by this treatment. Fumarase was inhibited by 13% in 0.2% Aminoxide. Each fraction was distributed into a series of smaller portions (0.3 or 0.8 ml) and stored either at 0 °C for immediate assay or in liquid N_2 for later assay.

Protein was estimated by the biuret method after precipitation with aqueous ethanol (80% v/v) followed by dissolution in NaOH (1.0 M).

Enzyme assays

All enzymes were assayed at 26 °C and pH 7.5 unless stated otherwise. For all assays except that of PEP carboxylase, enzyme was used in volumes of 10, 20, or 30 µl of Fractions 1 and 3 and both 50 and 100 µl of Fraction 2. Protein concentrations in these fractions lay within the ranges 3.1 to 5.3 mg · ml^{-1} for Fractions 1 and 3, and 0.8 to 1.4 mg · ml^{-1} for Fraction 2.

Ribulose bisphosphate carboxylase was assayed by the NADH-coupled procedure of Lilley and Walker [10] but with 30 mM $MgCl_2$ and 10 mM $NaHCO_3$ at pH 7.9. After 5 min preincubation, which fully activated the carboxylase, the reaction was initiated by the addition of 1 mM RuBP. Phosphoenolpyruvate carboxylase was assayed at pH 7.9 by the method of Stitt *et al.* [11]. Fumarase activity was measured by estimating fumarate formation spectrophotometrically [12].

Glycerol dehydrogenase was assayed according to Borowitzka and Brown [9] for glycerol oxidation and Borowitzka, Kessly and Brown [13] for DHA reduction except for the following reagent concentrations. For glycerol oxidation the concentration of Tris was 120 mM, that of $NADP^+$ was 1 mM and of glycerol, 2.4 M. In the reverse direction, DHA was used at 2 mM and NADPH at 0.1 mM.

Glycerol phosphate dehydrogenase was assayed in Tris buffer (120 mM) with other reagents as specified by Gancedo *et al.* [14]. Substrate concentrations were: DHAP, 1.2 mM; DL-glycerol-3-phosphate, 100 mM; NADH, 0.1 mM; NAD^+, 2 mM. The reaction was started by adding the appropriate pyridine nucleotide coenzyme after incubation of the enzyme for 3 min at 26 °C with all other reagents including one or other of the primary substrates. Reaction rates were determined from the change in absorption at 340 nm. "Blank" rates, determined without a primary substrate, were subtracted. In kinetic studies, which will be described in detail elsewhere, the enzyme was not preincubated with glycerol phosphate because of possible degradation of the substrate during this period by the glycerol phosphate phosphatase. Furthermore, Tris buffer was found slightly to inhibit the reduction of DHAP, apparently because of complex formation with it. This affected kinetics (in comparison with HEPES) but did not affect the standard assay reported here.

Dihydroxyacetone kinase was assayed by coupling the reaction to glycerol phosphate dehydrogenase according to Lerner and Avron [3]. Rabbit muscle glycerol phosphate dehydrogenase (Type 1, Sigma) was used in this assay. The method was modified from the original in the following respects: (i) the enzyme was preincubated for 3 min at 26 °C with all reagents except substrates; (ii) NADH was added and incubation was continued for long enough to determine the "blank" rate of NADH oxidation. The reaction was then started by adding ATP and dihydroxyacetone. (iii) The concentration of ATP used was 2.4 mM (instead of 0.6 mM). (iv) The reaction mixture had a total volume of 1.0 ml.

Glycerol-3-phosphate phosphatase was assayed essentially by the method of Gancedo *et al.* [14] in a total volume of 0.5 ml. The reaction mixture was buffered with imidazole (100 mM) and contained $MgCl_2$ (25 mM). Substrate (DL-glycerol-3-phosphate) concentration was 14 mM and the reaction was started by addition of the enzyme. Samples (50 µl) were withdrawn at intervals and assayed for inorganic phosphate by the method of Josse as described by Ames [15].

