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Subcellular Localization of a UDP-Glucose:Aldehyde Cyanohydrin β-Glucosyl Transferase in Epidermal Plastids of *Sorghum* Leaf Blades[1]

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ABSTRACT

Epidermal and mesophyll protoplasts, prepared from leaf blades of 6-day-old light-grown *Sorghum bicolor* seedlings were separated by differential sedimentation and assayed for a number of enzymes. The epidermal protoplasts contained higher levels of NADPH-cytochrome *c* reductase (EC 1.6.2.4), triose phosphate isomerase (EC 5.3.1.1), phosphoenolpyruvate carboxylase (EC 4.1.1.31), and a UDP-glucose:cyanohydrin β-glucosyl transferase (EC 2.4.1.85), but lower levels of $NADP^+$ triosephosphate dehydrogenase (EC 1.2.1.13) than did mesophyll protoplasts. When protoplast preparations were lysed and applied to linear sucrose density gradients, triosephosphate isomerase was found to be present in epidermal plastids. A significant fraction (41%) of the glucosyl transferase activity was also associated with the epidermal plastids.

In etiolated leaf blades of *Sorghum* seedlings, tyrosine is converted to *p*-hydroxy-[*S*]-mandelonitrile by a series of membrane-associated steps (Fig. 1) (4, 15). This cyanohydrin is then glucosylated by a soluble UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase to form the cyanogenic glucoside dhurrin (22). Dhurrin is enzymically degraded by dhurrin-β-glucosidase and hydroxynitrile lyase which are also present (but not in contact with the dhurrin) in *Sorghum* leaf blades (12, 14).

Recent work with isolated epidermal and mesophyll protoplasts and bundle sheath strands has provided a better understanding of the compartmentalization involved in the storage, synthesis, and degradation of dhurrin. Dhurrin is found predominantly in the vacuoles of epidermal cells (12, 24), while the mesophyll cells contain the catabolic enzymes dhurrin-β-glucosidase and hydroxynitrile lyase (12); these enzymes are located in the chloroplasts and cytosol, respectively (26). The biosynthetic enzyme UDP-glucose:aldehyde cyanohydrin glucosyl transferase, occurs predominantly in the epidermal cells (12).

Epidermal cells are of increasing interest because of accumulating evidence showing their ability to sequester metabolites, including secondary (natural) products (*e.g.* 12, 25). However, few quantitative studies have been made of the enzymes of epidermal cells and those which exist are concerned with primary metabolism including photosynthesis (3, 18, 19, 30). Although the production of protoplasts from epidermal tissue was reported several years ago (5), we are unaware of any attempts to examine subcellular localization of enzymes in epidermal cells. For this reason, we have prepared sucrose density gradients from epidermal-enriched protoplasts and examined the subcellular localization of the UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase contained in such protoplasts.

MATERIALS AND METHODS

Chemicals. UDP-[U-^{14}C]glucose (NH_4 salt) (specific activity = 288 mCi/mmol) was obtained from ICN. Unlabeled UDP-glucose and other biochemicals were purchased from Sigma. *p*-Hydroxy-(*R,S*)-mandelonitrile was synthesized by the method of Ladenburg *et al.* (13). Cellulysin was obtained from Calbiochem. β-D-Glucosylamine was prepared by the method of Isbell and Frush (10). The 44-μm mesh nylon net was purchased from Cistron, Lebanon, PA, and the 20-μm mesh nylon net was from Tetko Inc., Elmsford, NY.

Buffers. The Mes-mannitol buffer consisted of 30 mM Mes (pH 5.6) in 0.6 M mannitol; the breaking buffer contained 20 mM Tricine-NaOH (pH 7.5), 0.5% BSA (w/v), and 1% PVP 40 (w/v) in 0.6 M mannitol. The gradient buffer was identical to the breaking buffer, except that the mannitol was omitted.

Plant Material. Seeds of *Sorghum bicolor* (Linn.) Moench, var Redland X Greenleaf, were soaked in aerated water for 24 h at room temperature. They were then rinsed and allowed to germinate in water-saturated vermiculite under a 16:8 h (light:dark) regime. During the light period, fluorescent light (1,700 ft-c) was used at 26.5°C; in the dark, the temperature was 22°C. Leaf blades were harvested 2 to 8 h after beginning of the light period. After 6 d, the *Sorghum* shoots were 4 to 7 cm tall, with two expanded leaves.

