Isolation of Intact Chloroplasts from \textit{Dunaliella tertiolecta}\textsuperscript{1}

Received for publication March 22, 1988 and in revised form April 29, 1988

ARUN GOYAL, THOMAS BETSCHE\textsuperscript{2}, AND N. E. TOLBERT*  
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Cells of \textit{Dunaliella tertiolecta} from the log phase of growth were broken by rapid extrusion at low pressure through a Yeda press and the chloroplasts were isolated by centrifugation through a Percoll gradient. Osmolarity of the growth medium, the suspending media, and the Percoll gradient was kept identical to minimize change in chloroplast volume and mitochondrial entrapment. The isolated intact chloroplasts were obtained in a 30 to 50% yield based on chlorophyll and were stable to washing with buffered medium. Isolated chloroplast yield and purity was dependent on cell culture condition; a cycle of 16 hours light and 8 hours dark with continuous high CO\textsubscript{2} was optimum. Isolated chloroplasts were about 90% intact by microscopic examination, ferricyanide-dependent O\textsubscript{2} evolution, and the distribution of four stromal enzymes. Enzymes associated with glycolate metabolism were not in the chloroplast fraction. The isolated chloroplasts with 10 millimolar bicarbonate evolved 24 micromoles of O\textsubscript{2} and fixed 21 micromoles of CO\textsubscript{2} per hour per milligram of chlorophyll, which rates were about one-third of those by whole cells. The inhibition of oxygen evolution by 10 millimolar phosphate was reversed by P-glycerate. Whole chloroplasts were also isolated from cells adapted to low CO\textsubscript{2} in air for 24 hours. On low CO\textsubscript{2} the cells excreted more gelatinous material, which had to be removed with additional washing of the cells, before it was possible to obtain good chloroplast preparations.

The inability to isolate and purify physiologically intact chloroplasts from unicellular green algae has been a limitation for studies of algal photosynthesis and metabolism. Techniques for isolating active chloroplasts from leaves of higher plants (18, 25), when used with unicellular algae, have provided chloroplasts in very low yields and uncertain purity. Autolysis digestion and mild detergent treatment has been used to disrupt the cell wall of \textit{Chlamydomonas reinhardtii} (1, 12) and Euglena (21), and chloroplasts have been prepared from cell wall-less mutants of \textit{Chlamydomonas} (14, 16). The chloroplasts with some mitochondria were prepared in low yields of 1 to 2% by breaking the algal cells in a Yeda press and separating by centrifugation in an elutriation rotor (16, 22). Our report is about a 1 h procedure for the isolation and purification of chloroplasts in high yield from \textit{Dunaliella} by using a Yeda press and Percoll density gradient centrifugation. \textit{Dunaliella} has advantages over \textit{Chlamydomonas} and Euglena, because \textit{Dunaliella} cells lack a cell wall and are easily broken in a French pressure cell. In addition, the osmolarity of the chloroplast isolation media and the \textit{Dunaliella} growth medium can be similar, whereas \textit{Chlamydomonas} will not grow at high salt concentrations (10). In the present experiments 0.17 M NaCl has been used for cell growth and isosmotic 0.33 M sorbitol buffer for chloroplast isolation. For \textit{Chlamydomonas}, the chloroplast isolation media is hypertonic, and when the cells are resuspended in the isolation media, cell and chloroplast shrinkage occurs and the folding of the chloroplast may entrap mitochondria. This can cause contamination of the chloroplast preparations with mitochondria as observed by marker enzymes (our unpublished observations) and by electron microscopy (J Moroney, personal communication).

MATERIALS AND METHODS

Organism and Growth Conditions. \textit{Dunaliella tertiolecta} (CSIRO strain) (3) was maintained on agar slants at room temperature at low light. Cells were grown with rotary agitation at 25 to 26°C in white fluorescent light (80 µE·m\textsuperscript{-2}·s\textsuperscript{-1}) on a defined medium containing 0.17 M NaCl (11), but modified by omitting vanadium and by increasing the phosphate content to 0.73 mM. Stock solutions of KH\textsubscript{2}PO\textsubscript{4} and FeCl\textsubscript{3}·6H\textsubscript{2}O were sterilized separately and added aseptically to the medium. Slants of agar medium were prepared similarly with the addition of 1.5% agar.

