

Photosynthetic Activity of Spinach Chloroplasts after Isopycnic Centrifugation in Gradients of Silica¹

Received for publication December 20, 1973 and in revised form May 23, 1974

J.-J. MORGENTHALER² AND C. A. PRICE

Particle Separation Facility, Department of Biochemistry and Microbiology, Rutgers University, New Brunswick, New Jersey 08903

J. MICHAEL ROBINSON³ AND MARTIN GIBBS

Department of Biology, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT

Chloroplast suspensions from spinach (*Spinacia oleracea* L.) were clearly resolved into intact and stripped chloroplasts by isopycnic centrifugation in density gradients of silica sol ("Ludox") and polyethylene glycol. The intact chloroplasts fixed CO₂ and evolved O₂ more rapidly than the crude suspensions; the stripped chloroplasts were inactive. During the photosynthetic fixation of ¹⁴CO₂ in the intact chloroplasts recovered from the gradient, the ¹⁴C label was observed to spread through the photosynthetic intermediate pools, as well as into starch, which indicates that the purified chloroplasts are metabolically competent. This appears to be the first report of the retention of photosynthetic activity following the purification of chloroplasts in density gradients.

MATERIALS AND METHODS

Crude Chloroplasts. Selected, entire leaves from spinach, (*Spinacia oleracea* L.), purchased locally or obtained from a nearby farmer, were submerged in tap water and exposed to daylight and continuous aeration for half an hour. The mid-ribs were excised, and 15 g wet weight of leaves were diced into a stainless steel semimicro blending jar (Eberbach Corp., 360-ml capacity), driven by a fixed-speed Waring Blendor and containing 60 ml G-R medium (0.33 M sorbitol, 2 mM Na₂EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM Na₂P₂O₇, 5 mM araboascorbic acid, 50 mM HEPES/NaOH pH 6.8). The tissue was homogenized twice for about 5 and 3 sec each and strained through one layer of Miracloth into two centrifuge tubes. The brei was centrifuged in a No. 870 angle rotor (IEC), taken from 0 to 6,000 rpm and to rest in the shortest possible time (2). The pellets of crude chloroplasts were resuspended by repeated aspiration in a pipet in 1 ml each of G-R medium, pooled, and assayed immediately for O₂ evolution or CO₂ fixation. The preparation required no more than 6 min from the moment the blender was started (4).

Isopycnic Centrifugation. A 1.8-ml aliquot of crude chloroplast suspension was layered on top of a 10 to 80% v/v Ludox gradient and centrifuged in an SB-110 (IEC) swinging bucket rotor for about 4.8×10^5 rad²·sec⁻¹, corresponding to 7,000 rpm at 15 min: the maximum centrifugal field was 8,200g. After separation, the bands containing the intact and stripped chloroplasts were collected (roughly 5 ml each), diluted to 40 ml with G-R medium and centrifuged as described under "Crude Chloroplasts." The pellets were resuspended in 0.5 ml of G-R medium.

All components used in the preparation and separation of chloroplasts were kept in ice.

Preparation of Gradients. Ludox AM was purchased from E. I. du Pont de Nemours and Co. (Industrial and Biochemicals Department, Wilmington, Del. 19898) as a sol with 40% (w/v) SiO₂ and purified by the following procedure: the pH of the sol was adjusted to 6.8 by slow addition of the hydrogen form of a cation exchange resin (Biorad AG 50W-X8, 100-200 mesh); after the resin was removed by filtration through Miracloth 25 g/l of activated carbon (Darco Corp.) were added and the mixture was stirred or shaken overnight. The carbon was then removed by successive filtrations through Miracloth, a wet bed of diatomaceous earth (Sigma grade 1), prepared on top of a glass fiber filter (Gelman type E) fitted in a coarse glass frit, and finally through a paper filter (Whatman No. 5) in a Büchner funnel. To this purified material 10% w/v PEG

Most biochemical studies on chloroplasts and essentially all concerned with the carbon reduction cycle have been performed on particles purified by differential centrifugation (3, 6, 14). It is generally recognized that density gradient centrifugation yields chloroplasts of greater purity (11), but heretofore it has not been possible to recover from gradients chloroplasts which retain significant photosynthetic capacity.

The critical factor almost certainly lies in the choice of the gradient material. Sucrose and sorbitol have too high osmotic potentials; Ficoll is suitable for *Euglena* chloroplasts (13), but its solutions are extremely viscous and may be insufficiently dense for chloroplasts from higher plants. Silica sols appear more promising (5, 7, 10), but are normally toxic. We now find that a commercial silica sol, Ludox AM, is compatible with spinach chloroplasts when combined with certain organic polymers, especially PEG⁴ (8, 9).