Preparation of Protoplasts. Protoplasts from abraded (2) *Sorghum* leaf blades (4–8 g) were obtained using a modification of the method of Kojima *et al.* (12). A solution of 1.5% (w/v) Cellulysin in Mes-mannitol buffer was used as the digestion buffer. The protoplasts were harvested after 1.5 to 2 h at 30°C. After filtration through a 44-μm nylon net, the protoplasts were pelleted (220*g*, 3 min), washed with Mes-mannitol buffer, and pelleted again, essentially as described previously (12).

One drop of neutral red dye solution was added to 25 ml of Mes-mannitol buffer used for the washing (12) in order to stain the epidermal, and some of the mesophyll, vacuoles. For the experiments described herein, neutral red was added at a concentration just sufficient to lightly stain the epidermal vacuoles. Addition of higher concentrations of the dye resulted in the visualization of round, deep-red colored spherical or elliptical bodies several μm in diameter within the epidermal vacuoles; these bodies may reflect subcompartmentation within the vacuole (*cf.* 20).

Purification of Mesophyll and Epidermal Protoplasts. The protoplast pellet, containing 5×10^6 to 5×10^7 protoplasts consisting

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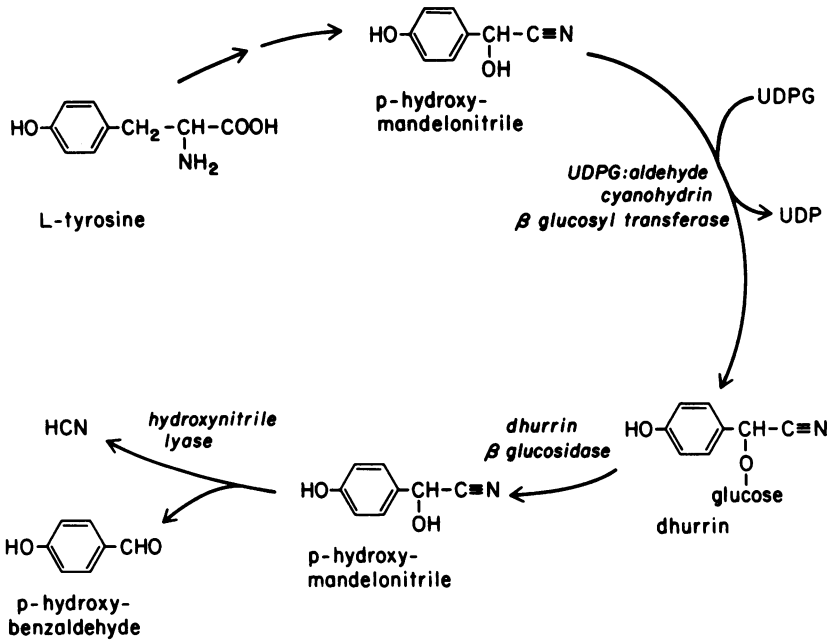

FIG. 1. Dhurrin metabolism in *Sorghum* seedlings.

of approximately 15% epidermal protoplasts and 85% mesophyll protoplasts, was resuspended in 5 ml Mes-mannitol buffer in a 30-ml test tube, and allowed to settle for approximately 35 min. During this time, the epidermal protoplasts preferentially settled out so that a layer of variable thickness consisting of about 20% epidermal protoplasts collected at the bottom of the tube. The top ml of supernatant, containing predominantly mesophyll protoplasts and fragments from broken cells, was discarded. The rest of the supernatant, which consisted of about 95% mesophyll protoplasts and 5% epidermal protoplasts, was used to obtain the mesophyll-enriched protoplast preparation. The settled protoplasts were used as the source of the epidermal-enriched preparation. Wide-lipped pipettes were used for all resuspensions and transferrals to avoid breakage of protoplasts due to excessive shearing force. After the initial settling, protoplasts were transferred to conical 12-ml glass centrifuge tubes which were not scratched.

After transfer, the mesophyll-enriched supernatant was allowed to stand for 20 min. The supernatant was then removed and the protoplasts which settled out were discarded. This process was repeated (two to four times) until the supernatant was 98 to 99.5% mesophyll protoplasts.

The partially epidermal-enriched preparation was resuspended in 2 ml Mes-mannitol buffer, and the mixture was allowed to stand for 25 min. The supernatant was discarded, and the cells in the middle of the settled layer, which was enriched in epidermal cells, were carefully removed and resuspended in 2 ml Mes-mannitol. This process was repeated usually five times, until the epidermal-enriched resuspension was 70 to 85% epidermal cells.

