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Communication

Simplified Procedure for the Isolation of Intact Chloroplasts from *Chlamydomonas reinhardtii*¹

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ABSTRACT

A simple procedure that yields highly purified intact chloroplasts from *Chlamydomonas reinhardtii* is described. This procedure involves breakage of cell wall-deficient cells by passing them through a narrow bore syringe needle. The intact chloroplasts are then purified from the crude homogenate by differential centrifugation and Percoll gradient centrifugation. This procedure generates relatively high yields of chloroplasts capable of CO₂ fixation. These chloroplasts were characterized by electron microscopy, marker enzyme analysis, and ferricyanide exclusion. Transmission electron microscopy indicates that these chloroplasts retain their pyrenoids and eyespots. Scanning electron microscopy confirms that the characteristic cup shape of *C. reinhardtii* chloroplasts persists *in vitro*. This rapid, inexpensive procedure produces chloroplasts that should be useful for researchers studying the biochemistry and cell biology of *C. reinhardtii* chloroplasts.

Chlamydomonas reinhardtii is a unicellular biflagellate green alga that possesses one cup-shaped chloroplast. *C. reinhardtii* is an excellent organism for the study of organelle-nuclear interactions due to its well-characterized genetics and the ability of researchers to transform either the chloroplast genome (1, 4) or the nuclear genome (5). *C. reinhardtii* can grow heterotrophically on acetate as well as photoautotrophically, allowing researchers to alter, delete, and study genes essential to photosynthesis, procedures that would normally be lethal to a higher plant. Mutants defective in photosynthetic electron transport (3), the chloroplast ATP synthase (3), the photosynthetic carbon reduction cycle (18), as well as the CO₂ concentrating mechanism (9, 13) can all be maintained on acetate in the dark. *In vitro* studies utilizing intact chloroplasts from *C. reinhardtii* have been hampered, however, by the difficulty of purifying active intact chloroplasts in high yield from cells and mutant strains grown on different growth conditions. In this communication, we describe a rapid and simple method for preparing intact chloroplasts from cell wall-deficient strains of *C. reinhardtii*. This method does not utilize detergents, so the intact chloroplasts are suitable for uptake studies and other transport experiments. These chlo-

roplasts retain their characteristic cup shape and are active in CO₂ fixation.

MATERIALS AND METHODS

Algal Culture Conditions

The cell wall-deficient mutant of *Chlamydomonas reinhardtii*, CC-400 *cw-15* *mt*⁺, was obtained from the Duke University *Chlamydomonas* Culture Collection. In liquid culture, the strains were inoculated at a cell density of 4×10^4 cells mL⁻¹ and were grown in Min medium² (17), aerated with 5% CO₂ in air, and continuously illuminated with 300 μ E m⁻² s⁻¹ of white light for 2 d. The cultures were then switched to synchronous growth by using a 12 h light/12 h dark cycle for 3 d and harvested.

Chloroplast Isolation

Chlamydomonas strain CC-400 cultures were grown in Min medium (3.0 L) on a 12 h light/12 h dark regimen to synchronize growth. The cells ($0.6\text{--}1.0 \times 10^7$ cells mL⁻¹) were harvested in the middle of the third light period by centrifugation, the pellets were washed in 100 mL of 20 mM Hepes-KOH (pH 7.5), and were resuspended in 2 mL of ice-cold Hepes-KOH (pH 7.5). The Chl concentrations were determined spectrophotometrically. Subsequent steps were also done at 4°C. Cells (3 mg Chl aliquots) were diluted to 0.3 mg Chl/mL with 10 mL breaking buffer (300 mM sorbitol, 50 mM Hepes-KOH [pH 7.5], 2 mM Na-EDTA, 1 mM MgCl₂, 1% BSA) and were broken immediately by one passage through a 27 gauge stainless steel needle at a flow rate of 0.5 mL · s⁻¹. A 10 mL syringe was used manually to pass the cells through the needle. This flow rate yields a pressure of about 80 p.s.i. The yield of intact chloroplasts was best when the cells were in the breaking buffer a very short period of time before passage through the syringe. The lysate was then centrifuged in a Sorvall HB-4 rotor for 2 min at 760g (2000 rpm) to pellet whole cells and intact chloroplasts. This pellet was resuspended in 2 mL breaking buffer and layered onto discontinuous Percoll gradients (20, 45, 65% Percoll) prepared according to Price and Reardon (2, 15). A 15-min centrifugation of the gradients was carried out in a Sorvall HB-4 rotor

