Isolation of Intact Chloroplasts of *Euglena gracilis* by Isopycnic Sedimentation in Gradients of Silica\(^1, 2\)

JEFFREY L. SALISBURY,\(^3\) AUREA C. VASCONCELOS, AND GARY L. FLOYD\(^4\)
Department of Botany and the Particle Separation Facility, Rutgers University, New Brunswick, New Jersey 08903

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**ABSTRACT**

A technique is described for the isolation of structurally intact and partially active chloroplasts from phototrophically grown *Euglena gracilis*. The separation of intact chloroplasts from stripped chloroplast membranes and other subcellular particles was achieved by sedimentation in continuous, isosmotic density gradients of Ludox AM, a silica sol.

The final preparations contained an average of 92\% intact chloroplasts and corresponded to approximately 10\% of the total chlorophyll of the original cell suspension and 20 to 30\% of the chlorophyll layered on the gradients.

The chloroplasts obtained were intact by the criteria of ultrastructure, their content of ribulose diphosphate carboxylase, and their activity in a modified Hill reaction assay (U. Heber and K. A. Santarius. 1970. Z. Naturforsch. 25b: 718-727). In addition, the isolated chloroplasts were capable of incorporating *amino acids* into protein in the light.

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*Euglena gracilis* has been a favorite organism for the study of chloroplast development because of the ready conversion of its proplastids to chloroplasts upon transfer of the cells from darkness to light (21). The chloroplasts of *Euglena* have been difficult to isolate with any reasonable degree of purity or structural and functional integrity.

Density gradient centrifugation is generally conceded to be the method of choice for the separation of pure organelles. Gradients of sucrose have been widely employed for the isolation of structurally intact chloroplasts of higher plants (10, 14, 22) and of *Euglena* (18). To achieve such a separation, sedimentation through sufficiently dense sucrose gradients also involves exposure to osmotic pressures which in effect "wrings out" or dehydrates the organelle. Upon subsequent dilution into isotonic sucrose, the organelles often lose their limiting membrane and soluble proteins. *Euglena* chloroplasts appear to be especially sensitive to high concentrations of sucrose.

The rate-zonal method utilizing isosmotic gradients of Ficoll, a polymer of sucrose, has been described for the separation of structurally intact *Euglena* chloroplasts (23). Although this was the first report of the separation of intact *Euglena* chloroplasts on density gradients, the method has several drawbacks, including the limited capacity of gradients in rate-zonal separations, the extreme viscosity of dense solutions of Ficoll, and the substantial expense of Ficoll in the case of large scale and routing separations.

Silica sols are an alternative, nonosmotic gradient material well suited to the separation of cellular (13, 17) and subcellular particles, including chloroplasts (8, 12, 15, 19). Indeed, Morgen-thaler *et al.* (15) have recently described the isolation of photo-synthetically competent, structurally intact chloroplasts from spinach by centrifugation in gradients of the silica sol, Ludox AM, supplemented with PEG.

Our initial attempts to isolate intact *Euglena* chloroplasts on the gradient of Morgen-thaler *et al.* (15) were largely unsuccessful, but we ultimately found, as described below, that a combination and modification of the procedures of Vasconcelos *et al.* (23) and Morgen-thaler *et al.* (15) yields *Euglena* chloroplasts which are structurally intact by several criteria and active in light-driven protein synthesis.

**MATERIALS AND METHODS**

**Preparation of Crude Chloroplasts.** *Euglena gracilis* (Klebs) strain Z (Pringsheim) was grown phototrophically on modified Hutner's medium as described previously (23).

Cells were harvested by centrifugation, washed twice in de-ionized water and once in the breaking mix (0.15 M sucrose, 0.15 M sorbitol, 1\% [w/v] Ficoll [Pharmacia], 2 \(\mu\)g/ml polyvinylsulfate, 15 mM NaCl, 5 mM mercaptoethanol, 5 mM HEPES-NaOH, pH 6.8) and weighed. The cells were resuspended in 2 ml of breaking mix/g wet weight, disrupted in a French pressure cell (not exceeding 105 kg/cm\(^2\)), and the eluate was collected in a reservoir containing 4 volumes of the breaking mix. All isolation steps were carried out at 0 to 4 C.

The crude brei was clarified by centrifuging at 1000 rpm for 1 min in a No. 870 angle rotor (IEC). The resulting supernatant was then centrifuged at 3000 rpm for 3 min to pellet the chloroplasts. The upper green portion of this pellet was resuspended by gentle aspiration in the breaking mix to approximately 1 mg Chl/ml and centrifuged into the gradients as described below.

**Preparation of Ludox Gradients.** Ludox AM (E.I. du Pont de Nemours and Co.) was purified as described by Morgen-thaler.

