FRACTIONATION OF SPINACH CHLOROPLASTS BY FLOW SEDIMENTATION–ELECTROPHORESIS

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ABSTRACT

A separation of spinach chloroplasts in vitro into fractions according to size (volume) and activity (light-dependent shrinkage and NADP reduction) has been achieved by stable-flow free boundary sedimentation-electrophoresis. The salient features of this chloroplast study are: (*a*) separation is achieved within 30 min; (*b*) only small density gradients are required, thus minimizing osmotic effects; (*c*) the fractions are collected continuously, with size fractionation being evidenced; and (*d*) particles are separated into fractions of higher and lower activities as compared with the control population.

INTRODUCTION

Spinach chloroplasts in vitro have revealed the existence of a variety of morphological forms: in particular, two are well characterized. One class is a population of highly refractive chloroplasts that do not show distinct grana; the second shows clear evidence of grana ordered in a regular array (1). In addition to these forms in vitro, chloroplast size and shape changes both in vivo (2) and in vitro (3–7) have been associated with variations in tonicity of suspension medium and of light level. Isolation of chloroplasts results in a heterogeneous population, as evidenced by size distribution studies of isolated particles performed in the Coulter counter by Orth and Cornwell (8), Itoh et al. (6), and Packer et al. (5). The fractions contain both whole chloroplasts and fragments of varying sizes. It is not understood how this variation in chloroplast size and integrity seen in vitro, when chloroplasts are studied by density gradient centrifugation (9), countercurrent distribution (10), and electrophoretic techniques (11), correlates with biochemical activity. The stable-flow free boundary (STAFLO) method (12) of sedimentation and/or electrophoresis has been used successfully for fractionating cell populations (13). Its application to fractionation of subcellular organelles (of more uniform size and activity) seemed promising, because separation may be achieved by a unique combination of the features of sedimentation and an electric field.

METHODS

CHLOROPLAST ISOLATION: Chloroplasts were isolated from spinach leaves by homogenization in a Waring Blendor for 30 sec in a medium containing 200 mM sucrose + 5 mM Na phosphate (pH 7.5). The homogenate was centrifuged at low speed (200 × *g*, 5 min) to sediment large particles and debris. The supernatant was recentrifuged (600 × *g*, 5 min) to sediment the chloroplasts. The chloroplast-containing pellet was resuspended and used as starting material for experiments.

STAFLO APPARATUS: The above suspension of partially purified chloroplasts was introduced at 25°C into one channel at the right end of the narrow rectangular cell of the stable-flow free boundary apparatus (12) (cf. Fig. 1). Media (e.g. sucrose solutions) of different density were introduced into all twelve inlets with solutions increasing in density from

top to bottom (channels 1–12) of the flow cell. Chloroplasts suspended in sucrose enter in the second channel from the top (Fig. 2). The over-all apparatus (Fig. 1) consists of a syringe drive mechanism (to pump the solutions contained in the twelve syringes simultaneously), a flow cell (through which the solutions are pumped and in which the density gradient is established and separation occurs), and a collection assembly (containing twelve tubes located within a lucite collection chamber kept at 0°C by circulating a coolant liquid). The flow cell is equipped with two platinum electrodes separated from the main chamber by a semipermeable membrane. The electrode compartments are flushed continuously by solutions which pass by gravity flow through the space separating the electrodes from the main chamber at a rate of 35 ml/min for each electrode. The particles, following stable patterns of flow, traverse the chamber and migrate under the influence of gravity and/or an electric field. Control samples of the same chloroplast preparation were located in a syringe in the syringe drive assembly, and were passed through a length of the same sized plastic tubing (for a distance equivalent to that for particles traveling through the flow cell) directly to a tube in the collection assembly. Details of the apparatus shown in Fig. 1 have been previously described (12), except for the arrangement of the collection assembly.