Gradients. Aliquots of the epidermal- and mesophyll-enriched preparations were examined microscopically for intactness, and the epidermal and mesophyll cells were counted. Each protoplast preparation was pelleted (270*g*, 2 min) and resuspended (0–4°C) in 1 to 3 ml breaking buffer. Protoplasts ($1–5 \times 10^5$) were ruptured by passage through a syringe covered with a 2-μm mesh nylon net and layered onto a 40-ml linear sucrose density gradient (30–55% sucrose, w/w) in gradient buffer. Gradients were centrifuged 30 min to a maximum speed of 100,000*g* in a SW27 rotor, and 1.3- or 2-ml fractions were collected.

Assays. Gradient fractions and/or epidermal- or mesophyll-enriched protoplast preparations were analyzed for triose-P dehydrogenase (6), PEP[2] carboxylase (27), and NADPH Cyt *c* reductase (29). Mesophyll-enriched preparations were analyzed for Chl (1); the neutral red in the epidermal vacuoles interfered with the Chl determination for the epidermal-enriched preparations.

The UDPG:aldehyde cyanohydrin β-glucosyl transferase was analyzed by measuring the formation of dhurrin from UDP-[^{14}C] glucose and *p*-hydroxy[*R,S*]-mandelonitrile using a modified procedure of Kojima *et al.* (12). For the incubations with mesophyll or epidermal protoplasts as the enzyme source, about 0.4 to 1×10^5 protoplasts and 1.2 μmol of UDP-glucose were used for each incubation. For analysis of the UDP-glucose transferase activity in the gradient fractions, 200 μl of each fraction and 6.9 nmol of UDP-glucose were used for each incubation. In each case, reaction mixtures contained 1 μCi UDP-[^{14}C]glucose, 0.5% BSA (w/v), 2 mM DTT, and 10 mM β-D-glucosylamine (a dhurrinase inhibitor) in 0.1 M Tricine (pH 8) in a total volume of 210 μl.

After 30 min incubation at 30°C with shaking, the reactions were terminated by addition of 50 μl of 20% acetic acid. Unlabeled dhurrin was then added, and the reaction mixtures were centrifuged for 8 min at 1,000*g*. The supernatants were applied directly to chromatography paper (4 mm thick Whatman No. 1). Alternately, for assays with gradient fractions, the supernatants were lyophylized and extracted (twice) with 500 μl 95% ethanol to separate the dhurrin from the sucrose; the ethanol extract was then applied to the chromatography paper.

The paper was developed in 2-butanone:acetone:water (15:5:3, v/v/v). The dhurrin was visualized by a modification of the method of Menzies *et al.* (16) using, sequentially, sprays of $AgNO_3$ (0.6% [w/v] in acetone), NaOH (0.5 N in 95% ethanol), and $Na_2S_2O_3$ (20% [w/v] aqueous) which stain dhurrin (R_F 0.76) bright pink and sucrose, glucose, UDP-glucose, or other glycosides brown. The papers were radio-scanned, and the peak of radioactivity corresponding to the dhurrin standard was counted by liquid scintillation.

Microscopic Examination of Protoplasts. Mesophyll and epidermal cells in each preparation were counted using an AO Spencer bright line hemocytometer as described previously (12).

[2] Abbreviation: PEP, phosphoenolpyruvate.