Gel electrophoresis

Subcellular preparations were centrifuged at $100000 \times g$ for 15 min at 0 °C and then analysed by discontinuous gel electrophoresis according to Aitken & Brown [16] with the omission of Solution 4 (sodium chloride). Ammonium persulphate was omitted from the catalyst and, as a result. Solution 3 contained only riboflavin (0.75 mg/100 ml). The gels contained 7% (w/v) acrylamide and were polymerised in the light of a fluorescent lamp. They were prepared with and without glycerol (2 M) in the gels and buffer systems. Protein was applied in solution in glycerol, the concentration of which was 1.5 M for gels that contained no glycerol and 3.0 M for gels and buffers containing glycerol. A current of 4 mA per tube was applied for 70–75 min at 5 °C.

Glycerol dehydrogenase was located specifically with a histochemical reagent [13]. Phosphatase was located specifically by incubating for 10 min at room temperature in a solution of DL-glycerol-3-

phosphate (100 mM) following by a molybdate reagent as used in the phosphatase assay (see above).

Results and Discussion

Marker enzyme distribution

Fig. 1. shows a section of *D. tertiolecta* from a 9-day culture. Algae from younger cultures were more extensively vacuolated but otherwise similar. The chloroplast occupies at least half the cell volume and has extensions, close to the plasmalemma, almost to the flagellar pole of the cell. The extensions are a major reason for the difficulty in isolating intact chloroplasts by any means dependent on mechanical breakage of the cell. It is of interest, and possible relevance to this project, that mitochondria were usually observed in the groove at the junction of the main body of the chloroplast and its extensions. Fig. 2 shows an unfractionated suspension after treatment in the Polytron. The suspension contains apparently whole cells, apparently intact cell-free chloroplasts, damaged chloroplasts and a range of small particles. The distribution of particulate matter in Fraction 3 (chloroplast-enriched) was essentially similar.

The total protein content of the supernatant (Fraction 2) showed that about one quarter of the total cellular protein was released by the homogenization, (Table I), the remainder being recovered in the pellet (Fraction 3). A slightly higher proportion (one third) of the total RuBP carboxylase activity was released; RuBP carboxylase is a marker enzyme for the chloroplast stroma [17]. A much higher proportion (two thirds) of the total PEP carboxylase activity, however, was released. Phosphoenolpyruvate carboxylase is a marker enzyme for the cytosol [11]. The partitioning of these two enzymes confirmed that Fraction 3 was enriched in chloroplast stroma and Fraction 2 in cytosol. The summed recoveries of PEP carboxylase activity (Table I) in the two fractions was slightly low (84%) compared with the crude homogenate, while that for RuBP carboxylase was almost complete.

Fumarase was assayed in some experiments in an attempt to determine the distribution of the soluble components of the mitochondrial matrix [17] between Fractions 2 and 3. The following problems were encountered:

1. Fumarase could not be detected to any appreciable extent in the crude homogenate although, after high-speed centrifugation, its activity was similar to that of PEP carboxylase. It seems, therefore, that the particulate fraction of the crude homogenate contained an inhibitor of the enzyme.

2. Fumarase activity in the high-speed supernatant of the crude homogenate or either of the fractions was unstable with a half-life of 1 – 2 h at 0 °C.

After high speed centrifugation about 90% of the fumarase activity was in the cytosolic fraction.

Glycerol cycle enzyme distribution

In the presence of Aminoxide WS-35 all four glycerol cycle enzymes were soluble. This is shown in Table II which lists activities in the supernatant solutions before and after ultracentrifugation. Fraction 2 ("cytosol") contained an inhibitor of DHA kinase associated with the particulate component (probably an ATPase). Centrifugation decreased the activity of the phosphatase by about 7% in Fraction 2 but only by about 3% in Fraction 3 ("chloroplast"). The other two enzymes were essentially unaffected by ultracentrifugation. The distribution of the four glycerol cycle enzymes together with that of the two major markers is shown in Table I where results are expressed as specific activity and as proportion (%) of the total activity found in Fraction 3. Glycerol dehydrogenase partitioned between fractions in the same proportion as PEP carboxylase, the cytosol marker. On the other hand, glycerol phosphate dehydrogenase partitioned similarly to RuBP carboxylase, the chloroplast marker. In this case however, there was an apparent difference in the distribution of the "forward" and "reverse" reactions, the proportion of DHAP-reducing activity in the chloroplast being higher than that of glycerol phosphate oxidising activity. The mean difference between the partitioning of the two reactions was significant at the 2% level.