RESULTS

The electrophoresis-sedimentation separation of the crude chloroplast suspension is illustrated in Fig. 2, which shows a close-up photograph of the migration pattern obtained. The linear rate of flow through the 30-cm chamber is 1 cm/min in this experiment. The chloroplast suspension enters the flow cell in a stream through channel 2 and immediately drops to channel 3, presumably due to the greater density of the chloroplast suspension. Thereafter the chloroplasts migrate as a heavy band which slowly drops and becomes more diffuse as the chloroplasts move toward the outlets. If no electric field is present, the chloroplasts sediment about one channel downward in the passage along the flow cell and remain as a narrow band. In the presence of the electric field, the upper boundary of the main band drops almost two channels between entry and exit (cf. Figs. 2 and 3).

Some particles move with much greater "mobility" than the average immediately upon entry

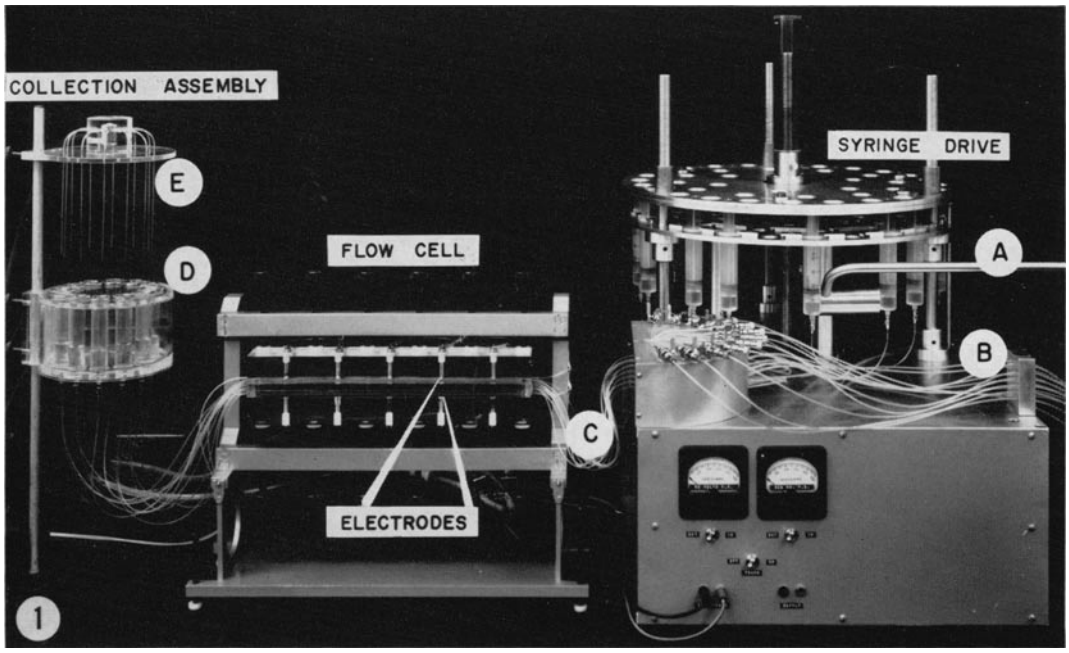


FIGURE 1 The STAFLO apparatus. The inside dimensions of the flow cell are 7 mm deep, 1.5 cm high, and 30 cm long, with 12 symmetrical inlet and outlet channels. *A*, magnetic stirrer used for sample syringe; *B*, tubing to gradient solution reservoirs; *C*, inlets for two electrode rinse and twelve gradient solutions; *D*, coolant jacket; *E*, suction device for emptying collection tubes. The electrode power supply and variable-speed syringe drive controls are not shown.

