Large-Scale Isolation of Intact Vacuoles and Isolation of Chloroplasts from Protoplasts of Mature Plant Tissues

Abstract. *Intact plant vacuoles were prepared in large numbers (10^6) from protoplasts of mature leaves, flower petals, stems, pedicels, filaments, styles, and young fruits. Treatment of protoplasts with 0.2 molar K_2HPO_4-HCl, pH 8, with slow stirring resulted in gentle osmotic rupture of the protoplasts and release of intact vacuoles. Particulate components of the protoplast, less the vacuole, were largely shed as an aggregate, which was removed by filtration. Vacuoles were recovered from the filtrate by low-speed centrifugation. The general procedure was also used to isolate chloroplasts with a high degree of integrity and excellent photochemical activity.*

The vacuole of the mature plant cell occupies 90 percent or more of the total cell volume. Despite its size, knowledge of this ubiquitous plant organelle has been largely limited to observations made by light and electron microscopy (*1*). A detailed understanding of the composition and function of the mature plant vacuole has been impeded by the lack of a method for preparing large quantities of the organelle. There have been a few reports of the small-scale isolation of mature vacuoles from protoplasts (*2*). In addition, isolation of immature vacuoles from meristematic plant tissue (*3*) and of mature vacuoles from yeast (*4*) have been reported. We describe here a procedure for isolating large numbers of intact vacuoles (10^6) from various mature tissues of several plants; to our knowledge, this is the first procedure for accomplishing this. The technique shows promise as a general method for gentle isolation of plant organelles since it does not involve shearing forces or aeration and the deleterious effects (*5*) of exposing plant cell proteins and organelles to vacuolar contents are at least partially avoided. Chloroplasts isolated by this method have been shown to have a high degree of integrity and excellent photochemical activity.

Protoplasts were prepared in large numbers (10^6 to 10^7) from leaves, petals, pedicels, filaments, and styles of *Hippeastrum*; leaves, petals, and stems of *Tulipa*; young leaves and petals of *Ipomoea*; young leaves and fruits of *Lycopersicum*; and *Pisum* leaves. Smaller numbers of protoplasts and vacuoles (10^5) were prepared from leaves of *Cichorium*, *Lactuca*, *Hordeum*, *Digitalis*, and *Petunia* and from petals of *Streptocarpus*, *Petunia*, *Pelargonium*, *Iris*, *Cosmos*, and *Coreopsis*.

Protoplasts were prepared by sterilizing the tissue surface with 70 percent alcohol; the epidermis was then removed by peeling or the tissue was sliced into 2-mm strips. The tissue was floated on a solution of 2 percent (weight to volume) Cellulysin (Calbiochem) and 0.6*M* mannitol adjusted to *p*H 5.8 with NaOH; the solution had been sterilized on a 0.22-μm filter (*6*). Incubation was at 25°C for 17 to 24 hours with gentle shaking (ten circular oscillations per minute). After incubation, undigested and cuticular materials were removed and a suspension containing 1.0×10^7 protoplasts was transferred to a 250-ml centrifuge bottle. The protoplasts were allowed to settle for 15 minutes or were centrifuged at 70*g* for 5 minutes and the supernatant was removed by aspiration. In a typical experiment the petals of two *Hippeastrum* flowers (average diameter, 16 cm) were sliced and distributed among ten petri plates (100 by 15 mm), each containing 12 ml of enzyme solution. Such an experiment yielded approximately 10^7 pigmented protoplasts (average diameter, 85 μm) which, when treated as described below, gave 1.0×10^6 to 1.5×10^6 vacuoles (average diameter, 85 to 90 μm).

