Isolation of Intact Chloroplasts from *Euglena gracilis* by Zonal Centrifugation¹

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**ABSTRACT**

Chloroplasts were separated from *Euglena gracilis* by zonal centrifugation at low speed in density gradients of Ficoll or dextran. The chloroplasts were intact by the criteria of ultrastructure and their content of ribulose diphosphate carboxylase and soluble protein. The chloroplasts also contained ribosomes and ribosomal RNA uncontaminated by the corresponding cytoplasmic particles.

Although the alga *Euglena gracilis* has been favored for the study of chloroplast development (cf. 1, 25, 26), the isolation of intact chloroplasts from this organism has proven to be very difficult.

The chloroplasts of some plants can be purified by isopycnic centrifugation in sucrose density gradients (11), but upon dilution into isotonic sucrose, the organelles often lose their limiting membrane and stromal proteins, leaving a skeleton of lamellae containing trapped or attached ribosomes and DNA. Indeed, Eisenstadt and Brawerman (4) purified such particles from *Euglena* by flotation through concentrated sucrose solutions and obtained ribosomes from them, but it is doubtful that the chloroplasts obtained were intact. Chloroplasts of spinach can be recovered intact after rate-zonal centrifugation into shallow sucrose density gradients (27) in which the osmolarity remains small. The procedure has been considerably improved by the substitution of gradients of sorbitol and adapted to continuous flow harvesting in the K-X zonal rotor (D. H. Brown, in preparation), but in our hands this procedure has never been successful with *Euglena*. While others have succeeded, we have never seen any intact *Euglena* chloroplasts after exposure to gradients of sucrose or sorbitol.

On the premise that *Euglena* chloroplasts are especially sensitive to high osmotic pressures, we tested their behavior in isosmotic gradients prepared from Ficoll or dextran. With gradients of these polymers we have obtained preparations of *Euglena* chloroplasts with ultrastructure indistinguishable from those in situ and which are pure and intact by several criteria.

**METHODS AND MATERIALS**

Cells. *Euglena gracilis* (Klebs) z strain (Pringsheim) was grown phototrophically at 25 C in a modified Huttner medium (23), but without added copper and with 0.1 M glucose as the carbon source. They were harvested 48 to 72 hr after inoculation when the turbidity reached 1200 to 1300 corrected Klett units. One liter of cells (about 15 g wet wt) was harvested, washed with a 3:5:1 solution (3% [w/w] sorbitol, 5% [w/w] sucrose, and 1% [w/w] Ficoll; pH adjusted to 7.6 with HEPES¹ buffer to a final concentration of 5 mM), and diluted to a final volume of 40 ml with 3:5:1 solution containing 2 μg of polyvinylsulfate per ml.

The cells were disrupted in a French pressure cell (1500 psi) and diluted to 60 ml with a 6:5:2 solution (6% [w/w] sorbitol, 5% [w/w] sucrose, and 2% [w/w] Ficoll, pH 7.6). The brei was clarified by centrifuging at 1000 rpm (121g) for 2 min.

**Zonal Centrifugation.** The general techniques of zonal centrifugation were those described by Price (22). Forty milliliters of the clarified brei were loaded into a B-XXXr rotor (International Equipment Co.) over 300 ml of gradient that was 0 to 10% (w/w) sucrose and 10% (w/w) sucrose and 5 mM HEPES throughout. The gradient was supported by 130 ml of a cushion or underlay of 18% (w/w) Ficoll, 10% (w/w) sucrose, 10% (w/w) sucrose, and 5 mM HEPES, pH 7.6. The sample was followed by an overlay of 60 ml of the 3:5:1 solution. The gradient and cushion were delivered from an IEC two-cylinder gradient pump. All solutions contained 2 μg of polyvinyl sulfate per ml.

The sample was centrifuged at a maximal speed of 7000 rpm for 5 × 10⁵ rad²·sec⁻¹ as indicated on an ′f′ω²t meter; this was equivalent to about 17 min.