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² Abbreviations: Min medium, phosphate-buffered medium that contains no carbon source other than CO₂; PEP, phosphoenol pyruvate.

at 4200g. The 20 to 45% and the 45 to 65% interface areas were collected and diluted fourfold with breaking buffer. Thylakoids (20–45% interface) and intact chloroplasts were collected by centrifugation at 670g for 1 min, and resuspended in 250 μ L of 50 mM Hepes-KOH (pH 8.0), 0.3 M sorbitol. These intact chloroplasts were used to measure marker enzyme activity and the ability to fix CO₂.

Electron Microscopy

For transmission electron microscopy, whole cells or chloroplasts at varying stages of isolation were fixed for 2 h at 4°C in 2.5% glutaraldehyde added to chloroplast isolation buffer containing 1% BSA or, in the case of whole cells, Hepes-KOH (pH 7.5) buffer. Samples were rinsed in the appropriate buffer, then postfixed overnight at 4°C in 1% osmium tetroxide. After a rinse in distilled water, the samples were dehydrated in a graded series of ethanol, with 2% uranyl acetate added to the 80% step and held overnight at 4°C, to a final concentration of 100% ethanol. Samples were pelleted by centrifugation between each change. The samples were slowly infiltrated at 4°C with hard grade LR White epoxy, with seven changes over a period of several days, then polymerized for 24 h at 60°C. Thin sections were cut using a diamond knife mounted on a Sorvall Porter-Blum MT-2 Ultramicrotome, poststained with uranyl acetate and lead citrate, and examined and photographed on a JEOL JEM 100CX transmission electron microscope.

For scanning electron microscopy, small samples of each suspension were removed from the 100% ethanol step, collected on a 13 mm membrane filter with 0.2 μ m pore size, critical point dried, sputter coated with 20 nm AuPd, and examined and photographed using a Cambridge S-260 scanning electron microscope.

Other Methods

PEP carboxylase (8), Cyt *c* oxidase (16), galactosyl transferase (11), and Rubisco (18) were assayed as previously described.

RESULTS

Chloroplast Isolation

In the past, two strategies have been employed to isolate chloroplasts from *C. reinhardtii*. Both procedures used cells that were cell wall-deficient either through mutation (*cw-15* or *cw-92*) or by treatment of the cells with autolysin, an enzyme released by *C. reinhardtii* during its mating sequence. The first method, described by Klein and coworkers (6, 7), used a short exposure to the detergent, digitonin, to lyse the plasma membrane, followed by a rapid dilution of the detergent. The second method was developed independently by Togasaki and his coworkers (20) and Mendiola-Morgenthaler and Boschetti (11, 12). These research groups used pressure and shear forces to gently break the cells, yielding some intact chloroplasts along with disrupted cells and thylakoids. The Yeda press is commonly used to break cells (11, 14, 20), although the Parr bomb has also been used (10). The Yeda press works best on cells grown photoheterotrophically on

acetate, but less satisfactorily on cells grown photoautotrophically (14, J. V. Moroney and R. K. Togasaki, unpublished observations). Alternate methods of breaking the cells were attempted and it was observed that passing *cw-15* cells rapidly through a 27 gauge needle (see "Materials and Methods") yielded a high percentage of intact chloroplasts. The chloroplasts in the homogenate retained the ability to fix CO₂, so the intact chloroplasts were purified from the homogenate by differential centrifugation on Percoll step gradients. The purified chloroplasts retained their cup shape (Fig. 1A and B) and their pyrenoid body and eyespot (Fig. 1C and D). The entire procedure takes about 2.5 h and the average yield of intact chloroplasts, based on Chl recoveries, was $13 \pm 1\%$ (average of five experiments).