Several possibilities, including isoenzymes, were considered as reasons for the different apparent distribution of the two reactions, forward and reverse, catalysed by glycerol phosphate dehydrogenase. The explanation apparently lies in an artifact caused by some kinetic peculiarities of the enzyme and the conditions of assay. True kinetic constants for DHAP reduction are shown in Table III. They are identical for each subcellular fraction and lend no support to an explanation based on isoenzymes which, if they were to produce the discrepancy shown in Table I, would necessarily have dif-

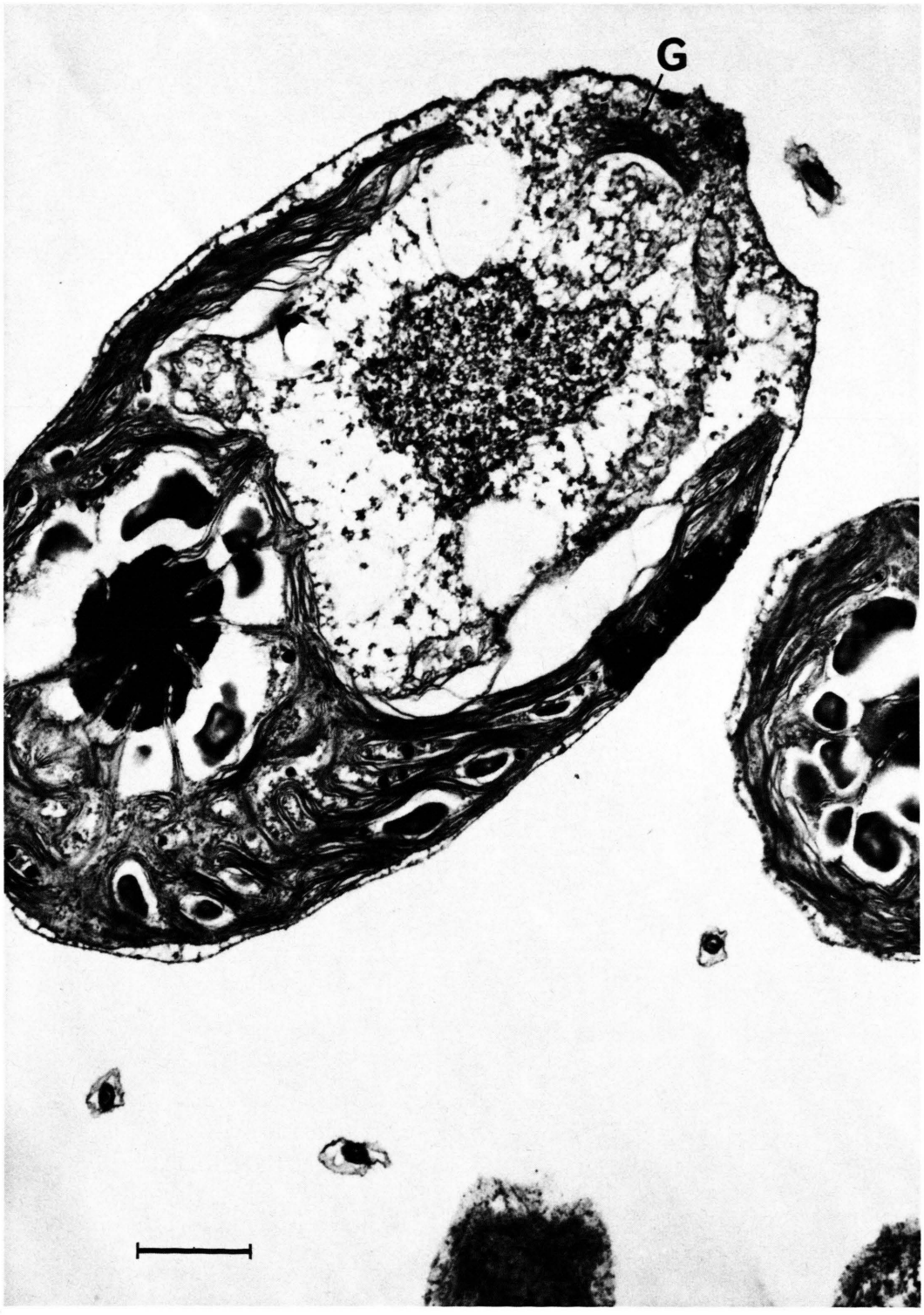

Fig. 1. A section of *Dunaliella tertiolecta* from a 9-day culture. The chloroplast occupies about half the cell volume. The extensions often approach the flagellar pole more closely than in this illustration. The number of vacuoles in the cytoplasm diminishes with increasing culture age. The nucleus is evident and a presumed Golgi body (G) is situated near the flagellar pole. The small, spherical electron-opaque granules within the chloroplast are thought to contain a carotenoid. The bar denotes 1 μm.

