Tissue Distributions of Dhurrin and of Enzymes Involved in Its Metabolism in Leaves of Sorghum bicolor

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ABSTRACT

The tissue distributions of dhurrin [p-hydroxy-(S)-mandelonitrile-β-D-glucoside] and of enzymes involved in its metabolism have been investigated in leaf blades of light-grown Sorghum bicolor seedlings. Enzymic digestion of these leaves using cellulase has enabled preparations of epidermal and mesophyll protoplasts and bundle sheath strands to be isolated with only minor cross-contamination. Dhurrin was located entirely in the epidermal layers of the leaf blade, whereas the two enzymes responsible for its catabolism, namely dhurrin β-glucosidase and hydroxynitrile lyase, resided almost exclusively in the mesophyll tissue. The final enzyme of dhurrin biosynthesis, uridine diphosphate glucose-p-hydroxymandelonitrile glucosyltransferase, was found in both mesophyll (32% of the total activity of the leaf blade) and epidermal (68%) tissues. The bundle sheath strands did not contain significant amounts of dhurrin or of these enzymes. It was concluded that the separation of dhurrin and its catabolic enzymes in different tissues prevents its large scale hydrolysis under normal physiological conditions. The well documented production of HCN (cyanogenesis), which occurs rapidly on crushing Sorghum leaves, would be expected to proceed when the contents of the ruptured epidermal and mesophyll cells are allowed to mix.

The biosynthesis and distribution of various cyanogenic glucosides within higher plants have been closely investigated during the past decades (8). Among such plants, the Sorghum genus which possesses the cyanogenic glucoside dhurrin [p-hydroxy-(S)-mandelonitrile-β-D-glucoside] has received special attention (1, 18, 24). This secondary product may constitute 5% or even more of the dry weight of young Sorghum bicolor seedlings (1, 22). When this tissue is crushed or otherwise disrupted, a large amount of free HCN is rapidly released, as has been reported for other cyanogenic plants (7). Under these conditions, the dhurrin is hydrolyzed by an endogenous β-glucosidase to yield p-hydroxymandelonitrile. This intermediate then dissociates to produce free HCN and p-hydroxybenzaldehyde (Fig. 1). Although this dissociation can occur nonenzymically, Sorghum seedlings also possess an hydroxynitrile lyase, which catalyzes this reaction.

Since free HCN can be detected only when the tissue becomes damaged, several possibilities regarding compartmentation under normal physiological conditions may be envisaged. First, dhurrin and its degradative enzymes may be sequestered in different tissues. Alternatively, dhurrin and the β-glucosidase may exist within the same cell, but in separate intracellular compartments. Finally, all components could exist even within the same intracellular compartment (e.g. the vacuole), if the activity of the β-glucosidase could be controlled by endogenous inhibitors.

The above alternatives have been investigated here using epidermal and mesophyll proplasts and bundle sheath strands isolated from light-grown Sorghum leaves. The level of dhurrin and the activities of UDPGp-hydroxymandelonitrile glucosyltransferase, dhurrin β-glucosidase, and hydroxynitrile lyase have been determined. A clearer understanding about the role of each tissue in dhurrin metabolism in Sorghum seedlings has thereby been obtained.

MATERIALS AND METHODS

CHEMICALS

Uridine diphospho-p-[U-14C]glucose (NH4 salt) was obtained from Amersham/Searle and diluted with unlabeled uridine diphosphoglucose from Sigma Biochemicals, Inc. Dhurrin was isolated from 4-day-old etiolated Sorghum shoots and purified as described (19), p-Hydroxy-(R,S)-mandelonitrile was synthesized according to the method of Ladenburg et al. (13). Cellulase (Onozuka R-10) was purchased from All Japan Biochemicals Co., Ltd., and almond emulsin was obtained from Sigma Biochemicals, Inc. Hydroxynitrile lyase was purified from Sorghum as described by Bové and Conn (5). The 20-μm nylon net was purchased from Teto Inc., Elmsford, N. Y., and the 44-μm and 149-μm nets were obtained from Cistron, Lebanon, Pa.

BUFFERS

The following solutions were commonly used: medium A: 0.5 M mannitol, 25 mM K-phosphate-citrate (pH 5.5); medium B: 10 mM Na-phosphate (pH 7.5); medium C: 10 mM Na-phosphate (pH 7.5), containing 1 mg/ml BSA.