¹ This work was supported in part by the United States Public Health Service Grant HD-05602 to C. A. P. and the United States Atomic Energy Commission Grant AT (11-1)3231 to M. G. Paper of the Journal Series, N. J. Agricultural Experiment Station, Cook College, Rutgers University, New Brunswick, N. J. 08903.

² Postdoctoral Fellow of the Foundation for Biomedical Fellowships, Switzerland.

³ Postdoctoral Fellow of the National Institutes of Health.

⁴ Abbreviation: PEG: polyethylene glycol.

(Carbowax 6000, Union Carbide) was added, and the solution kept at room temperature as a stock. Just prior to use, 1% (w/v) BSA (Sigma) was added. Starting and limiting solutions for the gradients were made 10 and 80% (v/v) in Ludox/PEG/BSA, respectively, and also contained the same ingredients as the G-R medium plus approximately 20 mg GSH/100 ml. Linear gradients, 25 ml each, were pumped into 24 × 90 mm centrifuge tubes of the SB-110 rotor, followed by 2 ml of a "cushion" of limiting solution.

O₂-Evolution. Assays were carried out in a YSI oxygen monitor (Model 53). The assay mixture contained 0.2 ml of chloroplast suspension containing from 30 to 160 μg of Chl in a final volume of 5 ml of A medium (same composition as G-R medium, but containing 5 mM NaHCO₃ and adjusted to pH 7.8). The assay solution was gassed with N₂ before the addition of the chloroplasts and equilibrated with the oxygen electrode at 25 C. A single 150-w lamp mounted 10 cm from the cell provided a light intensity of roughly 40,000 lux. Calculations of O₂-evolution are based on the assumption that 1 ml of water contains 0.28 μmoles of oxygen when saturated with air. Chlorophyll was determined according to Arnon (1).

CO₂-Fixation. The assays were carried out in a final volume of 1 ml of A medium to which were added 50 μl of the same chloroplast suspension used for measuring O₂ evolution. Unlabeled NaHCO₃ was replaced by the same amount of ¹⁴C-NaHCO₃ at a specific radioactivity of 0.34 c/mole. Samples of 0.1 ml were withdrawn at the stated time intervals and treated with 0.1 ml 24 N formic acid prior to liquid scintillation counting. The assay tubes were continuously gassed with a slow stream of N₂, immersed in an aquarium kept at 25 C, and illuminated from two sides with 300-w lamps 20 cm away. The light intensity within the reaction vessels was about 7000 lux.

Products of Photosynthesis. ¹⁴C-labeled photosynthetic intermediates were separated by one-dimensional descending chromatography on Whatman No. 1 paper employing Wood's GW₃ solvent (15) which contains *n*-butyl alcohol/*n*-propyl alcohol/acetone/30% (w/v)-trichloroacetic acid/80% (w/v) formic acid (40:20:25:15:25 v/v), and 0.3 g of EDTA per 100 ml. After solvent development, the chromatograms were allowed to dry at 20 C, and the dried chromatograms were sprayed with 1 M NaHCO₃ to convert glycolic acid to the less volatile salt form.

Kodak No-Screen x-ray film was exposed to the dried chromatograms in order to identify the ¹⁴C-labeled products. Product identification was confirmed by co-chromatography using both ¹⁴C-labeled and unlabeled, known standard intermediates. Unlabeled phosphate esters of three-carbon acids and five- and six-carbon sugars were positioned on chromatograms employing the methods outlined by Smith (12). Radioactivity in the labeled products which had been separated by paper chromatography was quantitated by excising the ¹⁴C-labeled areas from the chromatograms, placing the excised areas in scintillation vials with 10 ml of fluid containing 5 g of PPO per liter of toluene, and counting them in a Beckman LS-250 scintillation counter.