Vacuoles were prepared by rapidly adding to the protoplasts during 15 seconds at least 240 ml of 0.2*M* K_2HPO_4 or Na_2HPO_4, adjusted to *p*H 8 with HCl, containing 3 m*M* $MgCl_2$ and 1 m*M* dithiothreitol to give a uniform suspension. A multibladed stirrer was introduced and gentle stirring (15 rev/min) was continued for 2 to 4 minutes, during which time protoplasts underwent gentle osmotic rupture and released intact vacuoles. An aggregate of protoplasmic organelles accumulated on the stirrer blades. The stirrer was removed and the suspension was filtered through plastic screen (1-mm openings) and then through two layers of Pyrex glass wool; vacuoles were then recovered by centrifugation. Vacuoles prepared from tissues that contained anthocyanin or aurone were recovered by centrifugation of the suspension at 23*g* for 3 minutes, followed by removal of debris by stirring the suspension with a wood applicator stick (residual aggregated material adhered to the stick), and finally centrifugation at 100*g* for 3 minutes. This procedure resulted primarily in the sedimentation of vacuoles in the population that were densely pigmented. Colorless vacuoles were sedimented by centrifugation at 1100*g* for 3 minutes. Further purification of vacuoles could be achieved by resuspending the organelles in 0.3 ml of the same supernatant liquid from which they had been sedimented; passing the suspension through one layer of glass wool into 20 ml or more of a solution containing 0.55*M* sorbitol, 1 m*M* tris(hydroxymethyl)aminomethane–2-(*N*-morpholino)ethanesulfonic acid (tris-MES) buffer, *p*H 8, 3 m*M* $MgCl_2$, and 1 m*M* dithiothreitol; and recovering vacuoles by centrifugation at 100*g* for

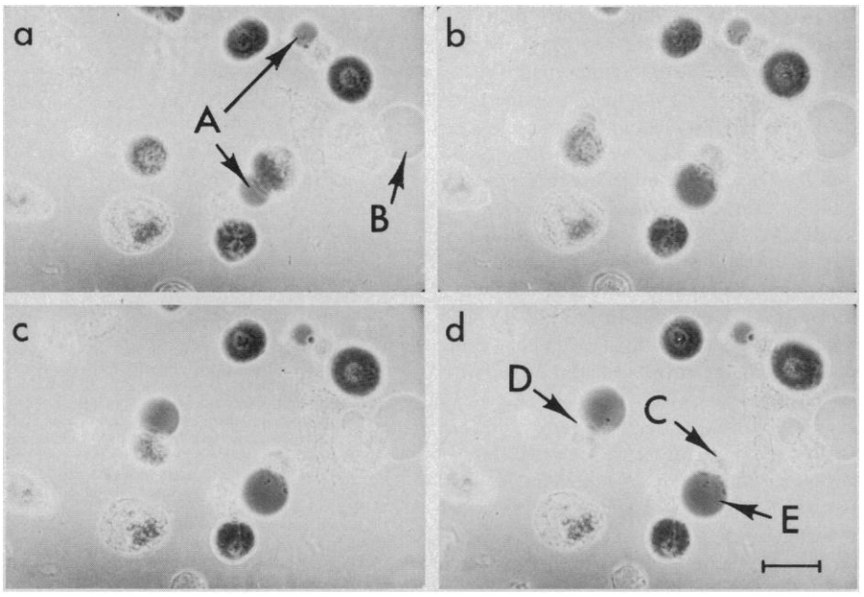

Fig. 1. Emergence of intact vacuoles from protoplasts of *Hippeastrum* flower petals. Equal volumes of protoplast suspension and 0.2*M* Na_2HPO_4-HCl, *p*H 8, were mixed and observed (a) immediately after mixing, (b) after 0.25 minutes, (c) after 0.5 minutes, and (d) after 1.0 minute. Shown are *A*, anthocyanin-containing vacuoles, emerging and completely emerged; *B*, colorless vacuole emerging; *C*, compact aggregate of particulate protoplasmic materials; *D*, one large and several smaller vacuoles released; and *E*, one large vacuole released. Scale bar, ~ 100 μm.

3 minutes for pigmented vacuoles and 1100*g* for 3 minutes for colorless vacuoles. Preparations of colorless vacuoles contaminated with chloroplasts were further purified by layering them on 5 percent Ficoll, 0.55*M* sorbitol, 1 m*M* tris-MES, *p*H 8, and then centrifuging at 500*g* for 5 minutes. This procedure resulted in sedimentation of chloroplasts and residual contaminants, while the vacuoles remained suspended.