PLANT MATERIALS

Seeds of Sorghum bicolor (Linn.) Moench, var. Redland × Greenleaf, were soaked in aerated water for 24 h at room temperature. They were then allowed to germinate under fluorescent light (1,700 ft-c) at 26°C in water-saturated Vermiculite, employing a 16:8 h (light:dark) photoperiod. Leaf blades were digested or extracted 3 to 4 h after the beginning of the light period. After 6 days, the Sorghum shoot was 9 to 10 cm tall and possessed two expanded leaves.

PREPARATION OF TISSUE FRACTIONS

Mesophyll and Epidermal Protoplasts. Approximately 1.5 g of leaves were excised at the base of the leaf blades and abraded with...
150 grit carborundum using a small artist brush according to the method of Beier (3). After the abraded leaves had been rinsed in distilled H$_2$O, they were incubated for 2 to 2.5 h in 18 ml medium A, containing 1.5% (w/v) cellulase, in a covered 9-cm Petri dish. The incubation was carried out at 30 °C in a shaking water bath at 50 oscillations/min. The partially digested leaves were filtered using a 44-μm nylon net and were rinsed with medium A to maximize the yield of protoplasts. The released protoplasts were harvested by centrifuging the filtrate at 220g for 3 min in a bench-top centrifuge (swing-out type). The pellet, which contained a mixture of mesophyll and epidermal protoplasts, was gently resuspended in 2 ml medium A. Following the procedure of Saunders and Conn (21), 1 or 2 drops of neutral red (dissolved in medium A) were added to the suspension. The dye was quickly taken up by the epidermal protoplasts and became sequestered in their vacuoles. By contrast, dye could be observed within the mesophyll protoplasts only after much longer time periods. Since the average diameter of the epidermal protoplast population significantly exceeded that of the mesophyll, their partial separation could be effected as follows. The protoplast suspension, after treatment with neutral red, was filtered without applied suction through one layer of 20-μm nylon mesh, which rested upon a single layer of Miracloth in a Büchner funnel (75 mm, i.d.). The mesophyll protoplasts passed unimpeded through the filtration system and were collected by centrifugation at 500g for 3 min in a bench-top centrifuge. The pellet was resuspended in medium A. The epidermal protoplasts were preferentially retained on the 20-μm net and were therefore collected by rinsing the latter with a small volume of medium A.

In addition to filtration, the cross-contamination of the mesophyll and epidermal protoplast fractions could be reduced as follows. Protoplast preparations were allowed to stand for 15 to 30 min at either 4 °C or at room temperature. Since the epidermal protoplasts sedimented more quickly, the overlying mesophyll protoplasts could be removed using a Pasteur pipette. Fractions were then concentrated by centrifugation. This procedure was repeated several times as necessary.

**Bundle Sheath Strands.** Bundle sheath strands were obtained using a method modified from that of Kanai and Edwards (10, 11). The leaves were cut across the veins into 0.5-mm segments using a Gilson gel slicer. Approximately 0.8 g of leaf segments was placed in a covered 9-cm Petri dish with 18 ml medium A, containing 2% cellulase, and the tissue was quickly evacuated twice (40 mm Hg) with a vacuum pump. Incubation was then undertaken at 31 to 32 °C in a shaking water bath at 60 oscillations/min. After 2 h, the cellulase medium was replaced by fresh medium, and digestion was continued for a further 2 h. The digestion process was accelerated at intervals by drawing the tissue fragments up and down through a Pasteur pipette. The contents of the Petri dish were then transferred to two 12-ml centrifuge tubes and allowed to settle for approximately 5 min. The overlaying medium, which contained mainly cuticular fragments, was removed using a Pasteur pipette. This procedure was repeated after resuspending the strands in 12 ml of medium A. The strands were then dispersed in medium A, collected by filtration on a 149-μm nylon net and were thoroughly washed using large volumes of this medium. The strands were then recovered by washing the net with medium A and by centrifugation at 300g for 1 min. The supernatant fluid was discarded and the pellet was taken up in 12 ml medium A. Further purification by sedimentation (settling), as described above, was undertaken to remove cuticular contaminants. Finally, the bundle sheath strands were collected by centrifugation at 100g for 3 min and were dispersed in 3 ml medium A.