RESULTS AND DISCUSSION

After chloroplasts were sedimented to equilibrium in gradients of Ludox-PEG-BSA, we found Chl in two clearly separated zones. The lower zone was seen under phase contrast microscopy to contain mostly intact chloroplasts; the upper, less dense zone contained only stripped chloroplasts. It is remarkable that in Ludox gradients containing no PEG, the intact chloroplasts are found at densities lower than the stripped ones (5, 7, 10). The addition of PEG not only reverses the banding order but also greatly increases the sharpness of

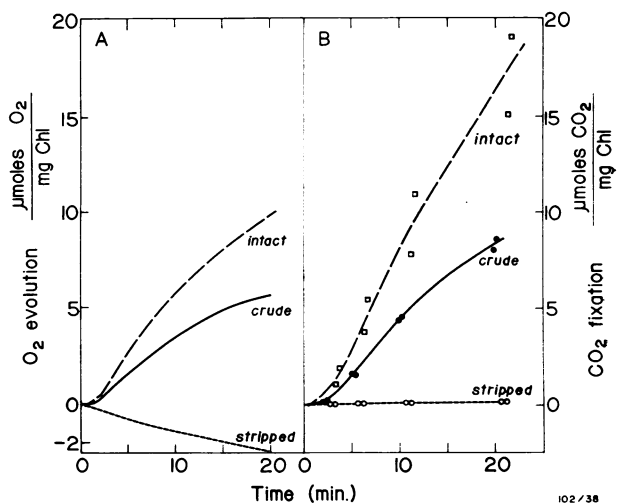


FIG. 1. O₂ evolution (A), and CO₂ fixation (B), spinach chloroplasts. The assay medium contained 0.33 M sorbitol, 2mM Na₂EDTA, 1mM MgCl₂, 1 mM MnCl₂, 1 mM Na₂P₂O₇, 5 mM ascorbic acid, 5 mM NaHCO₃, and 50 mM HEPES/NaOH pH 7.8. Duplicate assays were carried out when measuring CO₂ fixation.

Table I. ¹⁴C Label Distribution in Products of Photosynthesis from Intact Chloroplasts Separated on Ludox Density Gradients

The assay medium for ¹⁴CO₂ fixation was identical to that described for those studies shown in Fig. 1; 5.1 μmoles of NaHCO₃ (1.66 μC) and 41.9 μg of Chl were employed in a reaction mixture of 1 ml final volume. The samples used for paper chromatography were taken at 5, 10, and 15 min after the beginning of illumination, and ¹⁴C-labeled intermediates were determined as indicated under "Materials and Methods."

| ¹⁴ C Intermediates | Total cpm Fixed After | | |
|--|-----------------------|--------|--------|
| | 5 min | 10 min | 15 min |
| Fructose-1,6-diP | 2.6 | 2.8 | 7.6 |
| Ribulose-1,5-diP | 0.4 | 1.0 | 2.7 |
| Glucose-1-P, glucose-6-P, and fructose-6-P | 12.1 | 9.5 | 7.2 |
| Ribose-5-P | 2.3 | 2.7 | 2.8 |
| Triose-P ¹ | 2.8 | 4.9 | 8.4 |
| Glycerate-3-P | 56.8 | 52.6 | 38.5 |
| Glycolate | 1.5 | 1.5 | 2.2 |
| Insoluble ² | 19.7 | 23.5 | 29.4 |
| Unknown | 1.8 | 1.6 | 1.3 |
| Rate of CO ₂ fixation, μmoles/mg Chl-hr, 0 to . . . min | 103 | 87 | 74 |

¹ Mostly dihydroxyacetone-P.

² Polyglucan.

the separation. As an average, 35% of the total Chl was recovered in the zone of intact and 50% in the zone of stripped chloroplasts.

In no experiment did the stripped chloroplasts evolve O₂ or fix CO₂; on the contrary, this fraction consumed O₂ in the light. The intact chloroplasts, however, evolve O₂ (Fig. 1A) and fix CO₂ (Fig. 1B) at enhanced rates. This observation is in contrast to the findings of Heber and Santarius (5), who observed low rates of O₂ evolution and no CO₂ fixation after banding spinach chloroplasts in Ludox gradients.

All the data presented in Figure 1 were obtained with the

same chloroplast preparation. Although the ratio of CO₂ fixed to O₂ evolved is greater than 1, at least in the later stages of the experiments, both O₂ evolution and CO₂ fixation curves clearly show the intact chloroplasts to have higher rates of O₂ evolution and CO₂ fixation than the crude preparation on a Chl basis. The increase in activity in this experiment was 2-fold in both measures. From the pattern of recovery of Chl in this particular experiment (30% intact, 48% stripped) one would expect a 2.6-fold increase in activity. In no experiment did we observe the full increase in activity expected, which may indicate that the chloroplasts suffered some slight damage and that there is a possibility of further improving the method. The activities of a number of preparations averaged 90 μmoles CO₂ fixed/mg Chl·hr for the period from 0 to 15 min after the beginning of the light period; the highest activities were observed between 2 and 5 min after the light was turned on and reached a mean rate of 130 μmoles CO₂ fixed/mg Chl·hr in several experiments using fresh spinach. In the preparations used for assays, *i.e.*, chloroplasts banded in a Ludox gradient and washed twice, a minimum of 75% appeared intact in phase contrast microscopy.