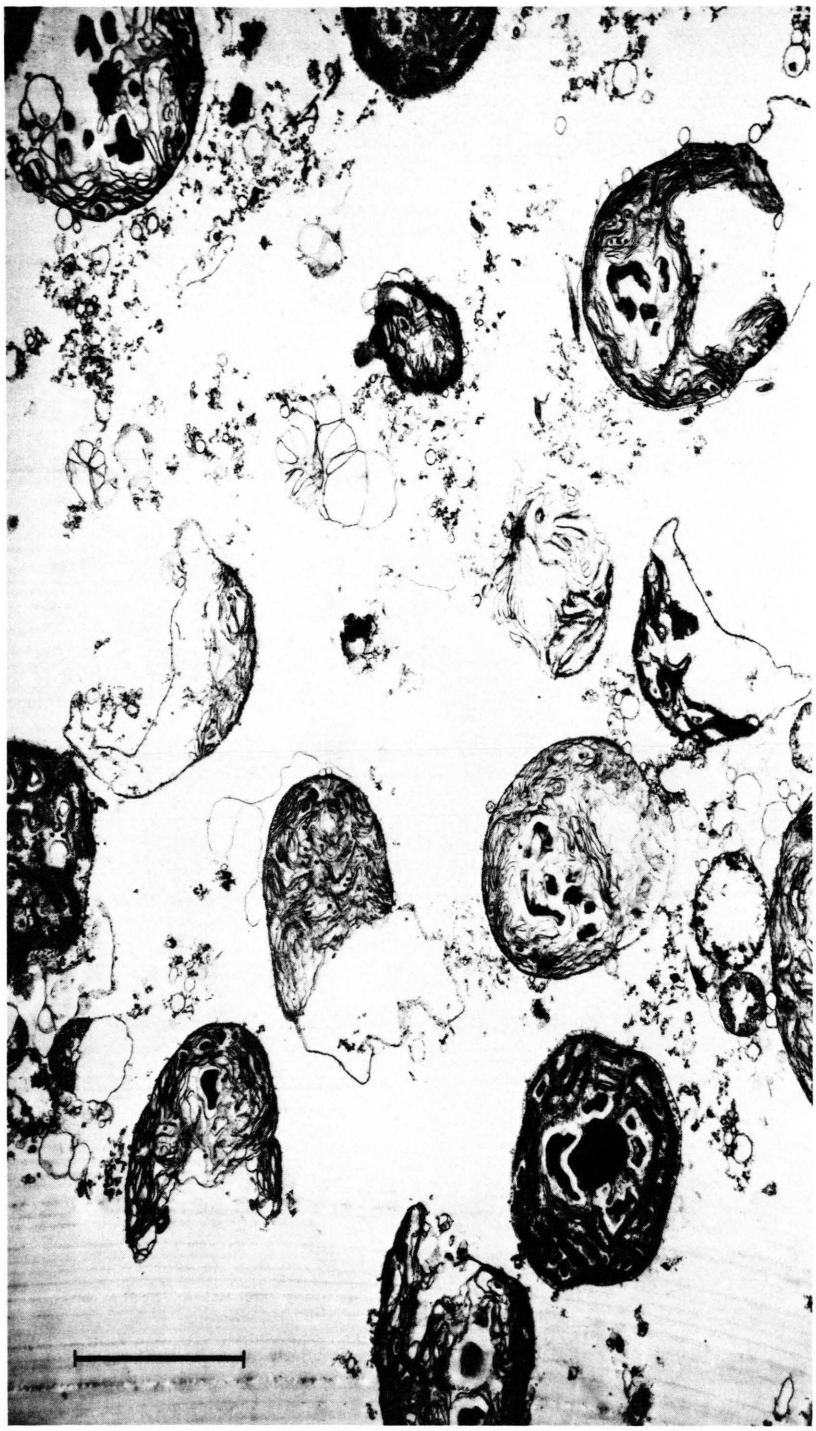

Fig. 2. A section of a *Dunaliella tertiolecta* suspension after treatment in the Polytron. The suspension has not been fractionated but contains a similar range of particles to Fraction 3, the chloroplast-enriched fraction. The particles include whole cells, apparently intact cell-free chloroplasts, damaged chloroplasts and a variety of smaller particles. The bar denotes 5 μm.

Table I. Distribution of enzyme activities and protein following fractionation.

Fraction	Specific activity [nmol · mg^{-1} protein · min^{-1} ± S.D.] 1	2	3	Partitioning[a] Proportion in "chloroplast enriched" fraction [%] ± S.D.	Total recovery [%] ± S.D. (2 + 3)/1
Glycerol dehydrogenase					
DHA reduction	26 ± 8 (6)	72 ± 25 (6)	10 ± 4 (6)	27.8 ± 10.8 (6)	100.8 ± 11.3 (6)
glycerol oxidation	29 ± 5 (6)	73 ± 9 (6)	11 ± 3 (6)	30.2 ± 6.5 (6)	94.9 ± 12.8 (6)
Glycerol-P Dehydrogenase					
DHAP reduction	89 ± 22 (6)	152 ± 39 (6)	75 ± 18 (6)	58.1 ± 4.9 (6)	104.9 ± 11.8 (6)
glycerol-P oxidation	11 ± 2 (4)	12 ± 2 (4)	9 ± 2 (4)	65.5 ± 3.9 (4)	90.5 ± 20.1 (4)