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\(^3\) Present address: Department of Botany, School of Biological Sciences, Ohio State University, Columbus, Ohio 43210.

\(^4\) Abbreviation: PEG: polyethylene glycol.
et al. (15). The purified Ludox was made to contain 10% (w/v) PEG (Carbowax 6000, Union Carbide Corp.) and 1% (w/v) BSA (Sigma). Linear gradients of 10 ml or 25 ml were generated from 20 to 70% (v/v) Ludox-PEG-BSA solutions with the same concentration and composition of the breaking mix (minus mercaptoethanol) and 5 mM glutathione throughout. The gradients were pumped into 12- or 36-ml polyallomer centrifuge tubes followed by a cushion of 0.80 or 2.5 ml, respectively, of 70% (v/v) Ludox-PEG-BSA.

In one experiment reported here the composition and limits of the gradients were exactly as described by Morgenthaler et al. (15).

Centrifugation. Two or 4 ml (2 mg or 4 mg of Chl) of the crude chloroplast preparations were centrifuged into the gradients in a SB206 or SB110 (IEC) swinging bucket rotor at 7000 rpm for an \( t_o \) of about 500 rad, corresponding to approximately 15 min.

In the case of the preparative gradients, the desired bands were removed from the gradients by aspiration, like bands pooled diluted with 2 volumes of breaking mix, pelleted, and washed by centrifugation with 40 to 50 ml of breaking mix. Alternatively, the 12-ml tubes were pierced, and the gradients were fractionated in an IEC (No. 3851) gradient fractionator and 0.55-ml fractions were collected. Absorbance profiles of the gradients were determined by monitoring at 680 nm in a Gilford Model 240 spectrophotometer equipped with a 2-mm flow cell (IEC, No. 3634).

Density Measurements. The density of each gradient fraction was determined by allowing drops to sediment to their equilibrium density in an organic solvent density column (11, 16) of 55% (v/v) water-saturated chloroform to 15% (v/v) water-saturated chloroform in water-saturated benzene. The gradients were calibrated in steps of 0.02 g/cm\(^3\) with sucrose of known densities.

**ASSAY PROCEDURES**

**Modified Hill Reaction.** The fraction of intact chloroplasts was measured by comparing ferricyanide-dependent Hill activity of untreated and osmotically shocked chloroplasts essentially according to Heber and Santarius (8). The reaction mixture was modified to contain the ingredients of the breaking mix and further modified according to D. A. Walker (personal communication) to measure \( \text{NH}_4 \text{Cl} \)-uncoupled ferricyanide-dependent \( \text{O}_2 \) evolution in the presence of \( \text{d}-\text{l} \)-glyceraldehyde. Oxygen evolution was monitored in a YSI Model 53 biological oxygen monitor.

Chlorophyll was determined by the method of Arnon (1) and Bruinsma (4).

Ribulose Diphosphate Carboxylase. Ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39) activity was determined by assaying 10-\( \mu \)l aliquot of each gradient fraction according to Chen et al. (5), except that the acidified assay mixture was diluted at 90 C for 90 min and then extracted with aquasol universal cocktail (New England Nuclear) as the scintillant.

**Electron Microscopy.** Gradient fractions were prepared for electron microscopy as previously described (23).

**Protein Synthesis.** Light-driven incorporation of L-(\( \text{PS} \))-methylene into trichloroacetic acid-insoluble material was determined as follows. Washed chloroplast preparations from the gradient were washed further in 0.33 m sorbitol, 1 mM MgCl\(_2\), 2 mM EDTA, 4 mM mercaptoethanol, and 50 mM Tricine-KOH, pH 8.4, and resuspended in the assay mixture composed of 0.33 m sorbitol and 50 mM Tricine-KOH, pH 8.4 (3). Reaction mixtures of 0.8 ml, containing chloroplasts corresponding to approximately 120 \( \mu \)g of Chl, were equilibrated with gentle agitation for 3 min in a constant temperature water bath at 20 C in the dark. At zero time, 10 \( \mu \)l (52 \( \mu \)Ci) of L-(\( \text{PS} \))-methylene (New England Nuclear, NEG-009), corresponding to a specific radioactivity of 158 Ci/millie containing the fraction of the day of the experiment, were added, and the system was illuminated with red light from four 250-w incandescent photoflood lamps filtered by cellophane that transmits above 640 nm, with an approximate light intensity of 2000 ft-c at the reaction vessels. At the indicated times, 50-\( \mu \)l aliquants were transferred in duplicate to Whatman No. 3 filter paper discs (2.5 cm diameter), processed according to the methods of Bollum (2), and assayed by liquid scintillation. \( \Phi -\text{three-Chloro} \text{amphenicol} \) (Sigma) was added to some trials at a final concentration of 300 \( \mu \)M. Dark controls were wrapped with aluminum foil. All solutions and glassware were sterilized with the exception of the Ludox-PEG-BSA solution, which was prepared fresh.