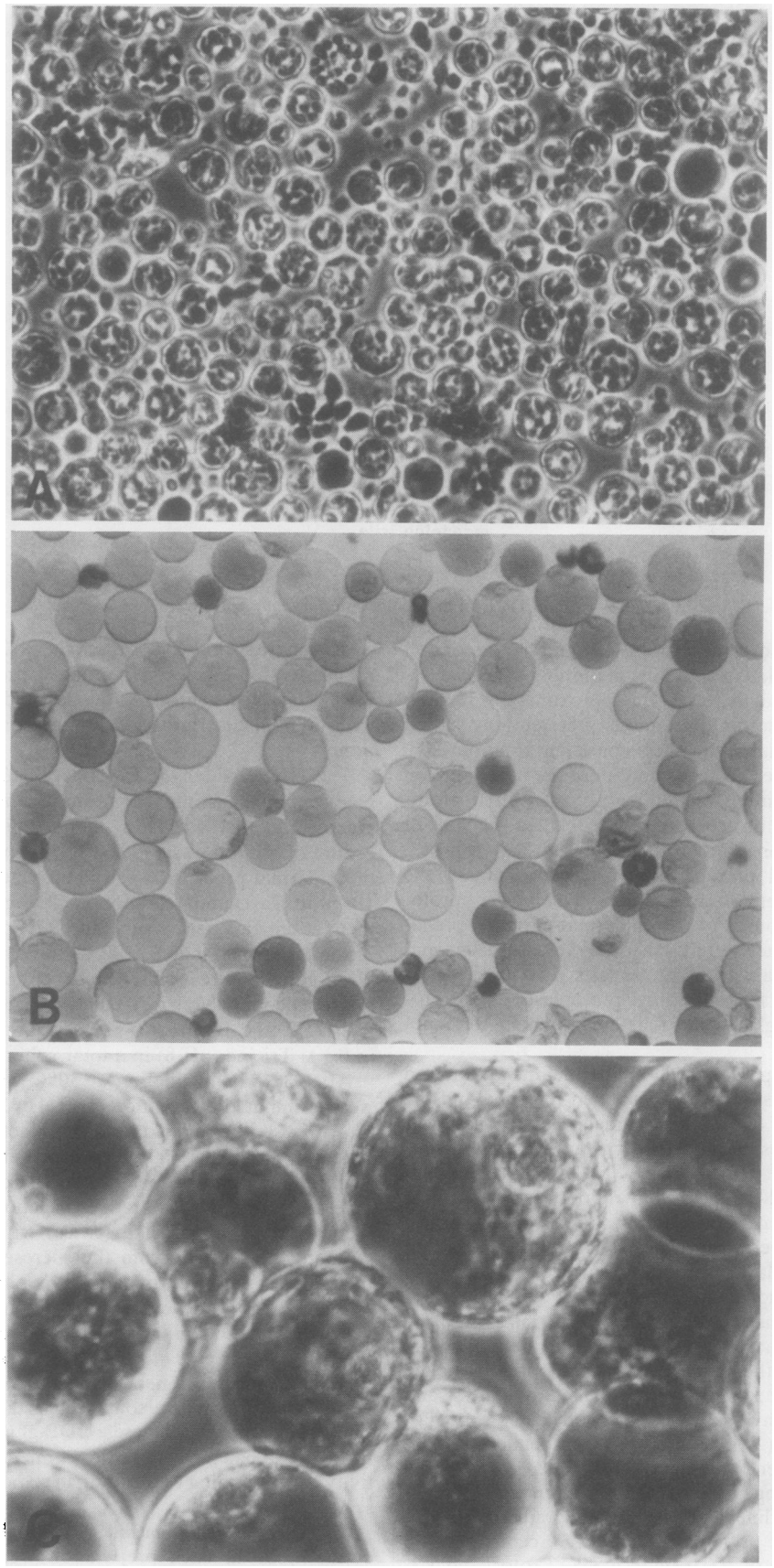

FIG. 2. Protoplasts from *Sorghum* seedling shoots. A, Phase contrast of mesophyll preparation (× 540). B, Epidermal preparation (× 540). C, Phase contrast of epidermal preparation; nuclei and organelles are clearly visible (× 2,160).

The intactness of the chloroplasts in the ruptured protoplast preparations was examined under phase-contrast (× 450).

RESULTS

Microscopic Examination of Protoplast and Chloroplast. The mesophyll-enriched protoplasts (Fig. 2A) contained 98.0 to 99.5% mesophyll protoplasts, with 2.0 to 0.5% contamination by epidermal protoplasts. (Guard cells were rarely observed in this or the epidermal-enriched preparation.) Microscopic analysis revealed little protoplast breakage, as indicated by the scarceness of free chloroplasts. A minimum of 1×10^6 mesophyll protoplasts were obtained from each g fresh weight of tissue digested.

The epidermal-enriched protoplasts (Fig. 2B) contained 70 to 85% epidermal protoplasts, with mesophyll protoplasts representing the balance. The majority of the epidermal protoplasts were intact as evidenced by observation of the protoplasts under high-power phase contrast (Fig. 2C). Each g fresh weight of tissue yielded from 5×10^4 to 1×10^5 epidermal protoplasts after purification.