A liquid growth medium (100 mL in 250 mL Erlenmeyer flask) was seeded with an axenic culture from an agar slant and incubated in continuous light with air but without shaking, to develop a preinoculum culture. After 1 week, other 100 mL portions of medium were inoculated with 10 mL of the preinoculum and incubated for another week in continuous light. Finally, a 100 mL inoculum was added to 1 L of the growth medium in a 3 L Fernbach flask. Cultures were aerated either with 5% CO\textsubscript{2} in air or with air. In order to partially synchronize the cells and to prevent excessive accumulation of starch, a light/dark regime of 16/8 h was selected. Before harvesting, cultures were examined microscopically for contamination. Exponentially growing cultures, about 6 d after inoculation, were harvested by centrifugation at 1000 g for 10 min. The cell pellet was resuspended in fresh growth medium and then pellet again by centrifugation at 1500g for 10 min. This was considered to be one washing, and it was repeated another time for algae which had been adapted on low CO\textsubscript{2} for 24 h to remove more of the extracellular slime. The cell pellet was resuspended in 15 to 20 mL ice-cold isolation medium containing 330 mM sorbitol, 50 mM Hepes, 2 mM EDTA, 1 mM MnCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 15 mM β-mecraptoethanol, and 0.5% (w/v) BSA (Sigma A4403) adjusted to pH 7.5 with KOH. The algal suspension contained about 0.5 mg Chl·mL\textsuperscript{-1} and was equivalent to about a 5% (v/v) cell suspension.

Breakage of the Cells and Density Gradient Centrifugation. A 10 to 15 mL cell suspension in an ice-cold Yeda press was kept under a low, 150 to 200, p.s.i. of nitrogen for 4 min. The outlet valve was then opened to a rapid flow rate of about 4 mL·s\textsuperscript{-1}, and the pressate was collected in an ice-chilled tube. After centrifugation at 1,000g for 30 s, the pellet was resuspended in

\textsuperscript{1} Supported by the McKnight Foundation and the Michigan Agricultural Experiment Station and published as journal article No. 12559 at the Michigan Agricultural Experiment Station.

\textsuperscript{2} Present address: Department de Biologie, Service de Radioagronomie, C.E.N. de Cadarache, 13108 Saint Paul Lez Durance, France.
the isolation medium to about 1 mg Chl·mL⁻¹. This chloroplast enriched suspension, containing 3 to 4 mg Chl, was placed on the top of a continuous Percoll gradient (15–75%, 30 mL) which was layered on a cushion of 3 mL of 100% Percoll. Percoll solutions contained 0.33 m sorbitol and 25 mM Hepes adjusted to pH 7.5 with KOH. Gradient centrifugation was run at 20,000g at 4°C for 15 min in a Sorvall SS-34 rotor. The gradient was collected from the bottom of the tube into 30 drop or 0.85 mL fractions. The chloroplast fractions (see “Results”) were pooled and diluted 10-fold with a resuspending medium containing 0.33 m sorbitol and 25 mM Hepes (pH 7.5). The chloroplasts were pelleted by centrifugation at 1,000g for 30 s and the rotor was stopped within 90 s. The chloroplast pellet was gently agitated to resuspend it in a small volume (1–2 mg Chl·mL⁻¹) of resuspending buffer. These steps to wash the chloroplasts once removed most of the Percoll and some mitochondrial membrane or mitochondria, as judged by decreased Cyt c oxidase activity.

Oxygen Exchange and ¹⁴CO₂-Fixation. Oxygen exchange was measured in a Clark-type oxygen electrode (Rank Brothers, Cambridge, U.K.). The reaction mixture of 2 mL contained 330 mM sorbitol, 1 mM MgCl₂, 2 mM EDTA, 50 mM Hepes (pH 7.5), 10 mM NaHCO₃, 1 mM KH₂PO₄, and 50 to 60 μg Chl·mL⁻¹ from the chloroplast preparation. When oxygen evolution and ¹⁴CO₂-fixation were measured simultaneously, the assay mixture contained 10 mM NaH¹⁴CO₃ (2 μCi·mL⁻¹) with a specific activity 0.2 mCi·mmol⁻¹. At time intervals, 50 μL samples were withdrawn and added to 500 μL of 0.5 mM glacial acetic acid, and the mixture was swirled several times in the next 3 h before fixed radioactivity was determined by liquid scintillation counting.