**ENZYME ASSAYS.**

**UDPG: Aldehyde Cyanohydrin β-Glucosyltransferase.** Glucosyltransferase activity was assayed using a modified method of Reay and Conn (20). Mesophyll (or epidermal) protoplasts were sedimented by centrifugation at 370g for 3 min, and the supernatant liquid was carefully removed. The pellet was resuspended in 100 μl 0.15 M Tris-HCl (pH 8.0) containing 100 μg BSA, 0.2 μmol DTT, and 10 μmol p-hydroxy-((R,S)-mandelonitrile. The protoplasts were ruptured by freezing the suspension three times using liquid N$_2$. After the addition in 20 μl of 0.6 μmol UDP-[U-14C]glucose, containing 1.6 μCi, the mixture was incubated at 30 °C for 25 min. The reaction was terminated by addition of 30 μl 20% acetic acid, and the precipitated material was removed by centrifugation at 1,050g for 10 min. The supernatant liquid was applied to a 4-cm-wide strip of Whatman No. 1 chromatography paper and developed with butanone-acetone-water (15:5:3, v/v). Radioactive dhurrin (R$_s$ = 0.74) was located using a Packard radiochro-
matogram scanner. The area on the chromatogram was cut up into a vial, and the radioactivity was determined by liquid scintillation counting.

In the case of the bundle sheath strands, homogenization could not be successfully effected by freezing and thawing. The strands were homogenized in a TenBroeck homogenizer with 650 μl 0.15 M Tris-HCl (pH 8.0) containing 500 μg BSA, 1.0 μmol DTT, 50 μmol p-hydroxy-(R,S)-mandelonitrile. The subsequent addition of radioactive UDPG and the incubation conditions were as described above. The enzyme activity is expressed as the amount of dhurrin synthesized during incubation for 60 min at 30 C.

Preparation of Extracts for β-Glucosidase and Hydroxynitrile Lyase Assays. Mesophyll protoplasts were washed with four 10-ml aliquots of medium A and were finally pelleted by centrifugation at 500g for 3 min. After discarding the supernatant liquid, the protoplasts were resuspended in a small volume (3–6 ml) of medium B and ruptured using a sonicator (model W185F, Heat Systems-Ultrasonics, Inc.) at 4 C for periods of 3 × 10 s. The sonicate was centrifuged for 1.5 min in a Beckman microfuge B centrifuge, and the supernatant liquid was used as enzyme source. Epidermal protoplasts were washed carefully using medium A and were then pelleted by centrifugation at 500g for 3 min. The pellet was resuspended in 200 to 500 μl medium B or medium C, and the protoplasts were ruptured by drawing them up and down through a narrow micropipette tip (Eppendorf, 200 μl).

The bundle sheath strands were washed and then pelleted by centrifugation as described above. The strands were resuspended in 2 to 6 ml medium B and thoroughly homogenized at 4 C using a tightly fitting TenBroeck homogenizer. After removal of an aliquot for Chl and protein determinations, the remainder was centrifuged for 1.5 min in a Beckman microfuge B centrifuge. The supernatant liquid was assayed for enzyme activity.

Dhurrin β-Glucosidase Activity. Dhurrin β-glucosidase activity was assayed by measuring the production of p-hydroxybenzaldehyde from dhurrin in the presence of extract and exogenous hydroxynitrile lyase (17). The enzyme extract was added to a quartz cuvette containing 1.8 μmol dhurrin, 55 milliunits purified hydroxynitrile lyase, and 80 μmol Na-acetate (pH 5.5) in a total volume of 1.2 ml. Incubation was carried out at 37 C and the A of the liberated p-hydroxybenzaldehyde was measured at 285 nm.

Hydroxynitrile Lyase Activity. The hydroxynitrile lyase activity of isolated fractions was followed by measuring the decomposition of the cyano-hydryn of p-hydroxybenzaldehyde (5). The assay was carried out at 37 C in a total volume of 1.2 ml which contained enzyme, 2 μmol p-hydroxy-(R,S)-mandelonitrile, and 80 μmol Na-acetate (pH 5.5). The reaction was initiated by addition of cyano-hydryn, and the A of the liberated p-hydroxybenzaldehyde was measured at 285 nm. Since the cyano-hydryn also decomposes slowly at pH 5.5 in the absence of enzyme, the rate of the apparent enzymic reaction had to be corrected for nonenzymic decomposition.