After mechanical rupture of the protoplasts by passage through a 20-μm nylon net until about 70% were broken, most chloroplasts were intact as observed under phase contrast.

Comparison of Enzyme Activities in Epidermal and Mesophyll Cells. The enriched epidermal and mesophyll preparations were assayed for UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase as well as for several enzymes of primary metabolism which had potential for use as organelle markers. The results, corrected for cross contamination, are presented in Table I. Bundle sheath preparations were not included in this study, as it had previously been shown (12) they were lacking in glucosyl transferase activity. Epidermal protoplasts contained much higher activities, per cell, of NADPH-Cyt *c* reductase, PEP carboxylase, and UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase per cell, but lower NADP-triose-P dehydrogenase activity, than did mesophyll protoplasts. Levels of triose-P isomerase were similar in both cell types.

Cytological examination has shown that *Sorghum* seedling leaf blades contain approximately 8 times as many mesophyll cells as epidermal cells (12). When this factor of 8 is taken into account, it can be calculated (Table I) that two of the enzymes studied, UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase and NADPH Cyt *c* reductase, predominate in the epidermis, relative to the mesophyll tissue.

Gradient Studies. In preliminary experiments, lysed, unpurified mixtures of epidermal and mesophyll protoplasts were applied to sucrose density gradients, and fractions were assayed for UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase, NADP triose-P dehydrogenase (a chloroplast marker), and Chl. From 25 to 35% of the glucosyl transferase activity applied to the gradient was recovered in those gradient fractions containing both NADP-triose-P dehydrogenase activity and Chl.

Since this indicated that the UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase was localized in the plastids, and because the majority of this activity was in epidermal protoplasts, it was decided to apply epidermal-enriched preparations and mesophyll-enriched preparations separately to linear sucrose density gradients. Short (30 min) centrifugations were used both to decrease plastid breakage, and because with short centrifugations, chloroplasts and etioplasts migrate to the middle portion of the gradient while all of the other organelles remain in the first few gradient fractions (26). It was expected that the epidermal plastids would behave similarly to chloroplasts and etioplasts and also migrate towards the middle of the gradient. Because no Chl and little NADP triose-P dehydrogenase activity (Table I) are present in epidermal cells, these could not be used as markers for epidermal plastids. It was decided to test triose-P isomerase, which is present in epidermal cells at high levels (Table I), as a possible marker for epidermal plastids in the gradient. Since triose-P isomerase has been found in chloroplasts, root and endosperm plastids, and shoot etioplasts, as well as in the cytoplasm of the cells from which these plastids were obtained (17), the enzyme could probably be used as a marker for intact epidermal plastids in the gradient, but not as a measure of the number of plastids which have broken during isolation.

Equal numbers of protoplasts of epidermal- and mesophyll-enriched preparations were applied to separate gradients, centrifuged, fractionated, and assayed. The percent recoveries of Chl, triose-P isomerase, and glucosyl transferase applied to the gradients are shown in Table II. The peak density of the mesophyll chloroplasts, indicated by the coincident location of Chl and triose-P isomerase activity on gradients from mesophyll-enriched protoplasts, was 1.203g/cm^3 (Fig. 3). The epidermal-enriched gradients had triose phosphate isomerase activity at a peak density of 1.201g/cm^3. It was concluded that the epidermal plastids contained triose-P isomerase activity, and that the epidermal plastid peak density (1.201g/cm^3) was slightly less than that of the chloroplasts (1.203g/cm^3). The slight shoulder on the triose-P isomerase activity of the epidermal gradient at 1.203g/cm^3 is probably due to triose-P isomerase activity in contaminating chloroplasts.

Gradient fractions were also assayed for the glucosyl transferase activity, but the levels of glucosyl transferase applied to the mesophyll-enriched gradient were too low for detection. On the

Table I. *Activities of Various Enzymes in Epidermal and Mesophyll Protoplasts*

Simultaneous equations were used to correct for cross-contamination of the epidermal and meosphyll preparations. The ratiò of epidermal to mesophyll cells in the *Sorghum* seedling leaf is 1:8. This is a comparison of mesophyll and epidermal tissues only; bundle sheath cells and guard cells were not assayed. The figures in this table are the means of several experiments.