Protoplasts and vacuoles were observed by phase contrast light microscopy throughout the procedure by use of a 2-mm-deep well-type slide. Counts were made with a hemacytometer.

After the protoplasts were treated with phosphate, the emergence of vacuoles was observed under the microscope (Fig. 1). Emergence was preceded by a slight swelling of the protoplast. Usually a single large vacuole was extruded from one point on the plasma membrane, but emergence of one large and several smaller vacuoles was also common. Vacuole emergence appeared to depend primarily on osmotic swelling, whereas preliminary experiments with salts other than K_2HPO_4 suggested that aggregation of the particulate protoplasmic materials during vacuole emergence was dependent on the type of salt and *p*H used. In protoplasts containing many small vacuoles, such as those from *Streptocarpus* flower petals, no fusion or fragmentation of vacuoles was observed during emergence. A phosphate concentration of 0.2*M* and *p*H of 8 were found optimal for most systems.

Vacuoles prepared from all tissues containing visible pigment retained their pigment on isolation (Fig. 2a). All vacuoles prepared by the procedure described here concentrated neutral red. They were observed to swell, then burst, in hypotonic mannitol solution, and to become flaccid in hypertonic mannitol solution.

After staining with acetocarmine in 45 percent acetic acid, vacuole preparations from *Hippeastrum* petals and *Tulipa* leaves were found to contain only traces of intact nuclei and protoplasts. In *Hippeastrum* petal but not *Tulipa* leaf preparations, starch grains were noted exterior to the vacuoles after staining with Lugol's iodine in 0.6*M* mannitol. Chloroplasts were observed in *Tulipa* leaf preparations (Fig. 2b) but not in preparations from *Tulipa* petals (Fig. 2a) or *Hippeastrum* petals. Contamination with other cellular organelles in low gravity fields is not likely.

Vacuoles remained intact and retained pigment for more than 20 hours when held in 0.55*M* sorbitol and 1 m*M* tris-MES, *p*H 8.

The general utility of this procedure for obtaining plant cell organelles was examined. Chloroplasts were obtained from *Tulipa* leaf protoplasts after gentle osmotic rupture in 0.2*M* $Na_4P_2O_7$-HCl, *p*H 8, 3 m*M* $MgCl_2$, and 1 m*M* dithiothreitol by filtering the suspension through one layer of Miracloth into sufficient sucrose, $MgCl_2$, and dithiothreitol to make the final solution 10 percent sucrose (by weight), 10 m*M* $MgCl_2$, and 1 m*M* dithiothreitol. Centrifugation of the resulting suspension at 4100*g* for 15 minutes at 4°C gave a green pellet and a supernatant containing many vacuoles (*7*). The pellet was resuspended in 15 percent sucrose (by weight), 5 m*M* tris-MES, *p*H 8, 10 m*M* $MgCl_2$, and 1 m*M* dithiothreitol, and layered on a discontinuous sucrose gradient consisting of zones of 20, 30, 40, 50, and 55 percent sucrose (by weight) all containing buffer, $MgCl_2$, and dithiothreitol in the same concentrations used in the resuspending medium. Centrifugation at 8000*g* for 20 minutes at 4°C in a swinging bucket rotor resulted in a single dark green band of chloroplasts at the interface of 40 and 50 percent sucrose. These chloroplasts were highly refractile under the phase contrast microscope. The only other green material in the gradient was a small faint green band located at the 30 to 40 percent interface and consisting primarily of nonrefractile chloroplasts. No pellet was observed.

The integrity and photochemical activity of the high-density chloroplasts were measured with an oxygen electrode. Chloroplasts recovered from the 40 to 50 percent interface of the sucrose gradient were resuspended in a medium containing 1.06*M* sucrose, 5 m*M* tris-MES, *p*H 8, 10 m*M* $MgCl_2$, 0.25 m*M* ferricyanide, and 2.5 μg of gramicidin D per milliliter; they consumed O_2 in the light at a rate of 242 microequivalents of O_2 per milligram of chlorophyll per hour. Those swollen in 0.27*M* sucrose, 50 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), *p*H 7.5, 2 m*M* $MgCl_2$, 20 m*M* NaCl, 0.25 m*M* ferricyanide, and 2.5 μg of gramicidin D per milliliter evolved O_2 in the light at a rate of 900 μeq of O_2 per milligram of chlorophyll per hour.