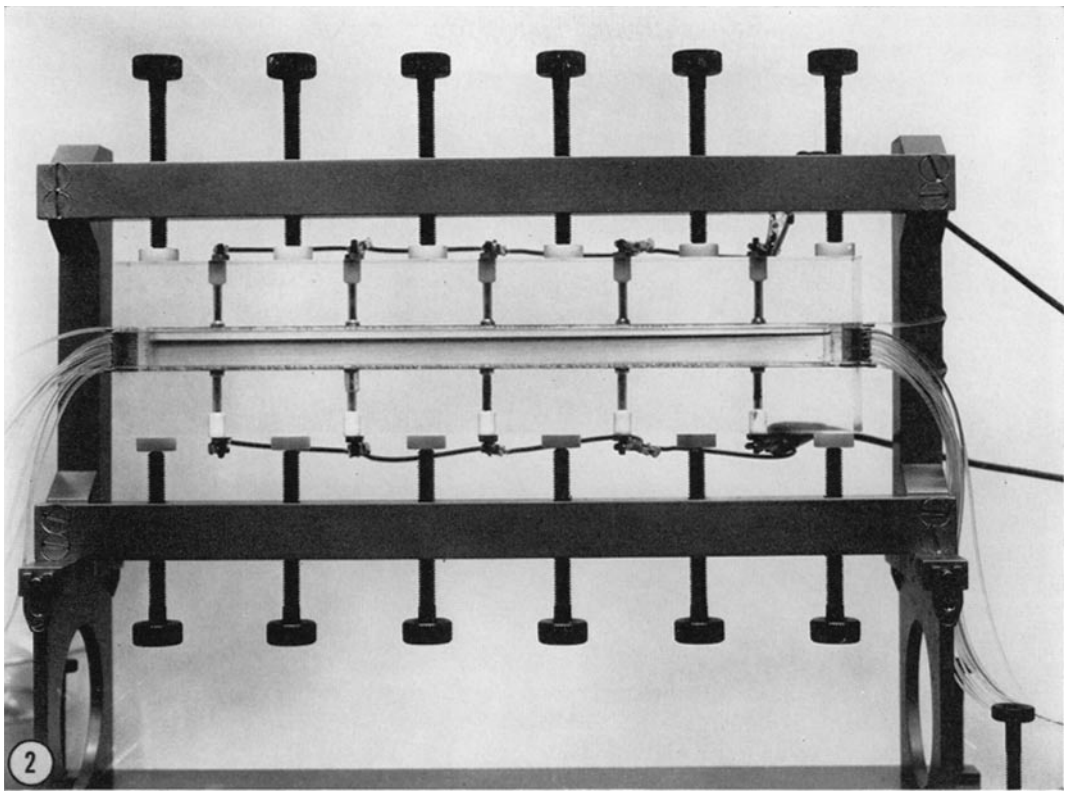


FIGURE 2 Pattern of chloroplast migration in flow cell. Chloroplasts enter at the right in channel 2 at a chlorophyll concentration of 0.5 mg/ml. The electric field strength is 3.3 v/cm with the positive electrode at the bottom. The 12 channels (reading from top to bottom) contained 5 mM Na phosphate (pH 7.5) plus the following concentrations of sucrose (mM): 200, 225, 255, 260, 265, 270, 275, 280, 285, 290, 295, and 300. The upper and lower electrode flush solutions contained 5 mM Na phosphate plus 200 mM sucrose (upper) or 300 mM sucrose (lower).

into the flow cell. In Fig. 2 this is observed as a gray, diffuse area that proceeds obliquely downward from the inlet. In the absence of an electric field this effect is not seen.

A plot of certain characteristics, representative of the Coulter counter size and biological activity of the chloroplasts in the isolated fractions obtained in this experiment, is given in Fig. 3. Fig. 3 *a* shows the distribution of chlorophyll, reflecting the concentration of chloroplasts, and the average volume of a chloroplast in the collected fractions. Most of the chlorophyll is collected in a broad peak between channels 4 to 7. Notice that as the fraction number increases, the average chloroplast size also increases; hence larger particles migrate faster in the downward direction. The chloroplasts under the conditions of separation and measurement employed in this experiment have average volumes in the range of 50 to 60 μ^3.