This method yielded active chloroplasts even when the cells were grown photoautotrophically on low CO₂, growth conditions that gave the poorest yields using the Yeda press. We obtained the highest yields (13% based on Chl yields) using synchronous cultures that were grown photoautotrophically for 3 d (72 h) on a 12 h light/12 h dark cycle. Our yields were considerably lower (3% or less based on Chl yields) when we attempted to isolate chloroplasts from cultures that had reached a density much greater than 1.2×10^7 cells mL⁻¹ (approaching stationary phase in our media) or higher. The results reported in this communication were from cells harvested 4 h after the onset of the light period, but chloroplasts were also obtained from cells harvested as early as 1 h into the light period to 6 h into the light period. Nonsynchronous cultures also yield intact chloroplasts, but these chloroplasts did not band as sharply on the Percoll gradients as chloroplasts isolated from synchronous cultures, probably due to the various sizes and starch contents of the heterogeneous population of chloroplasts. More importantly, Percoll gradient-purified chloroplasts prepared from nonsynchronous cultures were often significantly contaminated with whole cells or only partially disrupted cells.

Chloroplast Characterization

The photosynthetic properties of the intact chloroplasts generated by passage through a syringe agree with earlier published results (7, 11, 20). Chloroplasts isolated by this method retained moderate rates of CO₂ fixation and O₂ evolution (Table I). The chloroplasts retained about 30% of the activity of intact *cw 15* cells, and this activity was stable in the O₂ electrode for at least 20 min. On ice, in washing buffer the chloroplasts lost 15% of their photosynthetic activity after 2 h. We found O₂ evolution to be stimulated by both glycerate-3-P and HCO₃⁻ and, to a lesser extent, by ATP (Table I). In addition, high concentrations (≥ 10 mM) of Pi inhibited CO₂ fixation in agreement with earlier results (7, 11, 14). The phosphate effect indicates the presence of an operational phosphate/triose phosphate translocator and also implies that the envelope is intact. Chloroplasts prepared by this method also require an osmoticum to be present in agreement with some reports (11, 14, 20) but in disagreement with Klein *et al.* (6, 7) and Sültemeyer *et al.* (19). One reason for this difference might be that Klein *et al.* (6, 7) and Sültemeyer *et al.* (19) used digitonin when breaking the cells. This might alter the sensitivity of the chloroplasts to the

