Plant Physiol. (1978) 61, 154-157

Presence of the Cyanogenic Glucoside Dhurrin in Isolated Vacuoles from *Sorghum*[1]

Received for publication July 28, 1977 and in revised form October 4, 1977

JAMES A. SAUNDERS[2] AND ERIC E. CONN
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

Large numbers of vacuoles (10^6-10^7) have been isolated from *Sorghum bicolor* protoplasts and analyzed for the cyanogenic glucoside dhurrin. Leaves from light-grown seedlings were incubated for 4 hours in 1.5% cellulysin and 0.5% macerase to yield mesophyll protoplasts which then were recovered by centrifugation, quantitated by a hemocytometer, and assayed for cyanogenic glucosides. Mature vacuoles, released from the protoplasts by osmotic shock, were purified on a discontinuous Ficoll gradient and monitored for intactness by their ability to maintain a slightly acid interior while suspended in an alkaline buffer as indicated by neutral red stain. Cyanide analysis of the protoplasts and the vacuoles obtained there from yielded equivalent values of 11 μmoles of cyanogenic glucoside dhurrin per 10^7 protoplasts or 10^7 vacuoles. This work supports an earlier study from this laboratory which demonstrated that the vacuole is the site of accumulation of the cyanogenic glucoside in *Sorghum*.

The isolation of organelles may provide a convenient procedure for investigating the localization of secondary plant products. Until recently, however, the largest organelle in plant cells, the vacuole, has been available for study only in small numbers (4), in an immature form (10), or from unicellular organisms (6, 11). Wagner and Siegelman (13) and Lörz *et al.* (8) have described a technique for the isolation of large numbers of vacuoles which works well in petals of several species and the leaves of a few. However, the procedure requires long periods of incubation (12-24 hr) of the tissue involved in a digesting solution for the release of the protoplasts. Moreover, the procedure as described was not effective in obtaining large numbers of vacuoles from *Sorghum*. Leigh and Branton (7) have circumvented the long incubation of the tissue by using a mechanical slicer for the isolation of vacuoles. This technique works effectively on firmer tissues such as red beet root tissue; however, their technique would have limited use on leaf tissue.

This paper describes a technique which combines a relatively short incubation period (4 hr) for preparation of the protoplasts with a Ficoll discontinuous gradient to produce large numbers of vacuoles. The technique has been utilized to study the localization of the cyanogenic glucoside of *Sorghum*.

MATERIALS AND METHODS

Plant Material. Six-day-old light-grown shoots of *Sorghum bicolor* (Linn) Moench, variety Redland × Greenleaf, were harvested 1 cm above the caryopsis after germination at 24 C on water-saturated vermiculite under a 14-hr photoperiod.

Isolation of Vacuoles. The expanded leaves of approximately 3 g of tissue were excised above the caryopsis, abraded with 150 grit carborundum using a small artist brush after the technique of Beier and Bruening (2), rinsed in distilled H_2O, and incubated for 4 hr in 36 ml of 0.5 M mannitol, 25 mM K-phosphate-citrate buffer (pH 5.5), containing 5 mg/ml macerase and 15 mg/ml cellulysin. The incubation was carried out at 37 C in a shaking water bath at 18 oscillations/min. The partially digested leaves were filtered through one layer of cheesecloth, rinsed with 10 ml of cold 0.5 M mannitol in 25 mM K-phosphate-citrate buffer (pH 5.5), and the protoplasts released were harvested by centrifuging the combined supernatants for 3 min at 500*g* at 4 C in a HB-4 swinging bucket rotor of a Sorval RC2-B refrigerated centrifuge. The pellet was resuspended in 25 mM Tris-HCl buffer (pH 8) containing 0.4 M mannitol and a sample of the suspension was taken for counting of the protoplasts and analysis. Vacuoles were released from the protoplast preparation by diluting the medium to 0.15 M mannitol with 25 mM Tris-HCl (pH 8) containing 10 mg/ml BSA. This suspension was pipetted several times to facilitate lysis of the protoplasts and the solution was loaded onto the top of a discontinuous Ficoll 400 gradient composed of 7.5-ml fractions of 3, 9, 12.5, and 20% (w/w) Ficoll containing 0.5 M mannitol, and 25 mM Tris-HCl buffer (pH 8). The gradient was centrifuged for 2 hr at 26,000 rpm in a Beckman SW 27 rotor using a Sorval OTD-50 ultracentrifuge at 4 C. The vacuolar band which accumulated at the 3/9% (w/w) Ficoll interface was collected using a Büchler auto-density flow gradient fractionator.