Fig. 3 *b* shows the distribution of activity for two parameters: the reduction of NADP and the occurrence of light-induced volume change (shrinkage) upon illumination of the chloroplast. Clearly it is possible to isolate classes of chloroplasts in certain fractions, which have higher light-scattering activity than is present in the control. In Fig. 3 *b* it is seen that the light-scattering activity in channel 4 (5.7%) is higher than that in the control (4.5%), and that the activity has decreased in the bottom channels to values lower than that of the control. Qualitatively similar behavior was seen for five experiments under the above conditions; the highest activity was always in channel 4 and was always higher than that of the control. Fig. 3 *b* also shows

a clear fractionation according to NADP reduction; values range from 190 μmoles/mg chlorophyll/hr in channel 3 to values less than 40 in the lower channels, compared to a control value of 85. Hence the ability to reduce NADP decreases as the particle size increases, in agreement with Spencer and Unt who found that chloroplast fragments have a higher Hill reaction activity than whole chloroplasts (1).

Chloroplasts have also been separated in the STAFLO apparatus on the basis of sedimentation characteristics alone. In this case, it is found by microscope examination of the fractions that fragments and broken chloroplasts tend to float while chloroplasts which are clumped together tend to sediment. The fractions collected from channels 6 to 8 contain the most chlorophyll (chloroplasts enter at channel 4), the highest number of chloroplasts, and the most light-scattering activity. Although this method is slower than sedimentation plus the electric field, its advantage is that separation is based upon one parameter only (sedimentation behavior) and that size and density differences between the particles may dominate the pattern of fractionation. Since a longer time in the flow chamber may be required to achieve separation, there is a greater hazard of inactivation of the chloroplasts than if both sedimentation and electrophoresis are used for separation. For example, it is known that certain biochemical activities of chloroplasts, especially photophosphorylation, are quick to lose activity in vitro.

DISCUSSION

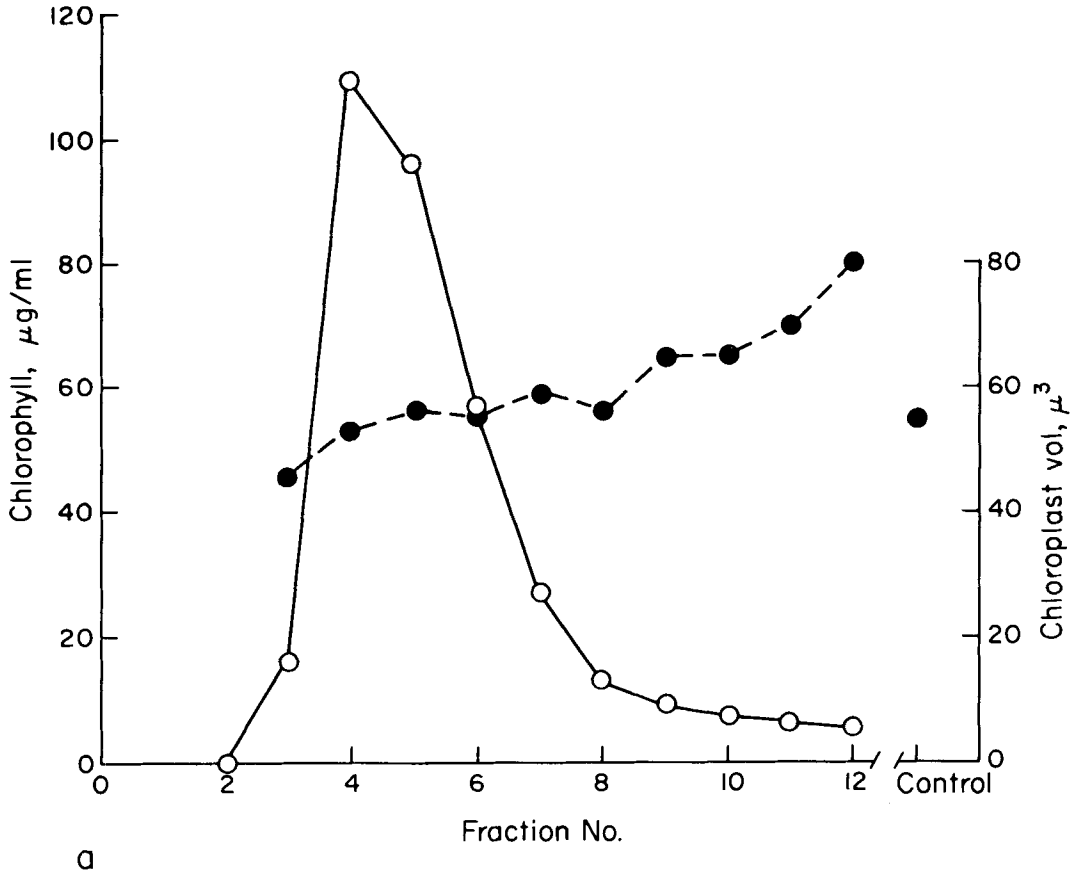
Stable-flow free boundary electrophoresis of chloroplasts in vitro in a density gradient system effects a rapid and reproducible separation of a population of chloroplasts into classes of varying activity and size in relation to the control population. Since this investigation constitutes the first report of separation of subcellular particles by such means, it seems worthwhile to summarize some of its salient features. These are: (*a*) separation is achieved within 30 min; (*b*) only small densiy gradients are required, thus minimizing osmotic effects; (*c*) the fractions are collected continuously; (*d*) the particles in the outlet channels are in a narrower size range than those in the entering suspensions; (*e*) particles are separated into higher and lower activity as compared with the control population.