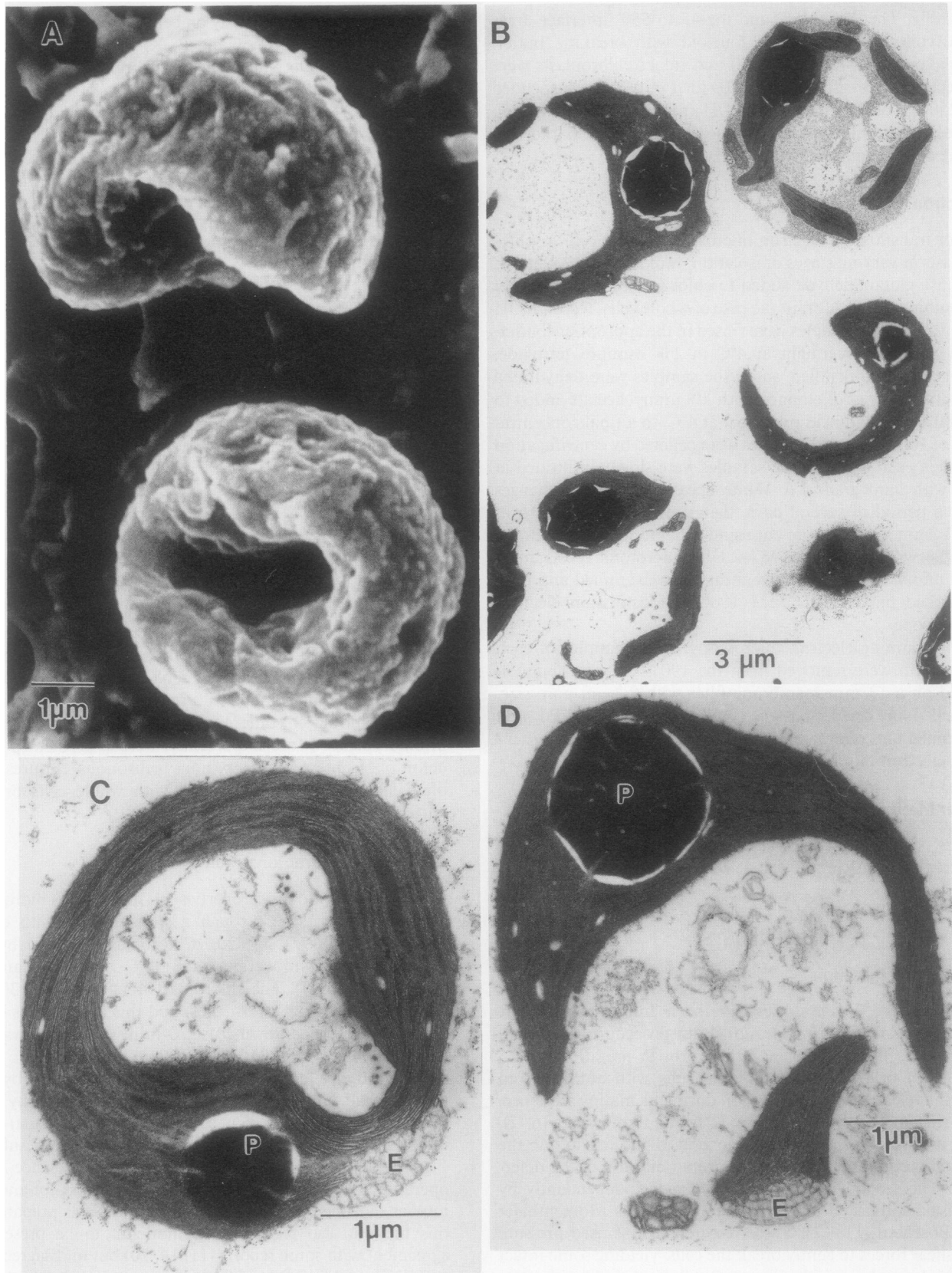


Figure 1. Electron micrographs of chloroplasts from *C. reinhardtii*. A, Scanning electron micrograph of two chloroplasts showing the cup shape. B, Transmission electron micrograph of a field of chloroplasts. C and D, Transmission electron micrographs showing the pyrenoid body (P) and eyespot (E).

osmotic concentration in the buffer. Other methods, including ours, rely on pressure and shear forces to break the cells and do not utilize detergents.

These chloroplast preparations also retained the enzymes of the C_3 cycle as well as galactosyl transferase (Table II). These chloroplasts retained high levels of Rubisco, as indicated by enzymatic assays (Table II) and the presence of the pyrenoid (Fig. 1). The presence of the envelope was determined by electron microscopy and by the presence of the marker enzyme galactosyl transferase (Table II). These chloroplast preparations had only low activities of PEP carboxylase, a cytosolic enzyme, low amounts of Cyt *c* oxidase, and low rate of O_2 uptake in the dark, indicating that these chloroplasts are not greatly contaminated with cytosolic enzymes or mitochondria (Table II). The low contamination by mitochondria was confirmed by electron microscopy. The percentage of intact cells or cell particles that retained a nucleus was estimated by electron microscopy to be under 6% of the total.