Enzyme Extraction Assay, and Analytical Methods. Intact cells or isolated chloroplasts were resuspended in 5 mL cold 50 mM Hepes-K⁺ buffer (pH 7.5) containing 5 mM DTT. The suspensions were placed into a cold Yeda press under nitrogen at a high, 1,500 p.s.i. for 6 min. The valve was slightly opened, and the homogenate was slowly collected over about 4 min. This procedure was then repeated with the lysate. An aliquot of the final homogenate was used to determine Chl and Cyt c oxidase activity. The homogenate was centrifuged at 35,000g for 15 min and the supernatant fluid was passed through a 0.22 μm membrane filter. The filtrates were designated as cell or chloroplast supernatants.

Enzyme assays at 25°C followed published procedures: NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (2), NADH:dihydroxyacetone phosphate reductase (6), ribulose-P₃ carboxylase/oxygenase (17), PEP-carboxylase (15), fumarase (9), and Cyt c oxidase (20). Analysis for enzymes associated with the C₃ cycle are found in Methods in Enzymology (23). The gluta-mate dehydrogenase assay (19) was modified to contain 200 mM Bicine buffer (pH 8.0), 0.12% Triton X-100, 1.2 mM CaCl₂, 50 μM NADPH or NADH, 20 mM 2-ketoglutarate, 40 μM KCN, and 125 mM ammonium sulfate. Chl was measured spectrophotometrically. Calculation of the specific activities of enzymes was based on the Chl content of the original cell or chloroplast homogenate.

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Distribution of intact chloroplast after isopycnic centrifugation in a Percoll density gradient. (●), A₆₅₂ is for the Chl in a 25 μL aliquot from each fraction which was diluted to 1 mL in ethanol for the absorbance reading.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>From total homogenate</th>
<th>From intact chloroplasts</th>
<th>Activity in Chloroplast Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>¹⁴CO₂ cpm × 10⁶ fixed·h⁻¹·mg Chl⁻¹</td>
<td>10⁻⁶·μmol·min⁻¹·mg Chl⁻¹</td>
<td>%</td>
</tr>
<tr>
<td>Ribulose-P₃ Carboxylase/oxygenase</td>
<td>20.3</td>
<td>17.2</td>
<td>85%</td>
</tr>
<tr>
<td>NADP-Glyceraldehyde-3-P dehydrogenase</td>
<td>13.8</td>
<td>11.1</td>
<td>86%</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate reductase</td>
<td>5.2</td>
<td>5.7</td>
<td>109%</td>
</tr>
<tr>
<td>NADPH:glyoxylate reductase</td>
<td>2.51</td>
<td>1.80</td>
<td>72%</td>
</tr>
<tr>
<td>NADP-Glutamate dehydrogenase</td>
<td>0.015</td>
<td>0.012</td>
<td>77%</td>
</tr>
<tr>
<td>PEP-Carboxylase</td>
<td>1.089</td>
<td>0.078</td>
<td>7%</td>
</tr>
<tr>
<td>Fumarase</td>
<td>0.204</td>
<td>0.021</td>
<td>10%</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>3.23</td>
<td>0.28</td>
<td>8%</td>
</tr>
<tr>
<td>Catalase</td>
<td>36.9</td>
<td>1.8</td>
<td>5%</td>
</tr>
<tr>
<td>Glycolate dehydrogenase</td>
<td>0.011*</td>
<td>0.001*</td>
<td>~9%</td>
</tr>
<tr>
<td>Serine:glyoxylate amino transferase</td>
<td>&lt;0.06</td>
<td></td>
<td>~9%</td>
</tr>
<tr>
<td>Glutamate:glyoxylate aminotransferase</td>
<td>&lt;0.017*</td>
<td></td>
<td>~9%</td>
</tr>
<tr>
<td>NADH:hydroxypropionate reductase</td>
<td>5.39</td>
<td>0.72</td>
<td>13%</td>
</tr>
<tr>
<td>NAD-glutamate dehydrogenase</td>
<td>0.39</td>
<td>0.13</td>
<td>33%</td>
</tr>
</tbody>
</table>