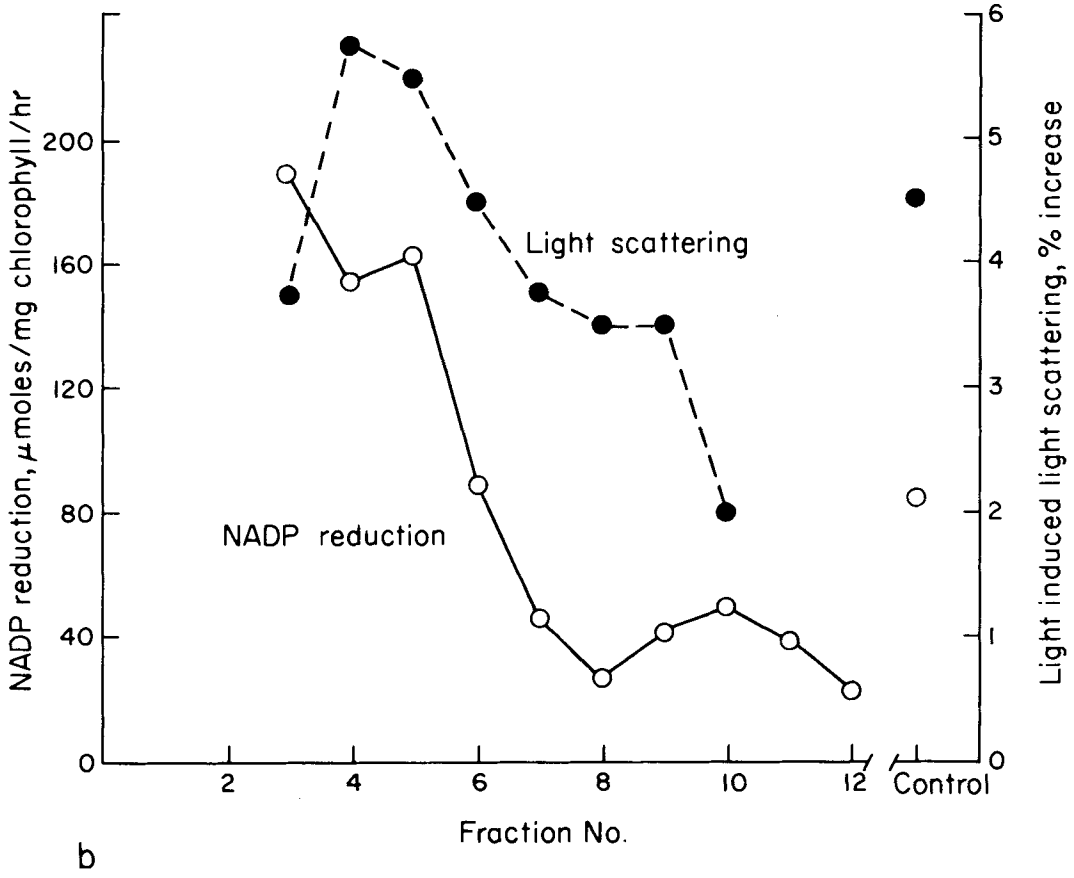
The results of this investigation do not show evidence for the existence of only two distinct classes of chloroplasts but rather for a broad spectrum of chloroplasts with respect to sedimentation rate and/or electrophoretic mobility (or the tendency to aggregate in an electric field) and biological activity. The enhancement of light-scattering activity and NADP reduction in the