Chloroplast Integrity

The chloroplasts prepared by this method are between 50 and 75% intact as judged by electron microscopy. The most common contaminating particles were chloroplasts that were unraveling. Although these unstacked organelles resembled the "thylakoid membranes" that band at the 20 to 45% Percoll interface, they still retain the envelope membrane as judged by galactosyl transferase assays.

The test often used for determining the integrity of intact chloroplasts, ferricyanide exclusion, indicated that the chloroplasts were greater than 90% intact. This test, which looks at ferricyanide-dependent O_2 evolution before and after osmotic lysis, determines whether an intact chloroplast preparation is excluding ferricyanide. This discrepancy between electron microscopic and the ferricyanide methods of estimating the integrity of chloroplasts may be due in part to the presence of the envelope surrounding the thylakoids even after the thylakoid membranes have begun to unstack. A

Table II. Enzyme Activities of Cell Homogenates and Intact Chloroplasts from *Chlamydomonas reinhardtii*

Activities shown are the averages of three separate chloroplast isolations.

Enzyme	Enzyme Activity in	
	Homogenate	Chloroplasts
	$\mu\text{mol} \cdot \text{mg}^{-1} \text{ Chl} \cdot \text{h}^{-1}$	
Rubisco	80	94
Galactosyl transferase	0.01	0.0095
PEP carboxylase	12.6	1.53
Cyt <i>c</i> oxidase	34.2	6.0

second explanation for the difference between these methods is that there is some additional damage to the chloroplasts in the initial fixing steps required for electron microscopy. Both methods agree that the chloroplasts prepared by this method are at least 50% intact and possibly as high as 90% intact.

DISCUSSION

The chloroplast isolation procedure described here is fast, simple, and reproducible. Unlike other published methods, it does not require a pressure cell (11, 14, 20) or detergent (6, 7, 19). Because no detergent is required, these chloroplasts are suitable for transport studies. We have used this method for cells grown under a variety of growth conditions and with different media. In particular, we obtained better intact chloroplasts from photoautotrophically grown cells with this method than were obtained with the Yeda press (14) or Parr bomb (10). The syringe method also works with cells grown on minimal media under high or low CO_2 conditions as well as acetate-grown cells.

In conclusion, this communication describes a rapid procedure to purify intact chloroplasts from *C. reinhardtii*. These chloroplasts appear intact as judged by electron microscopy, CO_2 fixation, and marker enzyme analysis.

LITERATURE CITED

1. Boyton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, Sanford JC (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* 240: 1534-1538
2. Goldschmidt-Clermont M, Malone P, Rochaix JD (1989) Preparation of *Chlamydomonas* chloroplasts for the *in vitro* impact of polypeptide precursors. *Plant Physiol* 89: 15-18
3. Harris EH (1989) The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego
4. Kindle KL, Richards KL, Stern DB (1991) Engineering the chloroplast genome: techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 88: 1721-1725
5. Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 87: 1228-1232
6. Klein U, Chen C, Gibbs M, Platt-Aloia KA (1983) Cellular fractionation of *Chlamydomonas reinhardtii* with emphasis on the isolation of the chloroplast. *Plant Physiol* 72: 481-487
7. Klein U, Chen C, Gibbs M (1983) Photosynthetic properties of

Table I. Photosynthetic Properties of Intact Chloroplasts Isolated from *Chlamydomonas reinhardtii*

The standard assay contained 25 μg Chl intact chloroplasts in 150 mM sorbitol, 50 mM Hepes-KOH (pH 7.5), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 3 mM KCl, 0.25 mM KH_2PO_4 , 4 mM glycerate-3-P, 2 mM HCO_3^- , and 1 mM ATP in a final volume of 1 ml. The light intensity was $400 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and samples were illuminated for 2 min before the addition of glycerate 3-P, ATP, and HCO_3^- . The O_2 evolution of the chloroplasts in the absence of sorbitol decayed within 2 min to less than $3 \mu\text{mol} \cdot \text{mg}^{-1} \text{ Chl} \cdot \text{h}^{-1}$.