In a separate experiment (Table I) the ¹⁴C-labeled products of photosynthesis by intact chloroplast recovered from Ludox gradients were determined as a function of time. After 5 min of light-dependent ¹⁴CO₂ assimilation, ¹⁴C is found principally in pools of glycerate-3-P and polyglucan or starch. Subsequently, the label shifted away from glycerate-3-P and spread into other compounds such as triose-P and fructose-1,6-diP; the label accumulation in polyglucan continued to increase. These ¹⁴C product distribution patterns are similar to those observed previously during photosynthetic ¹⁴CO₂ assimilation in "crude" chloroplast preparations (3). Our results indicate that intact chloroplasts from the Ludox gradient are metabolically competent; *i.e.*, they possess an active system of enzymes of the Calvin-Benson cycle as well as an active starch synthesis system.

The method presented permits the recovery of photosyn-

thetically active chloroplasts following isopycnic sedimentation and permits for the first time a full range of biochemical investigations on preparations of chloroplasts of greatly improved purity.

Acknowledgments—The authors wish to thank Mrs. Tirza Bamberger for excellent technical assistance and Dr. André Jagendorf for critical discussions.

LITERATURE CITED

1. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24: 1-15.
2. COCKBURN, W., D. A. WALKER, AND C. W. BALDRY. 1968. The isolation of spinach chloroplasts in pyrophosphate media. *Plant Physiol.* 43: 1415-1418.
3. GIBBS, M. 1971. Carbohydrate metabolism by chloroplasts. *In*: M. Gibbs, ed., *Structure and Function of Chloroplasts*. Springer-Verlag, Heidelberg. pp. 169-214.
4. GIBBS, M. AND J. M. ROBINSON. 1974. Photosynthetic CO₂ incorporation in isolated spinach chloroplasts. *In*: A. San Pietro, ed., *Experimental Plant Physiology*. C. V. Mosby Co., St. Louis, Mo. pp. 13-20.
5. HEBER, U. AND K. A. SANTARIUS. 1970. Direct and indirect transfer of ATP and ADP across the chloroplast envelope. *Z. Naturforsch.* 25 b: 718-728.
6. JENSEN, R. G. AND J. A. BASSHAM. 1966. Photosynthesis by isolated chloroplasts. *Proc. Nat. Acad. Sci. U.S.A.* 56: 1095-1101.
7. LYTTLETON, J. W. 1972. Use of colloidal silica in density gradients to separate intact chloroplasts. *Anal. Biochem.* 38: 277-307.
8. PERTOFT, H. 1969. The separation of rat liver cells in colloidal silica-polyethylene glycol gradients. *Exp. Cell Res.* 57: 338-350.
9. PERTOFT, H. 1970. Isopycnic zonal centrifugation in colloidal silica. *Anal. Biochem.* 38: 506-516.
10. PRICE, C. A., E. N. BREDEEN, AND A. VASCONCELOS. 1973. Isolation of intact spinach chloroplasts in the CF-6 continuous-flow zonal rotor; implications for membrane-bound organelles. *Anal. Biochem.* 54: 239-246.
11. ROCHA, V. AND I. P. TING. 1970. Preparation of cellular plant organelles from spinach leaves. *Arch. Biochem. Biophys.* 140: 398-407.
12. SMITH, I. 1969. *Chromatographic and Electrophoretic Techniques*. Vol. I, Ed. 3. Interscience Publishers, New York. pp. 305, 315-319.
13. VASCONCELOS, A., M. POLLACK, L. R. MENDIOLA, H. P. HOFFMANN, D. H. BROWN, AND C. A. PRICE. 1971. Isolation of intact chloroplasts from *Euglena gracilis* by zonal centrifugation. *Plant Physiol.* 47: 217-221.
14. WALKER, D. A. 1971. Chloroplasts (and grana): aqueous (including high carbon fixation ability). *Methods Enzymol.* 23: 211-220.
15. WOOD, T. 1961. A procedure for the analysis of acid-soluble phosphorus compounds and related substances in muscle and other tissues. *J. Chromatog.* 6: 142-154.