FIGURE 3 Properties of fractionated chloroplasts (see legend to Fig. 2).

FIGURE 3 *a* Chlorophyll concentration and average chloroplast volume. Chlorophyll was determined spectrophotometrically (14). Volume determinations were made with a Coulter counter model B with a 100 μ orifice and an automatic particle size distribution analyzer calibrated with ragweed pollen (3884 μ^3, Coulter Electronics, Hialeah, Florida). The chloroplasts in the collected fractions were diluted to 10 mμg chlorophyll/ml in the corresponding input solutions for that channel plus 35 mM Na phosphate (pH 7.5). Chloroplast volume was defined as the total volume of all particles greater than 12 μ^3, divided by the number of such particles.

FIGURE 3 *b* Shrinkage and NADP reduction. The reaction mixture for shrinkage determinations (light-scattering) contained Na phosphate (40 mM, pH 7.5), $MgCl_2$ (5 mM), Phenazine methosulfate, PMS, (20 μM), and chloroplasts (5 μg chlorophyll/ml). Changes in 90° scattering of the 546 mμ measuring beam in response to actinic light were measured as previously described (4). The NADP reaction mixture contained tris buffer (10 mM, pH 7.5), Na acetate (50 mM, pH 7.5), spinach ferredoxin (34 μg/ml), and chloroplasts (5 μg chlorophyll/ml). NADP reduction rate was calculated from absorbancy changes at 340 mμ in response to actinic light. Since chloroplasts in the collected fraction were diluted to 5 μg chlorophyll/ml in the appropriate reaction medium, small amounts of sucrose were also present in the test systems.





upper channels may, in fact, be partly due to the migration of aggregates into the lower channels (although these do not appear to arise from the low-field strength employed).

Gross et al. (11) have used electrophoresis to separate chloroplast fragments into classes of different Hill reaction activity. Albertsson and Baltscheffsky (10) have investigated the characteristics of spinach chloroplasts separated by countercurrent distribution by employing sucrose and high polymers to effect the phasic separation. Although these investigators were able to separate chloroplasts into two distinct classes according to appearance, the details of the activity of these chloroplasts are unavailable. Unfortunately, the inhibition of certain light-induced activities (NADP reduction and shrinkage) have been observed when chloroplasts are incubated in the presence of high concentrations of sucrose and polymers,[1] such as those employed for the countercurrent distribution studies.

Since this STAFLO apparatus is fabricated of a translucent plastic material, it is possible to illuminate chloroplasts during migration. Indeed, a change in migration pattern has been observed upon illumination of chloroplasts under sedimentation[1] and electrophoresis (15) conditions, a fact which indicates that patterns of migration established during STAFLO electrophoresis can be useful in detecting physical changes, e.g., in size, density, or charge caused by actinic light illumination. Thus, it may be possible to further explore the heterogeneity in physical properties as well as in metabolic events in populations of isolated chloroplasts.

The authors would like to thank Arthur Taurog, Roy Colombe, Richard C. Norman, and Elmer Wieking for skilled assistance in the design and construction of the STAFLO electrophoresis apparatus. Patricia Borther and Kerstin Nordenbrand also performed much valued technical assistance. This research was supported by grants from United States Public Health Service (AM 6438-03) and The National Science Foundation (GB 1550).

Received for publication 14 September 1965.

[1] Unpublished results.

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