Experimental Condition	Rate of O_2 Evolution $\mu\text{mol} \cdot \text{mg}^{-1} \text{ Chl} \cdot \text{h}^{-1}$
Standard assay	25.7 ± 1.4
Standard minus glycerate-3-P	11.6 ± 3.6
Standard minus HCO_3^-	9.3 ± 1.9
Standard minus sorbitol	7.1 ± 2.8
Standard minus ATP	20.5 ± 4.0
Standard plus 10 mM Pi	8.4 ± 1.3

- chloroplasts from *Chlamydomonas reinhardtii*. *Plant Physiol* **72**: 488–491
8. Lane MD, Maruyama H, Easterday RL (1969) Phosphoenolpyruvate carboxylase from peanut cotyledons. *Methods Enzymol* **13**: 277–283
 9. Manuel LJ, Moroney JV (1988) Inorganic carbon accumulation in *Chlamydomonas reinhardtii*. New proteins are made during adaption to low CO₂. *Plant Physiol* **88**: 491–496
 10. Mason CB, Manuel LJ, Moroney JV (1990) A new chloroplast protein is required for growth on low CO₂ in *Chlamydomonas reinhardtii*. *Plant Physiol* **93**: 833–836
 11. Mendiola-Morgenthaler L, Leu S, Boschetti A (1985) Isolation of biochemically active chloroplasts from *Chlamydomonas*. *Plant Sci* **38**: 33–39
 12. Mendiola-Morgenthaler L, Eichenberger W, Boschetti A (1985) Isolation of chloroplast envelopes from *Chlamydomonas*. Lipid and polypeptide composition. *Plant Sci* **41**: 97–104
 13. Moroney JV, Husic HD, Tolbert NE, Kitayama M, Manuel LJ, Togasaki RK (1989) Isolation and characterization of a mutant of *Chlamydomonas reinhardtii* deficient in the CO₂ concentrating mechanism. *Plant Physiol* **89**: 897–903
 14. Moroney JV, Kitayama M, Togasaki RK, Tolbert NE (1987) Evidence for inorganic carbon transport by intact chloroplasts of *Chlamydomonas reinhardtii*. *Plant Physiol* **83**: 460–463
 15. Price CA, Reardon EM (1982) Isolation of chloroplasts for protein synthesis from spinach and *Euglena gracilis* by centrifugation in silica sols. In M Edelman, RB Hallick, NH Chua, eds, *Methods in Chloroplast Molecular Biology*. Elsevier, Amsterdam, pp 189–209
 16. Raphael J (1987) Cytochrome c oxidase. In HU Bergmeyer, ed, *Methods of Enzymatic Analysis*, Vol 3. Verlag Chemie, VCH Publishers, Weinheim, pp 266–273
 17. Sueoka N (1960) Mitotic replication of deoxyribonucleic acids in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* **46**: 83–91
 18. Spreitzer RJ, Al-Abed SR, Huether MJ (1988) Temperature sensitive photosynthesis-deficient mutants of *Chlamydomonas reinhardtii*. *Plant Physiol* **86**: 773–777
 19. Sültemeyer DF, Klöck G, Kreuzberg K, Fock H (1988) Photosynthesis and apparent affinity for dissolved inorganic carbon by cells and chloroplasts of *Chlamydomonas reinhardtii* grown at high and low CO₂ concentrations. *Planta* **176**: 256–260
 20. Togasaki RK, Brunke K, Kitayama M, Griffith M (1987) Isolation of intact chloroplasts from *Chlamydomonas reinhardtii* with the Beckman centrifugal elutriation system. In J Biggins, ed, *Progress in Photosynthesis Research*, Vol 3. Martinus Nijhoff, Dordrecht, pp 499–505