The gradient was unloaded from the center with the use of 55% (w/w) sucrose at 30 ml/min, and 30-ml fractions were collected. The absorbance at 260 nm and the refractive index of the gradient were monitored and recorded continuously. There were some advantages in diluting the gradient fractions immediately with equal volumes of 3:5:1 containing 30 mM MeCl.

**Electron Microscopy.** Chloroplast fractions were fixed in an over-all concentration of 1% (v/v) glutaraldehyde before sedimentation. The pellets were postfixed in 2% osmium tetroxide in Palade's (18) veronal acetate buffer, pH 7.2, for 2 to 4 hr at about 2 C. After a gentle rinse with cold water, the pellets were embedded without mixing in warm 2% agar and

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¹ Abbreviations: DOC, deoxycholate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate; RuDP, ribulose diphosphate.
subsequently dehydrated in a rapidly graded acetone series. Usually pellets were also stained in a 1:1 mixture of acetone and saturated aqueous uranyl acetate before undergoing dehydration. All pellets were embedded in Luft's mixture of Epon 812 (12), polymerized at 50 °C, and sectioned with an LKB Ultramicrotome or a Sorval MT2 ultramicrotome equipped with diamond knives. Sections post-stained with uranyl acetate and lead citrate (24) were viewed on copper grids with an RCA-3G electron microscope at 50 kv.

Whole cells of *Euglena* were fixed at 0 °C in a glutaraldehyde-paraformaldehyde mixture in cacodylate buffer (8) at pH 7.2 for 2 hr, washed three times with water, and then post-fixed with 2% OsO4 in Millonig's (15) buffer for 4 hr. All subsequent steps were identical to the treatment of the fractions.

**Ribulose Diphosphate Carboxylase.** Ten-milliliter samples of each 30-ml fraction recovered from the gradient were centrifuged in an IEC 870 rotor first at 7,000 rpm for 10 min and then at 15,000 rpm for an additional 10 min. Both pellets and supernatant were analyzed by the method of Chen et al. (3), except that the acidified assay mixture was dried at 90 °C for 90 min, and then extracted with dioxane for scintillation.

**Ribosomes and RNA.** For the analysis of ribosomes, portions of the fractions corresponding to peaks I and II were pooled, divided into two parts, and centrifuged for 10 min each at 7,000 and 15,000 rpm. Chloroplast pellets were drained, and the tubes were wiped free of extra gradient solution. One part of each peak was resuspended by hand with a ground glass homogenizer in approximately 1 ml of solution containing 10 mM tris-HCl, pH 7.7; 10 mM KCl; 10 mM MgCl₂; 7 mM β-mercaptoethanol; 2 µg/ml polyvinylsulfate (10:10:10); and 0.5% DOC. According to Kohler et al. (9), the DOC chelates enough Mg²⁺ to reduce its effective concentration in the resuspension medium to about 4 mM. The other part of each peak was resuspended in 1 ml of solution containing 10 mM tris, 10 mM KCl, 2 mM MgCl₂, 7 mM β-mercaptoethanol, 2 µg/ml polyvinylsulfate, and 1.0% Triton X-100. After clarification at 10,000 rpm for 10 min, the MgCl₂ concentration of the Triton X-100 lysates was adjusted to 10 mM. Between 0.2 and 0.4 ml portions of lysates were layered over isokinetic (17) gradients in 10:10:10 and centrifuged at 35,000 rpm for 4½ hr in the IEC 283 swinging bucket rotor. Tubes were punctured in a closed system, with water displacing the gradient at a rate of 1.15 ml/min, and effluents were monitored at 260 nm with a Gilford spectrophotometer. Ribosome profiles from chloroplast lysates were compared with those from the original sample before zonal purification (diluted with 10:10:10 medium containing 0.5% DOC) and with those of *Escherichia coli* ribosomes (kindly donated by Dr. T. Chase).