The vacuoles and protoplasts were counted using an AO Spencer Bright Line hemocytometer. This technique was facilitated when appropriate, by staining the preparation with neutral red. Vacuoles could be concentrated in the final band by dilution of the fraction with 0.4 M mannitol in 25 mM Tris-HCl buffer (pH 8) and centrifuged at 3 min for 1,000*g* in a HB-4 swinging bucket rotor.

Chemicals. Macerase and cellulysin were obtained from Calbiochem, BSA from Sigma, Ficoll 400 from Pharmacia, and carborundum 150 grit from Fisher Chemical Co. All other chemicals were of reagent grade or better.

Assays. Chl was measured by the technique of Arnon (1).

Dhurrin in the isolated protoplast and vacuolar preparations was hydrolyzed by the addition of 0.8 mg of almond emulsin/sample and the HCN released was then determined by the spectrophotometric method of Epstein (5) as described earlier (12).

RESULTS

Protoplasts isolated by the procedure described under "Materials and Methods" assume a spherical outline which consists of one central vacuole surrounded by the chloroplasts and the remainder of the cytoplasm (Fig. 1A). As the concentration of

[1] This work was supported in part by National Science Foundation Grant BMS 74-11997-A01 and United States Public Health Service Grant GM 05301-19 to E.E.C.

[2] Present address: Tobacco Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, Md. 20705.