* Activity too low to be significant.
RESULTS AND DISCUSSION

Growth of Cells. Successful chloroplast isolation from Dunaliella was dependent on several factors. (a) All experiments were run with Dunaliella in the late exponential log phase of growth. When cultures became denser and entered a stationary phase of growth, much slime or gelatinous material surrounded the cells. This material hindered cell breakage and occluded in part the chloroplast fraction to cause clumping, and during centrifugation it retained organelles and thus prevented good particle separation. (b) When older cultures were diluted with fresh medium to continue the culture, serious clumping also occurred during isolation of the chloroplasts, probably due to increased extracellular material. Thus new cultures were used for each chloroplast preparation. (c) Dunaliella cultures were grown in 0.17 M NaCl and the isolation buffers were adjusted to the same osmoticum with 0.33 M sorbitol to limit chloroplast changes in volume during isolation. Without this precaution entrapment of other subcellular fractions by the chloroplast fraction greatly increased. (d) Cultures were grown in a 16 h light and 8 h dark cycle and harvested 4 to 6 h after the dark period. This cycle reduced the starch content so that the plastids were not broken by pelleting of dense starch granules during centrifugation. (e) During the growth cycle the cultures were continuously aerated with 5% CO2 in air which greatly reduced the extracellular slime, as judged by low viscosity in the top part of the Percoll gradient. In addition, cells grown continuously in high CO2 were more completely ruptured and more freely release intact chloroplasts by the low pressure Yeda press extrusion. It was best to continue the aeration with 5% CO2 during the dark period to prevent excretion of extracellular slime. Slow growth of Dunaliella on low CO2 produced too much slimy material for chloroplast isolation. However, chloroplasts could be isolated from cells that had been grown on high CO2 for 5 d and then adapted for 1 d to low CO2 (air). During this time Dunaliella cells and their isolated chloroplasts had developed a CO2 concentrating mechanism (our unpublished observations).

Chloroplast Isolation. The cell envelope of Dunaliella was easily broken when cells were rapidly extruded from the Yeda press with low pressure (150-200 p.s.i.). From microscopic examination, only a few cells remained unbroken and motile. A few were partially damaged, but most (about 90%) had lost their plasma membrane. For nearly complete cell breakage, it is important to release the cells from the Yeda pressure chamber quickly; slow release of the cell suspension from the press at low p.s.i. resulted in insufficient breakage. Higher pressure could not be used due to chloroplast rupture.

During Percoll gradient centrifugation, intact chloroplasts equilibrated at a density of 1.139 to 1.280 g·mL⁻¹ in fractions 3 to 6 out of a total of 39 fractions collected from the developed gradient (Fig. 1). Whole cells and broken chloroplasts equilibrated at a density of 1.1231 to 1.1071 g·mL⁻¹ in fractions 10 to 16. Whole cells were placed on the gradient to confirm this, and microscopic inspection of each fraction during chloroplast isolations indicate that the whole cells or broken chloroplasts were spread between fractions 10 to 16. A third peak of Chl in fractions 35 to 39 contained membranes and chloroplasts trapped in gelatinous material at the top of the gradient.

The intact chloroplast band on the Percoll gradient was very compact in fraction 3 to 6. The yield of whole plastids on a Chi basis was about 30 to 50%, and they contained less than 2%...
broken or resealed cells as judged by microscopic examination. The contamination from other subcellular compartments was about 5 to 8% (Table I). Similar results were obtained with cells grown for 5 d with 5% CO₂ and then adapted to low CO₂ (air) conditions for a day, except that the yield of chloroplast was about 20%. Lower yields were attributed to increased extracellular gelatinous material, even though these air adapted cells were washed a second time.