For analysis of ribosomal RNA, chloroplast lysates used for ribosome analysis were centrifuged overnight at 30,000 rpm. Pellets of these chloroplast ribosomes as well as those of *E. coli* and *Euglena* cytoplasmic ribosomes (Pollack, Eikenberry, and Price, in preparation) were resuspended in 50 mM NaCl-1 mM EDTA at pH 6.2. Sodium dodecyl sulfate was added to a final concentration of 1% (w/w) and 60% (w/w) sucrose containing bromphenol blue as a marker dye was added to the sample. Samples were applied to a mixed 3% polyacrylamide-
FIG. 3. Ultrastructure of particles recovered from the gradient. Particles were collected from the gradient shown in Figure 2. a: Peak I; b: peak II; c: peak III; d: peak III at greater magnification. Note that the external membranes of most of the chloroplasts are intact, and their shape in situ is preserved; chloroplasts from peak III are largely free of other particulate contaminants.

0.5% agarose gel (19) and were subjected to electrophoresis at 200 V, pH 8.3, and 5°C for 90 min in an E-C vertical gel unit. Molecular weights and S values of the RNA bands were estimated by comparison of mobilities with those of E. coli RNAs, which were assumed to be 23 and 16 S (1.1 and 0.56 x 10^6 daltons, respectively).

Protein Analysis. The proteins were analyzed by the Lowry protein assay, as modified by Price (21).

Chlorophyll. Chlorophyll in the pelleted chloroplasts was extracted with 0.8 ml of 100% acetone per 0.2 ml of suspension and centrifuged, and the absorbance of the supernatant was read at 652 nm (2).

RESULTS

Ultrastructure in Situ. We thought it important that the ultrastructure of chloroplasts in situ serve as the standard of reference for the intactness of isolated chloroplasts. An electron photomicrograph of a photoheterotrophic Euglena collected from a suspension prior to cell disruption is shown in Figure 1. We see a portion of the nucleus, mitochondria, the peculiar ridged pellicle, paramylon bodies which also contain "volutin" or polyphosphate (5), and chloroplasts. As described by Gibbs (5), the whole of the Euglena chloroplast stains heavily. The lamellae run the length of the organelle, and there
is no differentiation into grana. Between the lamellae, the stroma is dark and packed with particles the size of ribosomes.

We do not see pyrenoids in the chloroplasts, but as Gibbs (5) and others before her had noted, pyrenoids in a number of Euglena clones are formed only during auxotrophy. The pyramion bodies are scattered around the cell, rather than pressed to the chloroplast as is usual in photoauxotrophic Euglena.

Centrifugation. A suspension of harvested and washed cells was passed through the French Press at 500 kg·cm⁻² (1500 psig). The pressure was a compromise between efficient cell breakage and disruption of the chloroplasts. After clarification by centrifugation at low speed the brei was loaded into a B-XXXa rotor over a 0–10% (w/w) Ficoll gradient and a cushion of 18% (w/w) Ficoll with 10% (w/w) sorbitol and 10% (w/w) sucrose throughout.

After centrifugation at 7000 rpm for 5 × 10⁶ rad²·sec⁻², the cell particles resolve into three or four peaks as shown in Figure 2.

The original sample zone and peak I contain small particles including intact and broken mitochondria and chloroplasts (Fig. 3a). Peak II contains some stripped chloroplasts and intact chloroplasts containing ribosomes (Fig. 3b). Peak III, which is mostly in the steep gradient formed by the cushion, contains mostly intact chloroplasts (Fig. 3c). We think that this peak is the leading edge of peak II that has been compressed against the cushion. A close comparison of electron photomicrographs of chloroplasts in peaks II and III (Fig. 3, b and c) with those in situ shows a virtually complete identity: compact, parallel lamellae, densely staining stroma, and limiting membranes (Fig. 3d). Compared to their state in situ, the thylakoids of the separated chloroplasts are slightly swollen and the stroma somewhat less dense, but it is difficult to know if these differences are real or due to unavoidable differences in fixation and staining. Some of the chloroplasts in peak II show degrees of swelling and disruption extending to that of fully stripped chloroplasts. There is also a scattering of small particles and debris in electron photomicrographs in peak III. Because such a size of particle would not have migrated there by itself, it is quite possible that they are the remains of chloroplasts that became disrupted after sedimentation into the gradient, and that the stripped chloroplasts seen in peak III are artifacts.

The only other objects regularly seen in peak III are the densely staining globules of what we take to be polyphosphate, presumably from polyphosphate vesicles.