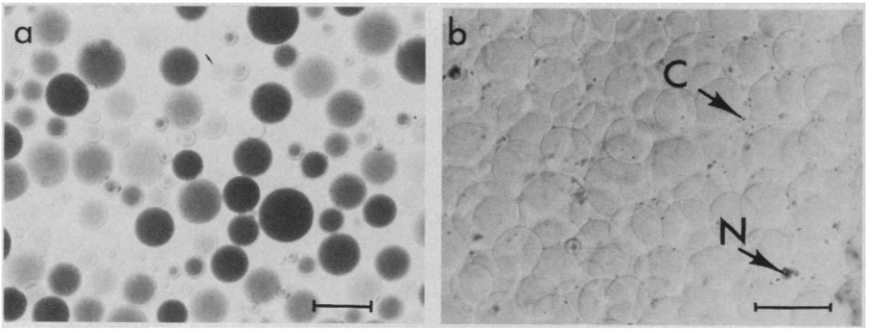

Fig. 2. (a) Mixture of vacuoles from protoplasts of two colored *Tulipa* species after sedimentation from 0.2*M* K_2HPO_4-HCl, *p*H 8. Scale bar, ~ 90 μm. (b) Vacuoles from protoplasts of *Tulipa* leaves after sedimentation from 0.2*M* K_2HPO_4-HCl, *p*H 8. Chloroplasts (*C*) and traces of nuclei (*N*) are present. Scale bar, ~ 80 μm.

The inability of unswollen chloroplasts to reduce ferricyanide in the light and their appearance under the phase contrast microscope suggested that they were type A chloroplasts (*8*). The high rate of O_2 evolution in the light for swollen chloroplasts indicated that their photochemical activity was maintained.

GEORGE J. WAGNER
H. W. SIEGELMAN

Biology Department,
Brookhaven National Laboratory,
Upton, New York 11973

References and Notes

1. R. Buvat, in *Origin and Continuity of Cell Organelles*, J. Reinert and H. Ursprung, Eds. (Springer-Verlag, New York, 1971), pp. 127–157.
2. E. C. Cocking, *Nature (London)* **187**, 962 (1960); D. W. Gregory and E. C. Cocking, *J. Exp. Bot.* **17**, 57 (1966).
3. P. Matile and A. Wiemken, in *Methods in Enzymology*; S. Fleischer and L. Packer, Eds. (Academic Press, New York, 1974), vol. 31, part A, p. 572.
4. K. L. Indge, *J. Gen. Microbiol.* **51**, 441 (1968); K. D. Nakamura and F. Schlenk, *J. Bacteriol.* **120**, 482 (1974).
5. T. Galliard, in *Methods in Enzymology*, S. Fleischer and L. Packer, Eds. (Academic Press, New York, 1974), vol. 31, part A, p. 520; W. D. Loomis, in *ibid.*, p. 528.
6. The enzyme preparation used to prepare protoplasts was recycled by dialyzing against distilled water for 2 hours at 4°C in VisKing cellulose casing pretreated by boiling in 1 percent Na_2HCO_3 for 10 minutes [H. Taniuchi and C. B. Anfinsen, *J. Biol. Chem.* **241**, 4366 (1966)]. The casing was changed after 1 hour of dialysis and the final retentate was lyophilized. This procedure removed approximately 85 to 90 percent of the mannitol. Recycling the enzyme twice did not substantially reduce protoplast-producing ability.
7. This procedure may be useful for preparing chloroplast-free vacuoles.
8. D. O. Hall, *Nature New Biol.* **235**, 125 (1972).
9. We especially wish to thank G. Hind for his assistance in the chloroplast studies and for useful criticism and suggestions on preparing the manuscript. We thank R. Evans for his help in part of this work; J. Tilley, H. Nakatani, and B. Peltz for their technical assistance; and M. Bull for assistance with microphotography. Research was carried out at the Brookhaven National Laboratory under the auspices of the Energy Research and Development Administration.

3 July 1975; revised 6 August 1975