In the first part of this investigation Dunaliella cells were aerated with 5% CO₂ during the light phase, but not during the dark. These cells were used for data in Table I. Because these cells produced more gelatinous extracellular protein, the whole chloroplast fraction from the Percoll gradient was spread over in fractions 4 to 9 (data not shown). The lower fractions (4 and 5) of the intact chloroplast band were nearly free of whole cells, while the upper part (fractions 6 to 9) was slightly more contaminated. Intact chloroplasts could also be separated on a two-step Percoll gradient of 45 and 75% Percoll, but such chloroplasts were more contaminated with whole cells.

**Intactness of Chloroplasts and Photosynthetic Capacity.** The 80 to 90% intactness of the isolated chloroplasts was estimated by their structural integrity as indicated by a bright halo under phase contrast microscopy (Fig. 2). Based on ferricyanide-dependent oxygen evolution by chloroplasts before and after osmotic lysis (13), the intactness of the chloroplasts was 80 to 90%. The chloroplast preparations exhibited bicarbonate dependent oxygen evolution (Fig. 3) as well as ¹⁴CO₂ fixation. With 15 different preparations, the bicarbonate dependent oxygen evolution varied between 15 to 35 μmol h⁻¹ mg⁻¹ Chl (24.1 ± 6.5). The washed isolated chloroplasts without additions other than the assay medium with 1 mM phosphate were also able to fix ¹⁴CO₂ at a rate of over 21 μmol h⁻¹ mg Chl⁻¹. These photosynthetic rates by the isolated chloroplasts were about one-third that of whole cells, which had rates of about 70 μmol h⁻¹ mg Chl⁻¹. Addition of ATP to isolated chloroplasts from CO₂ grown cells did not stimulate CO₂ fixation, as reported for Euglena chloroplasts (21). As with higher plant chloroplasts (5, 8) and chloroplasts from *Chlamydomonas* (1), photosynthetic oxygen evolution was inhibited by 10 mM phosphate, but this inhibition was reversed by 2.5 mM P-glycerate (Fig. 3).

**Enzymes in Intact Chloroplasts.** The isolated washed chloroplasts retained 80 to 100% of their stomatal enzyme activity (Table I). Besides glycolaldehyde-3-P dehydrogenase and ribulose-5-P carboxylase/oxygenase, *Dunaliella* chloroplasts contained NADH:dihydroxyacetone phosphate reductase (4, 7). The presence of NADPH:glyoxylate reductase in chloroplasts from *Dunaliella* is consistent with its presence in other isolated chloroplasts (24). A low level of NADP-glutamate dehydrogenase activity appeared to be in the *Dunaliella* chloroplasts, but a higher level of NAD-glutamate dehydrogenase was outside of the chloroplasts.

The extent of contamination of the isolated intact washed chloroplast preparation with other organelles or enzymes was examined by determination of the marker enzymes, Cyt c oxidase and fumarase of the mitochondria and PEP-carboxylase of the cytoplasm (Table I). The specific activities were based on the Chl content of the homogenate or isolated chloroplast. About 8% of the Cyt c oxidase and PEP-carboxylase were associated with the chloroplasts fraction. These contaminations were due in part to the presence of some whole cells and could be reduced to less than 5% by only using fraction 4 and 5 from the Percoll gradient. These data are from the intact chloroplast fraction from cells grown without CO₂ during the 8 h dark period, but purer chloroplast preparations are obtainable from cells kept continuously on high CO₂. The enzymes associated with glycolate metabolism (Table I) were also not present in the chloroplasts. These enzymes, except catalase, would be expected in the mitochondria of unicellular algae (24). The total activity of catalase was very low relative to plant or algal tissue containing peroxisomes (24). Glycolate dehydrogenase was extremely low in activity as was the activity of other enzymes associated with the C₂ cycle.

**LITERATURE CITED**

1. BELNAP JR 1983 Partial purification of intact chloroplasts from *Chlamydomonas reinhardtii*. Plant Physiol 72: 1130-1132
3. BOROWITZKA LJ, AD BROWN 1974 The salt relations of marine and halophilic species of the unicellular alga *Dunaliella*. The role of glycerol as a compatible solute. Arch Microbiol 96: 37-52
7. GIMMLER H, G LOTTER 1982 The intracellular distribution of enzymes of the glycerol cycle in the unicellular alga *Dunaliella parva*. Z Naturforsch 37c: 1107-1114