Ribulose-DIP Carboxylase Activity. The ribulose-diphosphate carboxylase activity of the bundle sheath strands was assayed as described (11, 15). The strands were broken by grinding them in the “activation” buffer of Lorimer et al. (15) in a tightly fitting TenBroeck homogenizer at 4 C. After filtering the homogenate through a 37-μm nylon net, the filtrate was assayed for carboxylase activity (15).

OTHER METHODS

Estimation of Dhurrin. Dhurrin was assayed using a modification of the Lambert procedure (14). The mesophyll and epidermal protoplasts (in medium A) were pipetted into small incubation vials (45 × 15 mm i.d.) and centrifuged at 540g for 3 min. After carefully removing the supernatant liquid, the protoplasts were quickly resuspended in 0.5 ml 0.1 M Na-phosphate (pH 6.8) initially incubated at 37 C for 3 h. The protoplast mixture was containing 0.3 g emulsin. A container holding 300 μl 1 N NaOH was suspended inside the vial above the protoplast mixture, before the vial was closed off using an air-tight rubber seal. The vial was initially incubated at 37 C for 3 h. The protoplast mixture was then frozen twice using liquid N2 and incubation was continued at 37 C for a further 19 h. In order to promote HCN release from the mixture and its subsequent absorption by the NaOH “trap,” 75 μl 5 N H2SO4 was injected into the mixture and incubation was continued for 4 h. The subsequent analysis of the liberated HCN was undertaken as previously reported (14).

This method had to be modified in the case of the bundle sheath strands, since the latter were not efficiently disrupted by freezing and thawing procedures. Instead, a bundle sheath strand preparation was centrifuged at 500g for 3 min, and the supernatant liquid was removed. The strands were lyophilized and then quickly ground using a pre-cooled pestle and mortar. The resultant fine powder was weighed and a sample thereof was retained for protein determination. The remainder was introduced into a small incubation vial and treated as described above for the HCN analysis of mesophyll and epidermal protoplasts.

Determination of Protein and Chl. The protein content of isolated fractions was determined by the Lowry method (16) after precipitating twice from solution by 10 ml of 10% (w/v) trichloroacetic acid. BSA treated similarly served as a standard. Chl was estimated by the technique of Arnon (2).

Determination of Cell Number and Microscopic Appearance. Mesophyll and epidermal protoplasts were counted using an AO Spencer Bright Line hemocytometer. This technique was facilitated by staining the preparation with neutral red as described above. The degree of contamination of the various fractions was estimated visually using an AO Spencer microscope at low (× 100) and high (× 450) power magnification. Photographs were taken on a Zeiss universal photomicroscope.

RESULTS

INTACT LEAVES: HCN ANALYSIS AND ENZYME ACTIVITY DETERMINATION

The two expanded leaf-blades of 6-day-old light grown Sorghum seedlings were analyzed to determine if they differed significantly with respect to dhurrin content or metabolism. As seen in Tables I and II, the first (older) leaf blades contained somewhat larger amounts of dhurrin and higher activities of the dhurrin β-glucosidase and hydroxynitrile lyase than the second (younger) leaf blades. Inasmuch as no major differences were observed in these preliminary experiments, both leaf blades were utilized in the production of protoplasts and bundle sheath strands.

ISOLATION OF TISSUE FRACTIONS USING DIGESTIVE ENZYMES

Digestion of six-day-old Sorghum leaf blades (Fig. 2A) as described under “Materials and Methods” allowed the isolation of large numbers (5–10 × 106/g tissue) of mesophyll protoplasts (Fig. 2C). These were spherical in shape, ranging from 10 to 30 μm in diameter.

Table I: The distribution of dhurrin within leaf blade sections derived from six-day-old sorghum shoots.

<table>
<thead>
<tr>
<th>Leaf age</th>
<th>Section of leaf</th>
<th>Dhurrin content (μmol/g fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>Upper half (older)</td>
<td>81.1</td>
</tr>
<tr>
<td>First</td>
<td>Lower half (younger)</td>
<td>68.4</td>
</tr>
<tr>
<td>Second</td>
<td>Upper half (older)</td>
<td>58.6</td>
</tr>
<tr>
<td>Second</td>
<td>Lower half (younger)</td>
<td>55.9</td>
</tr>
</tbody>
</table>

The following sections of the first and second leaves of six-day-old light-grown sorghum seedlings were analyzed for dhurrin content using previously described methods (12).
Table II. The distribution of dhurrin β-glucosidase and hydroxynitrile lyase activities in the leaf blades of six-day-old sorghum shoots.