Glycerol-P phosphatase	438 ± 72 (6)	746 ± 149 (6)	349 ± 68 (6)	56.6 ± 8.9 (6)	99.1 ± 7.5 (6)
DHA kinase	10 ± 5 (5)	17 ± 9 (5)	9 ± 4 (5)	57.9 ± 10.5 (5)	103.1 ± 14.7 (5)
PEP carboxylase	12 ± 3 (6)	21 ± 11 (6)	4 ± 1 (6)	33.0 ± 4.9 (5)	84.1 ± 9.5 (5)
RuBP carboxylase	293 ± 54 (6)	372 ± 120 (6)	264 ± 85 (6)	66.3 ± 5.0 (6)	95.7 ± 14.0 (6)
Protein (mg/total fraction)	57.6 ± 8.7 (6)	15.0 ± 3.6 (6)	41.5 ± 9.3 (6)	73.4 ± 2.4 (6)	97.3 ± 9.2 (6)

The bracketed numeral denotes the number of observations, specifically the number of algal extracts assayed.
[a] "Partitioning" is total activity in fraction (3)/total activity in fractions (2 + 3) expressed as a percentage.

Table II. Effects of ultracentrifugation on the specific activity of enzymes of the glycerol cycle.

Fraction	Enzyme Activity [nmol · min^{-1} · ml^{-1} extract] 2	2S	3	3S
DHA kinase	12	20	41	40
DHAP reduction	116	118	318	327
DHA reduction	65	66	67	65
Glycerol oxidation	53	56		
Glycerol-P phosphatase	228	213	259	252

Fractions 2 and 3 were centrifuged at 100000 × *g* for 15 min at 2 °C. Enzyme activities were assayed on these fractions before (2, 3) and after (2S, 3S) centrifugation.