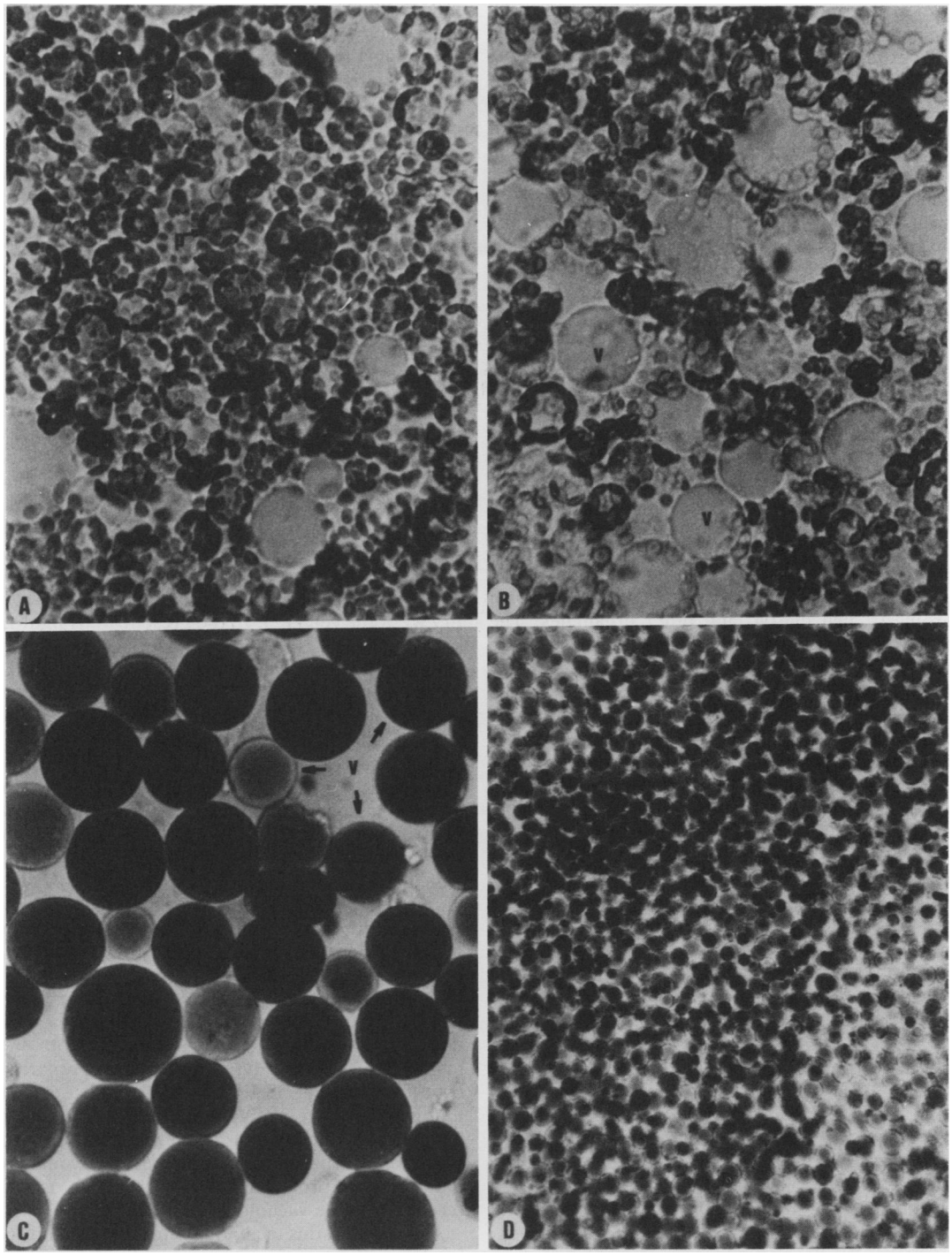

FIG. 1. A: Photomicrograph depicting a typical protoplast suspension prepared by incubation of the abraded *Sorghum* leaf tissue in the digesting medium for 4 hr. The protoplasts assume a spherical outline indicative of the loss of the cell wall (× 420). B: As the mannitol concentration is decreased from 0.4 M to 0.15 M, a single large vacuole emerges through the ruptured plasmalemma. The vacuole is often two to three times the diameter of the parent protoplast (× 420). C: After a 2-hr centrifugation of the lysed protoplasts at 100,000*g* in a discontinuous Ficoll gradient, the vacuoles equilibrate at the 3/9% (w/w) Ficoll interface. There is a conspicuous absence of nonvacuolar membranes adhering to the tonoplast (× 420). D: Large numbers of vacuoles can be obtained using the described technique with little contamination by cells or other organelles (× 85).

the mannitol in the medium was lowered the plasmalemma ruptured and a single vacuole was seen to emerge from the lysed protoplast (Fig. 1B). If the preparations were stained with neutral red at this point, the dye was first taken up by the liberated vacuoles and subsequently observed in the vacuoles of the unlysed protoplasts. Upon emergence from the protoplasts, which ranged from 15 to 20 μm, the vacuoles expanded in diameter from 30 to 60 μm.

Figure 1,C and D represents a typical vacuolar preparation obtained at the 3/9% (w/w) Ficoll interface (which has been stained with neutral red) and suspended in 25 mM Tris-HCl buffer (pH 8) containing 0.4 M mannitol. The vacuoles were visible because of their uptake of the neutral red. At this pH, the neutral red represents a vital stain (14) and easily distinguishes the vacuole from other cellular components. Very little cytoplasmic adherence is seen on the tonoplast membrane.

After centrifugation the vacuoles ranged in size from 30 to 60 μm and were shown to swell and contract, respectively, in hypotonic and hypertonic media.

The amount of dhurrin in the protoplast preparation was compared with that in the vacuoles isolated from those protoplasts by measuring the HCN released upon the addition of almond β-glucosidase (Table I). The results, which are expressed as μmol of HCN/10^7 organelles, indicate that all of the cyanogenic glucoside in the protoplasts is located in the vacuoles. This conclusion is based on the assumption that one vacuole is obtained per protoplast under our conditions. This assumption is supported through microscopic observation of protoplasts stained with neutral red which show one staining body, and through the additional observation in this study, supported by Indge (6), that one vacuole emerges from one protoplast. Butcher *et al.* (3) have also made this assumption in studying the amount of acid phosphatase in vacuoles isolated from *Hippeastrum* petals.