**RESULTS**

When crude chloroplasts were sedimented into the silica gradients, four Chl-containing zones could be discerned (Fig. 1). Chloroplasts were present principally in zones III and IV while the minor component, zone II, and the sample zone, I, were composed mainly of chloroplast fragments and smaller particles. The mean densities of zones III and IV were 1.1 and 1.127 g/cm\(^3\), respectively. The linearity of the density gradient is shown in the lower portion of Figure 1.

On the basis of several criteria (see below) the chloroplasts of zone IV were 85 to 93% intact whereas chloroplasts of zone III were less than 50% intact. Yields of intact chloroplasts (zone IV) corresponded to approximately 20 to 30% of the Chl layer on the gradients.

The presence of the soluble enzyme ribulose diphosphate carboxylase can be used as a measure of the structural integrity.
of the chloroplast. The distribution of this enzyme along the gradient shown in Figure 1 indicate that most of the activity was associated with chloroplasts of zone IV. Low but repeatable activities were found in zone III, the sample zone, and in the pellet, which contained large cell fragments and paramylum.

Electron photomicrographs (Fig. 2) of zone IV preparations show intact chloroplasts with electron dense stroma between agranal lamellae, numerous ribosomes, and entire envelope membranes (6). In contrast, the membranes of zone III, while clearly derived from chloroplasts, appear highly distended. The major contaminants of zone IV were thylakoid membranes and swollen chloroplasts, some of which may have resulted from fixation and embedding procedures (10). Pellicle fragments and mitochondrial profiles were seen occasionally on some grids.

An independent biochemical measure of intactness can be obtained by comparing ferricyanide-dependent O₂ evolution by chloroplast preparations before and after osmotic shock (8). This assay depends on the impermeability of intact chloroplast envelopes to ferricyanide. Table I lists the relative amounts of intact chloroplasts in various preparations as determined by this assay. We interpret these data to indicate at least a 2-fold increase of intact chloroplasts in zone IV over the amount in the crude chloroplast material. Zone IV chloroplasts isolated during separate experiments show a consistently high percentage intactness by this assay. Table I also lists data which indicates that Euglena chloroplasts isolated on the gradient of Morgenthaler et al. (15) show a low degree of structural integrity.

Light-dependent protein synthesis can be used as a further measure of the structural and functional integrity of chloroplasts. Chloroplasts from zones III and IV were tested for the incorporation of L-(3⁵S)methionine into trichloroacetic acid-insoluble material in the light and in the dark. The largely intact chloroplasts from zone IV incorporated at a rate of 0.16 nmole/mg Chl-hr during the interval of most rapid uptake (3–10 min in Fig. 3). On the basis of Chl content, the final level of light-driven incorporation by zone IV chloroplasts is more than double that of zone III chloroplasts. This value agrees closely with the fractions of intact chloroplasts in these two preparations,
Table I. Ferricyanide-dependent O₂ Evolution of Chloroplast Preparations before and after Osmotic Shock

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Chloroplast Preparation</th>
<th>Rates of O₂ Evolution</th>
<th>Mean Percentage Intact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated</td>
<td>Shocked</td>
</tr>
<tr>
<td>90/55</td>
<td>Crude chloroplasts</td>
<td>25.9</td>
<td>46.8</td>
</tr>
<tr>
<td></td>
<td>Zone III</td>
<td>45.4</td>
<td>71.4</td>
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<tr>
<td></td>
<td>Zone IV</td>
<td>4.2</td>
<td>151.6</td>
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<tr>
<td></td>
<td>Zone IV aged 30 min</td>
<td>7.3</td>
<td>111.6</td>
</tr>
<tr>
<td>90/54</td>
<td>Zone IV</td>
<td>12.1</td>
<td>118.5</td>
</tr>
<tr>
<td>90/56</td>
<td>Zone IV</td>
<td>2.8</td>
<td>131.5</td>
</tr>
<tr>
<td>90/49</td>
<td>Zone IV from gradient</td>
<td>128.4</td>
<td>165.6</td>
</tr>
<tr>
<td></td>
<td>of Morgenthaler et al.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Mean value for three separate experiments.  
² Mean value for zone IV experiments 90/54, 55, and 56 assayed immediately after recovery.

Fig. 3. Incorporation of 35S-methionine into tri chloroacetic acid-insoluble material by isolated Euglena chloroplasts. Assay procedures were as outlined in text. Light controls (△, □); dark controls (▲, △). Incorporation by zone IV chloroplasts (△, ▲); incorporation by zone III preparations (□, △).

as estimated from the Hill activity assay (Table I). Dark controls of zone IV chloroplasts incorporated methionine at rate less than 20% of those in the light. When treated with D-threo-chloramphenicol, zone IV chloroplasts incorporated methionine in the light at rates less than 13% of the untreated controls.