	Epidermal Protoplasts	Mesophyll Protoplasts	Epidermal Tissue	Mesophyll Tissue
	/10^6 protoplasts	*/mg Chl*	*% activity*	
Chlorophyll (mg)		0.048		
Triosphosphate isomerase (μmol/min)	1.14	0.73	15.2	16.1	83.8
PEP carboxylase (nmol/min)	15.9	4.3	89.5	30.4	68.4
NADPH Cyt *c* reducase (μmol/min)	45.1	1.8	37.5	75.8	24.2
NADP triose phosphate dehydrogenase (nmol/min)	3.5	19.0	39.5	2.3	97.7
UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase (μmol/h)	0.563	0.029	0.6	70.8	29.2

Table II. *Peak Density of Enzymes and Markers and the Percentage of Their Activity Recovered from Linear 30 to 55% (w/w) Sucrose Gradients of Ruptured Mesophyll- and Epidermal-Enriched Protoplasts*

UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase activity in the mesophyll-enriched preparation and Chl in the epidermal-enriched preparation were not detectable on the gradients. The protoplasts were prepared from 6-d-old light-grown *Sorghum* leaf blades.

Protoplast Preparation	Marker or Enzyme	Peak Density	Amount Recovered from Gradients
		g/ml	%
Mesophyll-enriched	Chl	1.170 (broken plastids) 1.203 (intact plastids)	84
	Triosephosphate isomerase	1.203	76
Epidermal-enriched	Triosephosphate isomerase	1.201	93
	UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase	1.201	61

other hand, a significant amount (41%) of the glucosyl transferase activity recovered from the epidermal-enriched gradient was associated with the epidermal plastids, as indicated by its cosedimentation to a density of 1.201g/cm^3 (Fig. 3). This value (41%), which does not take plastid breakage during centrifugation into account, must represent a minimum figure for the amount of UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase activity located in the epidermal plastids.

DISCUSSION

The compartmentation of dhurrin in the epidermal tissue, and of the dhurrin-degrading enzymes (dhurrin glucosidase and hydroxynitrile lyase) in the mesophyll tissue of *Sorghum* leaves has previously been established (12). In that paper, it was also found that at least 68% of the UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase activity in a *Sorghum* shoot was associated with the epidermal tissue; this was confirmed by our data, in which 71% of this glucosyl transferase activity was found to be localized in the epidermal tissue (Table I). On a cellular basis, this difference is much more dramatic: 0.56 μmol of dhurrin was synthesized per h/10^6 epidermal protoplasts compared to 0.03 μmol/h·10^6 mesophyll protoplasts.

Plants contain glucosyl transferases of varying specificities (8) and a UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase, which was isolated from leaves and roots of young *Sorghum* seedlings by Reay and Conn (22), glucosylated substrates other than *p*-hydroxy-(*S*)-mandelonitrile (*e.g.* hydroquinone and *p*-hydroxybenzyl alcohol). It was, however, stereospecific for the *S* epimer of its cyanohydrin substrate and synthesized dhurrin. In the absence of purification of the epidermal transferase described in this study and an earlier one (12), we cannot claim identity with the enzyme studied by Reay and Conn (22).

The subcellular localization of other glucosyl transferases has been examined. Hrazdina *et al.* (9) found that in *Hippeastrum* petals and *Tulipa* petals and leaves, the UDP-glucose:anthocyanidin 3-*O*-glucosyl transferase, along with other enzymes of anthocyanin synthesis, was low or absent from a fraction described as particulate cytoplasm, which presumably contained plastids. In another study, Poulton *et al.* (21) found that the UDP-glucose:*ortho*-coumaric acid glucosyl transferase activity was absent from chloroplasts of mesophyll cells of *Melilotus alba*. Thus, our finding that the UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase activity is associated with plastids from *Sorghum* epidermal cells contrasts with the studies on *Hippeastrum*, *Tulipa*, and *Melilotus* where plastids originated from a variety of cell types. In all of the above experiments, the specificity of the glucosyl transferases was not established; however, in each case a physiological substrate was used (*i.e.* malvidin [9], *o*-coumaric acid [21], and *p*-hydroxy(*R,S*)-mandelonitrile [this publication]). In *Sorghum*, subcellular compartmentation of glucosyl transferases could provide an additional mechanism for control of the biosynthetic pathways leading to different glycosides.

If the dhurrin is synthesized in the epidermal plastids, the earlier steps of dhurrin synthesis may also be plastid-associated, since *p*-hydroxy-(*R,S*)-mandelonitrile, the substrate for the UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase, is unstable at pH values above 7.0 (4). A study by Saunders and Conn (23) indicated that these early steps are associated with the ER; however, their study was performed, by necessity, on etiolated and therefore younger tissue.