Table II presents data on the extent of contamination of the vacuolar preparation by Chl, and the ratio of HCN (released from dhurrin) to Chl in different fractions obtained by centrifugation on a discontinuous Ficoll gradient. Examination of the interface bands showed vacuoles equilibrating at the 3/9% (w/w) interface, a mixture of vacuoles and protoplasts at the 9/12.5% (w/w) Ficoll interface, a large band of protoplasts at the 12.5/20% (w/w) Ficoll interface, and a large pellet which contained chloroplasts, cell debris, and other organelles. The ratio of HCN to Chl increases more than 16-fold from the 12.5/20 protoplast interface to the 3/9 vacuole band. These data indicate that the cyanogenic glucoside which is recovered at the vacuolar band is not associated with unlysed protoplasts which may contaminate the vacuolar preparation to a small degree.

The yield of intact vacuoles recovered from the 3/9% (w/w) Ficoll interface ranged between 5 and 10% based on the number of protoplasts before lysis. The lysed protoplast preparation contains a significant quantity of low mol wt compounds (including the hydrolysis products of the cyanogenic glucoside), which are normally in the vacuolar sap but are released when the vacuole is burst. Compounds of this size did not enter the gradient whether the lysed preparation was loaded onto the top of the 3% (w/w) Ficoll or if the preparation was resuspended in 25% (w/w) Ficoll and layered at the bottom of the gradient. In either case the vacuoles banded at the same 3/9% (w/w) Ficoll interface of the gradient and had equivalent levels of cyanogenic glucosides associated with them.

During the initial studies on the osmotic lysis of the protoplasts, cytoplasmic membranes could occasionally be observed adhering to the exterior of the tonoplast as the vacuole was extruded from the cell. Since dhurrin is soluble, the adherence of these membranes to the tonoplast did not represent a serious contamination of the preparation by the cyanogenic glucoside. However, the application of this technique to enzyme localization studies would be seriously hampered by the adhering membranes. When BSA in a final concentration of 5 to 8 mg/ml was included in the lysing media, the aggregation of nonvacuolar elements on the tonoplast could be largely prevented (Fig. 1C).

TABLE I

Quantitation of HCN in Vacuoles and Protoplasts of Sorghum

Source	HCN/10^7 cells of vacuoles	Standard Deviation	Dhurrin in Protoplasts
	μmol		%
Protoplasts	10.9	(1.9)	100
Vacuoles	11.3	(4.5)	103

Protoplasts were isolated from Sorghum shoots by 4 hr incubation in 1.5% cellulysin and 0.5% macerase at 37 C and counted with a hemocytometer. The protoplasts were then lysed and the vacuoles recovered as described in Materials and Methods. The μmol of the HCN measured in 10^7 protoplasts or 10^7 vacuoles is shown as the average of 10 separate experiments with three replicates each.

TABLE II

Analysis of Chlorophyll and HCN in Ficoll Gradients

Ficoll Interface	Component	Chlorophyll	HCN	HCN/Chlorophyll
		μg	μmol	Ratio
3/9	Vacuoles	9	172.5	16.4
9/12.5	Vacuoles & Protoplasts	14.5	44.3	2.6
12.5/20	Protoplasts	169	197.4	1.0

A protoplast preparation which was osmotically shocked by lowering the mannitol concentration from 0.4 M to 0.15 M, was layered onto the top of a discontinuous Ficoll gradient complex of: 3, 9, 12.5 and 20% (w/w) Ficoll. After the gradient was centrifuged at 100,000 x g for 2 hr, the vacuolar band (3/9 interface), a mixture of vacuoles and protoplast (9/12.5 interface), and the protoplast band (12.5/20 interface) were collected and analyzed for chlorophyll and HCN.

During the initial studies on the osmotic lysis of the protoplasts, cytoplasmic membranes could occasionally be observed adhering to the exterior of the tonoplast as the vacuole was extruded from the cell. Since dhurrin is soluble, the adherence of these membranes to the tonoplast did not represent a serious contamination of the preparation by the cyanogenic glucoside. However, the application of this technique to enzyme localization studies would be seriously hampered by the adhering membranes. When BSA in a final concentration of 5 to 8 mg/ml was included in the lysing media, the aggregation of nonvacuolar elements on the tonoplast could be largely prevented (Fig. 1C).