We have not determined the permissible range of concentrations of sucrose and sorbitol in the gradient, but we do find that Ficoll or dextran is indispensable. We were never able to recover other than stripped chloroplasts from gradients of sucrose, sorbitol, or both.

We know from experiments with simple step gradients in swinging bucket rotors that the equilibrium density of Euglena chloroplasts is less than that of 20% (w/w) Ficoll in 10% (w/w) sucrose and 10% (w/w) sorbitol, but the fact that the chloroplasts may be pelleted from the gradient simply by centrifugation tells us that the organelles are not at their isopycnic point. The separation observed therefore is probably an approach to equilibrium.

Ribosomes and RNA. We can test for the purity of the chloroplasts by determining what kinds of ribosomes are in the preparations. All but a small fraction of the ribosomes of Euglena are cytoplasmic ribosomes; the remainder of those detected so far are chloroplastic. We found earlier (13) that chloroplast ribosomes occur principally as subunits or polysomes and that the subunits (approximately 30 and 50 S) can be distinguished in the presence of cytoplasmic subunits (approximately 46 and 64 S) by sedimenting under conditions of low salt; the cytoplasmic subunits and monomers then aggregate into a broad zone of perhaps 120 S. We find that the chloroplast preparations yield subunits and a small amount of monomer, but negligible amounts of cytoplasmic particles (Fig. 4b).

Similarly, we tested for purity by analyzing chloroplastic RNA on acrylamide-agarose gel electrophoresis, by which one can detect unique species of cytoplasmic ribosomal RNA (13). None was detected in chloroplasts recovered from gradients.

Protein and Chlorophyll. We expected that sedimentation profiles of protein and chlorophyll would distinguish between the amount of stripped and intact chloroplasts. This is shown in Figure 2b where protein to chlorophyll ratios are much greater in peak III than in peak II.

Ribulose Diphosphate Carboxylase. The presence of ribulose diphosphate carboxylase is a sensitive indicator of the integrity of chloroplasts. We found not only that the intact chloroplasts of peaks II and III contain this enzyme (Fig. 2c), but that the activity remains with the chloroplasts upon pelleting.

DISCUSSION

We have described a method for the separation of chloroplasts from phototrophie Euglena gracilis that are intact by the criteria of ultrastructure and composition. Contamination is limited to stripped chloroplasts, some of which may be artifacts, and some polyphosphate. The first significance of this separation lies in the importance of Euglena for the study of chloroplast development, since it becomes feasible to work with intact cells and isolated chloroplasts of the same organism.
There are substantial possibilities in addition for studying photosynthesis.

Our method does not depart from established conditions for isolating chloroplasts: mildly hypertonic sucrose or sorbitol and rapid removal of the organelles from the remainder of the brei (7, 28). We attribute our success with this method to the use of isosmotic gradients. It is also possible that Ficoll and dextran exert a positive effect (cf. 6, 10, 14). Nobel (16) also employed Ficoll gradients for the isopynic separation of pea chloroplasts, but he reported that an overwhelming fraction of chloroplasts became stripped. It is possible that the high centrifugal field he employed, 35,000 rpm in the B-XIV rotor, may have been responsible. In contrast, we used 7,000 rpm in a rotor of essentially the same geometry.

We expect that our general method will be adaptable to other kinds of density gradient centrifugation. We chose the B-XXXa simply because it is the smallest available zonal rotor. The Z-15 and A-XII zonal rotors would be suitable, and possibly swinging bucket or angle rotors. Large scale separations could easily be performed in continuous flow rotors, a method from which our present procedure was adapted.

One cautionary note: extremely viscous Ficoll and dextran solutions (>20 centipoise) place serious restrictions on the kind of gradient generator that may be employed. The Spinco 131 generator was able to cope with these solutions only at unacceptably low pumping rates; furthermore, air embolisms occurred in the pump and resulted in unpredictable distortions in the programmed gradient. One needs a powerful, positive displacement pump, such as the IEC 10825 gradient former.

The same considerations of high viscosity make unloading from the edge advantageous but not essential.

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LITERATURE CITED