Epidermal plastids of *Sorghum* contain triose-P isomerase, as do other nonphotosynthetic plastids (*e.g.* from etiolated and endosperm tissue) (17, 28). It has been suggested (7, 28) that the dihydroxyacetone phosphate produced in the cytosol is transported into the plastids, converted to 3-P-glyceraldehyde, and used to generate reducing power in the form of NADPH via gluconeogenesis and the pentose-P pathway. (In this connection, glucose-6-P dehydrogenase has been found to occur in epidermal protoplasts of *Sorghum* [11]). Gluconeogenesis in the epidermal plastids could also supply the glucose needed for UDP-glucose in dhurrin synthesis. The low level of NADP triose-P dehydrogenase in the epidermal plastids was expected. This enzyme has no known function other than photosynthesis, and proplastids from castor bean endosperm and tobacco root also contain little detectable NADP triose-P dehydrogenase activity (28). The presence of PEP carboxylase activity in epidermal peels and isolated epidermal cells from several species of plants has previously been detected, and its possible roles in stomatal responses and CO_2 transport discussed (4, 30).

To our knowledge, this is the first report in the literature of the isolation of epidermal plastids. With the increasing evidence for the involvement of epidermal cells in secondary metabolism, it will be of interest to study further the primary and secondary metabolism which occur in this tissue and the subcellular localization of the enzymes involved.

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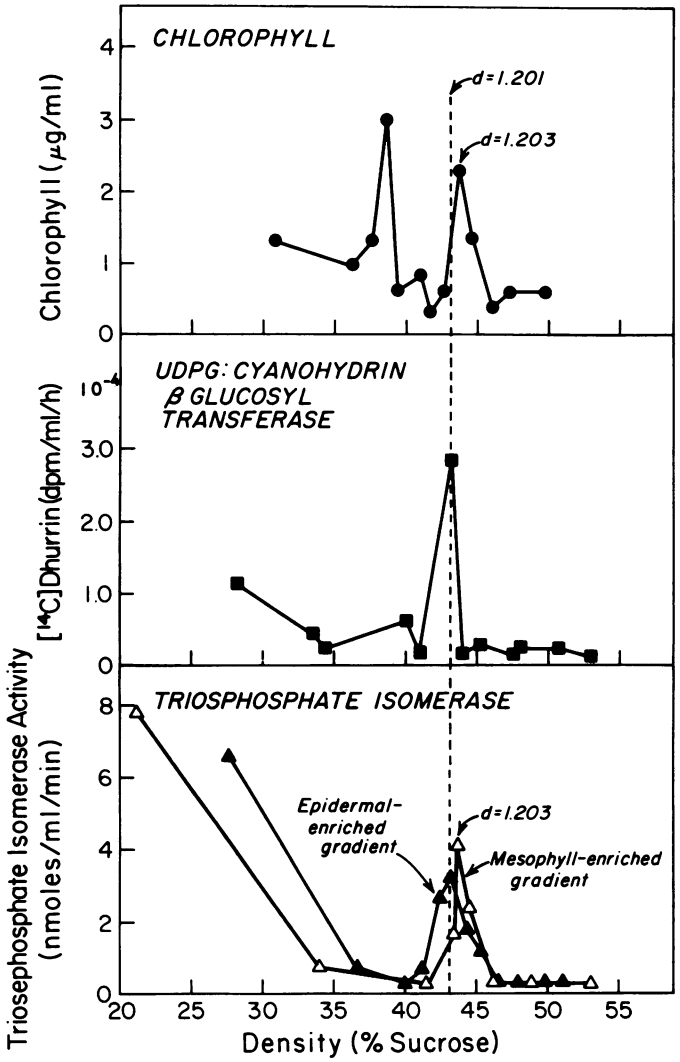

FIG. 3. Localization of UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase, Chl, and the plastid marker enzyme, triose-P isomerase in fractions of 30 to 55% (w/w) sucrose density gradients (linear, 40 ml) from ruptured protoplasts. UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase (■) and triose-P isomerase (▲) were from gradients of epidermal-enriched (70–85% pure) protoplasts; Chl (●) and triose-P isomerase (△) were from gradients of mesophyll-enriched (98–99% pure) protoplasts.

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