DISCUSSION

The isolation of organelles has been an important method for investigations of the localization of natural products in plant cells. Until recently, however (13), the largest organelle of the plant cell, the vacuole, has not been available in sufficient numbers and/or in a mature form for studies on the variety of natural products that are thought to be associated with the vacuole.

Dark-grown *Sorghum* seedlings contain high levels of the cyanogenic glucoside dhurrin, approximately 25 to 30% of the dry wt of the cell (12). In the present study light-grown *Sorghum* seedlings were used as the expanded leaf blade facilitated the isolation of the vacuoles. Using a microautoradiographic technique, Saunders *et al.* (12) have concluded that 90% of the cyanogenic glucoside in *Sorghum* is located in the vacuoles of such seedlings. As *Sorghum* also contains active β-glucosidases (9), it is necessary to postulate a spatial separation between the cyanogenic glucoside and those enzymes (12). No β-glucosidase activity has been recovered in the vacuoles of *Sorghum* in the present study nor from vacuoles isolated from *Hippeastrum* flower petals (3). The present paper describes a procedure by which large numbers (10^6) of mature vacuoles are isolated from mesophyll protoplasts of light-grown *Sorghum* seedlings and confirms the earlier conclusion that the vacuole is an important site for the concentration of dhurrin.

A serious problem in the preparation of vacuoles from protoplasts has been the adherence of cytoplasmic membranes to the exterior of the tonoplast. The results presented herein show that this problem can be largely prevented by the use of 5 to 8 mg/ml BSA in the lysed protoplast preparation. The high concentration of BSA apparently coats the exterior to the tonoplast thus preventing nonspecific adherence by cytoplasmic membranes. For this reason the quantitation of the cyanogenic glucoside was based on the number of vacuoles recovered from the isolation procedure rather than the amount of protein recovered in the vacuole preparation.

In summary, this technique represents an improved procedure for the isolation of mature vacuoles from plant tissue which does not lend itself well to other techniques of slicing (7) or extended digestion periods (8, 13, 14). Vacuoles isolated by this technique are suitable for studies on the constituents of the vacuolar sap or biochemical investigations of the vacuolar membrane. The present investigation has demonstrated that the vacuole represents the major site for the accumulation of the cyanogenic glucoside dhurrin in *Sorghum* seedlings.

LITERATURE CITED

1. Arnon DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1-15
2. Beier H, G Bruening 1975 The use of an abrasive in the isolation of cowpea leaf protoplasts which support the multiplication of cowpea mosaic virus. Virology 64: 272-276

3. Butcher HC, GJ Wagner, HW Siegelman 1977 Localization of acid hydrolases in protoplasts. Examination of the proposed lysosomal function of the mature vacuole. Plant Physiol 59: 1098-1103
4. Cocking EC 1960 A method for the isolation of plant protoplasts and vacuoles. Nature 187: 962-963
5. Epstein J 1947 Estimation of microquantities of cyanide. Anal Chem 19: 272-274
6. Indge KJ 1968 The isolation and properties of the yeast cell vacuole. J Gen Microbiol 51: 441-446
7. Leigh RA, D Branton 1976 Isolation of vacuoles from root storage tissue of *Beta vulgaris* L. Plant Physiol 58: 656-662
8. Lörz H, CT Harms, I Potrykus 1976 Isolation of vacuoplasts from protoplasts of higher plants. Biochem Physiol Pflanzen 169: 617-620
9. Mao CH, L Anderson 1967 Cyanogenesis in *Sorghum vulgare*. III. Partial purification and characterization of two β-glucosidases from *Sorghum* tissues. Phytochemistry 6: 473-483
10. Matile P 1968 Lysosomes of root tip cells in corn seedlings. Planta 79: 181-196
11. Nakamura KD 1973 The isolation of vacuoles rom *Candida utilis*. Prep Biochem 3(6): 553-561
12. Saunders JA, EE Conn, CH Lin, CR Stocking 1977 Subcellular localization of the cyanogenic glucoside of *Sorghum* by autoradiography. Plant Physiol 59: 647-652
13. Wagner GJ, HW Siegelman 1975 Large-scale isolation of intact vacuoles and isolation of chloroplasts from protoplasts of mature plant tissues. Science 190: 1298-1299
14. Zirkle C 1937 The plant vacuole. Bot Rev 3: 1-30