**DISCUSSION**

We have described a method for the isolation of structurally intact and partially active chloroplasts from Euglena gracilis in substantial yield and high purity. We attribute the success of this method to several factors. Rapid removal of the chloroplasts from the crude cell brei and maintenance of the chloroplast preparation in the mildly hypertonic braking mix throughout the isolation procedure were essential. The use of isoticom gradients of the silica sol eliminated osmotic shrinkage and subsequent damage to the chloroplasts upon dilution to isotonic conditions, a problem that appears to be unavoidable when sucrose gradients are used for isotypic separations. The inclusion of Ficoll in the breaking mix (23) is essential for the maintenance of Euglena chloroplast integrity during isolation. We recommend omitting Mg in the breaking mix because this ion causes clumping of the chloroplasts which results in severe reductions in yield.

The failure of the gradient of Morgenthaler et al. (15) to be referred to as the spinach gradient to yield intact Euglena chloroplasts may have resulted from several factors. First, the spinach gradient, which was designed for the separation of higher plant chloroplasts, has density limits (10-80% [v/v] Ludox) that define a steeper slope than the gradient we have described and thus has a lower resolving capacity for organelle and organelle fragments with similar sedimentation behaviors. Second, the spinach gradient contains Mg, which causes clumping of Euglena chloroplasts, and includes EDTA, which has been reported to cause structural damage to the chloroplasts of Euglena upon prolonged exposure (18). Finally, as the chloroplasts of Euglena sediment in the spinach gradient, they are removed from the protective effect of Ficoll.

On the basis of ultrastructure, the intact chloroplasts obtained in this study were indistinguishable from chloroplasts of whole cells. Although intact chloroplasts are partially functional, as indicated by light-driven protein synthesis (discussed below), light- and CO₂-dependent O₂ evolution or 14CO₂ incorporation could not be demonstrated. Intact chloroplast preparations generally consumed O₂ in the dark while neither consumption nor evolution occurred in the light. Broken chloroplast preparations consumed O₂ in the light at a greater rate than in the dark. The failure to elicit photosynthetic competence may be due to the loss of low mol wt intermediates and ions during the isolation procedure. In this respect, an adequately supplemented assay mixture is difficult to define experimentally. Photosynthesis in Euglena may be under a tighter cellular control than has been found with chloroplasts from higher plants in vitro.

Rates of protein synthesis by Euglena chloroplasts in vitro have been reported as high as 12.9 nmoles/mg Chl (40 min) (7). These rates, however, were obtained in the dark with a highly supplemented assay mixture including an ATP-generating system and 13 hot amino acids. The over-all low rates of methionine incorporation in this study may be explained by the total reliance of the chloroplasts on endogenous amino acids (except methionine) and inorganic phosphate pools, as well as other protein synthesis factors. Energy requirements for protein synthesis in this assay system may be supplied mainly through photosystem I (20), since photosystem II was not operational under these assay conditions. We believe this assay system gives a more reliable indication of chloroplast functional integrity with regard to protein synthesis than a highly supplemented assay mixture such as that used by Harris et al. (7).

Protein synthesis by the intact chloroplasts was almost completely inhibited by D-threo-chloramphenicol, which is known to be specific against the 70S ribosomes. This level of inhibition coupled with the light dependence for incorporation are indications of protein synthesis by chloroplasts rather than by cytoplasmic ribosomes.

Kahn and von Wettstein (9) first reported that higher plant chloroplasts when viewed by light microscopy could be distinguished as either intact, with clearly visible grana and a definite outline, or as broken, showing distinct grana and no definite outline. These observations have been routinely used to obtain a firsthand approximation of the relative amounts of intact chloroplasts in given preparations (10, 22), and with many higher plant chloroplasts, this criterion holds quite well. However, our experience with Euglena chloroplasts in vitro has been that, while a chloroplast that was clearly blebbing off membranes could be readily distinguished as broken, the remainder of the chloroplasts could not always be classified as intact. Indeed, an apparently broken chloroplast which appeared dark and indefinite in outline may upon changing its orientation in the field appear to be highly refractile and definite. While others have re-
ported the use of phase contrast microscopy as a means of estimating the fraction of intact chloroplasts from *Euglena*, we have found this method unreliable.

The method described for the isolation of *Euglena* chloroplasts has several advantages over previously reported ones. The non-osmotic properties of the inexpensive silica sol gradient material and the consistently high yields of structurally intact and partially active chloroplasts should make this method generally applicable for further studies of *Euglena* chloroplasts *in vitro*.

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**LITERATURE CITED**