Table III. True kinetic constants for dihydroxyacetone phosphate reduction by glycerol-3-phosphate dehydrogenase from the chloroplast- and cytosol-enriched fractions of *Dunaliella tertiolecta.*

	Variable Substrate NADH		DHAP	
	K_m[mM]	V_{max}	K_m[mM]	V_{max}
"Cytosol"	0.048	354	0.108	305
"Chloroplast"	0.042	336	0.101	297

The enzyme was assayed at pH 7.5 in Hepes buffer. The protein content of the assays was 21 µg and 32 µg for the "cytosol" and "chloroplast" fractions respectively. V_{max} units: nmol · mg^{-1} protein · min^{-1}.

ferent kinetic constants. Corresponding constants for the reverse reaction are not shown because the enzyme gave sigmoidal double reciprocal plots with glycerol phosphate as variable substrate. The shape of these plots varied with protein concentration, however, and they were linear at high protein concentrations (about 300 µg/ml). Differences in protein concentration were sufficient to cause different responses to glycerol phosphate concentration in the two fractions and thus the apparently different partitioning of the enzyme in each direction. The partitioning based on the kinetic assays was 77–23, chloroplast-cytosol and identical for each direction of the reaction. This ratio is not strictly comparable with those in Table I since the kinetics were done on a 100000 × *g* supernatant solution from each fraction but they leave no doubt that glycerol phosphate dehydrogenase is predominantly a chloroplast enzyme. The kinetics will be reported in full elsewhere but, at this stage, we should point out that the optimal pH for DHAP reduction is about pH 7 whereas that for glycerol phosphate oxidation is about pH 9. Activity in that direction is low at pH 7.5, at which it was assayed in these experiments. These optima are similar to those of the glycerol dehydrogenase [9, 13].

The other two enzymes, the phosphatase and the kinase, were much less clear-cut in their distribution. In fact they partitioned similarly to DHAP reduction but the argument for assigning this to the

chloroplast cannot be applied to the two irreversible enzymes. Attempts to detect the kinase after gel electrophoresis were unsuccessful but the phosphatase was readily detected and produced one band with identical mobility from each fraction (Fig. 3). Furthermore each enzyme gave normal Michaelis-Menten kinetics so that, if isoenzymes were present, they evidently had identical constants. Isoenzymes cannot be excluded on that account, however, since some are known which normally occur in different cellular compartments and have identical kinetic constants although their electrophoretic or chromatographic mobility is usually different [18].

A second possibility is that they were derived from a different compartment such as the mitochondria. This cannot be evaluated because of the uncertainties in fumarase partitioning already described. In earlier experiments (not reported) we had a similar problem with NAD-linked isocitrate dehydrogenase in that its specific acitivity in all fractions was very low and partitioning was ambiguous.

Although the results are not completely unambiguous for all four enzymes of the glycerol cycle, the two dehydrogenases are clearly located predominantly in different cellular compartments. The glycerol phosphate dehydrogenase, which is involved (in the present context) in glycerol synthesis, is located in the chloroplast whereas glycerol dehydrogenase, involved in glycerol dissimilation, is cytosolic. Thus transport (in its widest sense) of substrates across the chloroplast envelope is an integral part of the glycerol cycle and might itself be a regulatory step. It can be assumed, however, that the phosphorylated intermediates cross the chloroplast envelope in either direction by specific translocators whereas the non-phosphorylated compounds might do so by diffusion, the latter not being susceptible to specific regulation.

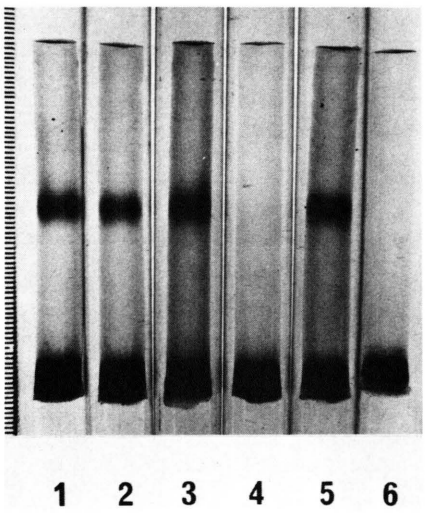

Fig. 3. Polyacrylamide gels stained specifically for glycerol-3-phosphate phosphatase after electrophoresis of cell-free preparations of *Dunaliella tertiolecta*. Tube 1, Fraction 1, (whole extract, 150 µg). Tubes 2, 3, 4, Fraction 2 ("cytosol"), tube 2, 50 µg; tube 3, 100 µg; tube 4, 100 µg with DHAP as substrate (or with no substrate). Tubes 5 and 6, Fraction 3 ("chloroplast"), 150 µg. Tube 6, DHAP or no substrate. Migration was from top to bottom. The grid or the left is graduated in 1 mm divisions.

Uncertainties in the distribution of the two irreversible enzymes prevent a conclusion about which substrates are transported. Either glycerol-3-phosphate or glycerol is evidently transported out of the chloroplast during operation of the cycle, a process that might complement or substitute for the better known efflux of DHAP during photosynthesis [19]. Completion of the cycle as written (Scheme 1) requires transport into the chloroplast of either DHA or DHAP but this might not occur under some conditions in the light when the chloroplast would normally be expected to export photosynthetically generated DHAP [19]. During dilution stress, however, either DHA or DHAP should be taken up by the chloroplast for starch biosynthesis.

The synthesis and degradation of starch in illuminated chloroplasts from leaf cells of higher plants occur simultaneously [20]. These processes are greatly affected by changes in metabolic fluxes across the chloroplast envelope, mediated by changes in the concentration of inorganic phosphate external to the chloroplast [20]. Given the ability of inorganic phosphate to regulate also the rate of photosynthetic carbon fixation [21], the cytosolic inorganic phosphate concentration in *Dunaliella* must be considered likely to be involved in the mediation of glycerol synthesis and dissimilation, especially since it is affected by water stress [22].

Certain physiological and biochemical consequences should flow from the spatial separation of the two dehydrogenases. The location of the glycerol phosphate dehydrogenase in the chloroplast implies that this organelle must be able to sustain a major NADH-dependent pathway in addition to the NADPH-dependent Benson-Calvin cycle. The apparent requirement by glycerol synthesis and carbon

fixation for different reduced pyridine nucleotide pools might, itself, have important regulatory implications. Another result of the separation, however, is that the continuous operation of the cycle will have the net effect of transferring reducing equivalents from NADH in the chloroplast to $NADP^+$ in the cytosol.

The other two enzymes of the cycle, the kinase and the phosphatase, partitioned ambiguously in our fractionation procedure. Even though these enzymes are soluble, at least in the presence of the detergent, however, we cannot exclude the possibility that, *in vivo*, they are associated in some way with the chloroplast envelope, or for that matter, another membrane system. Thus it might not be a coincidence that an inhibitor of the kinase was associated with particulate material and there was evidence of some small degree of association of the phosphatase with sedimentable material. If the enzymes are indeed loosely associated with the chloroplast envelope they might also be involved in the transport by a type of group translocation.

Direct experimental testing of this possibility is not easy because of the difficulty in isolating the *Dunaliella* chloroplast, notwithstanding a claim by Kombrink and Wöber [23] to have done so from *D. marina* by inducing lysis of the plasma membrane with DEAE-dextran. Their report was based on the recovery from sucrose density gradient centrifugation of a substantial proportion of the total cellular RuBP carboxylase activity in a chlorophyll-containing "heavy" fraction. This fraction, however, also contained fumarase and catalase in proportions which, when recalculated from Kombrink and Wöber's Table I, amounted respectively to about one quarter and one half of the total activities recovered from the gradient. Such high proportions of non-chloroplast enzymes [17] suggest that Kombrink and Wöber's "H Fraction" comprised a chloroplast-enriched fraction similar to our own rather than intact chloroplasts as claimed by those authors.

Gimmler (personal communication) has made a thorough study of the lysis of *Dunaliella* cells by DEAE-dextran and has demonstrated selective release of cellular enzymes depending on the conditions of incubation. His conclusions about the distribution of enzymes of glycerol cycle are similar to those we have reached in this report.

Acknowledgements

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Note added in proof: Gradient gel electrophoresis also confirmed the electrophoretic identity of the phosphatase from chloroplast- and cytosol-enriched fractions and assigned to it an apparent molecular weight of 147000. Under the same conditions the glycerol dehydrogenase had an apparent molecular weight of 225000. Taking all factors into account we consider that the most likely explanation of the partitioning of the phosphatase and kinase is that each enzyme occurs in both the cytosol and chloroplast stroma.

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