Twenty leaf blades (from either first or second leaf) were weighed, washed and blotted dry. They were then cut into approximately 0.5 cm strips using a razor blade and homogenized at 4°C in a mortar with 2 ml of medium B and a little sand. The homogenate was centrifuged for 1.5 min in a Beckman Microfuge B centrifuge to remove the larger debris. The activities of β-glucosidase and hydroxynitrile lyase were determined as described in the Methods section.

<table>
<thead>
<tr>
<th>Leaf age</th>
<th>Dhurrin β-glucosidase activity (µmol/h/g fresh weight)</th>
<th>Hydroxynitrile lyase activity (µmol HCN/h/g fresh weight)</th>
<th>Ratio (hydroxynitrile lyase/β-glucosidase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First leaf</td>
<td>313</td>
<td>929</td>
<td>3.0</td>
</tr>
<tr>
<td>Second leaf</td>
<td>217</td>
<td>617</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Fig. 2. A: transverse section of a 6-day-old Sorghum leaf blade fixed in 4% glutaraldehyde, dehydrated in ethanol, embedded in Spurr’s plastic (23), and stained with toluidine blue. Lower epidermis is toward the top of the photograph. M, mesophyll; E, epidermis; BS, bundle sheath cells (×420). B: preparation of purified epidermal protoplasts, stained with neutral red (×540). M, mesophyll. C: preparation of purified mesophyll protoplasts (×540). D: preparation of purified bundle sheath strands, showing three veins with interconnections (×370).

μm in diameter, and consisted of one central vacuole surrounded by 10 to 20 chloroplasts and the remainder of the cytoplasm. In contrast, the accompanying epidermal protoplasts (Fig. 2B) were larger, having diameters ranging from 25 to 50 μm, and did not contain visible plastids. The yield of epidermal protoplasts was only about 2 to 3 × 10⁵/g tissue. The separation procedure for the two protoplast populations, achieved by filtration and sedimentation techniques, produced preparations having a 5 to 10% cross-contamination within each fraction.

Accumulated evidence proved that the colorless protoplasts were indeed derived from the epidermal layer of the leaf. First, these protoplasts were actually observed being released from the
epidermal layers of leaf segments during cellulase digestion. In other experiments, the anthocyanin content of the Sorghum seedlings was increased specifically in the epidermal cells by growing the plants under continuous illumination (5,000 ft-c). No anthocyanins were visible in the mesophyll or vascular bundles in hand-cut sections of these seedlings. Upon digestion of the leaves, the epidermal protoplasts were easily distinguishable from the green mesophyll protoplasts by their larger size and anthocyanin content; moreover, they behaved identically with those colorless protoplasts which were routinely obtained from the control seedlings. Additional microscopic evidence demonstrated that the epidermal protoplasts were not vacuoles, as described by Saunders and Conn (21). The presence of nuclei was indicated using the nuclear stain acetocarmine, while ER and other cytoplasmic organelles were recognized within the protoplasts using electron microscopy.

Bundle sheath strands (Fig. 2D) were prepared in high yield from thin slices of leaf blades as described by Kanai and Edwards (10, 11). These strands, which are resistant to the action of cellulase (Onozuka), were almost completely stripped of the adjoining mesophyll tissue. Microscopic examination revealed some mesophyll contamination only in those fragments originating from the leaf tip and the leaf margins.

Before undertaking biochemical analysis of these three tissue fractions, it was necessary to determine which precautions were compulsory in order to avoid contamination by enzymes in the digestion mixture. Trial experiments indicated that the cellulase (Onozuka) preparation itself possessed dhurrin β-glucosidase but no hydroxynitrile lyase activity. However, the latter activity could be detected in the medium after digestion of the leaves and presumably originated from mesophyll protoplasts which became broken during the incubation process. This contamination could be completely eliminated by washing the protoplasts and bundle sheath strands four times with medium A prior to homogenization.

ISOLATED FRACTIONS: HCN ANALYSIS AND ENZYME ACTIVITY DETERMINATIONS

Dhurrin Distribution. As indicated in Table III, large amounts of dhurrin (expressed here as μmol/10⁶ protoplasts) were detected within epidermal protoplasts. By contrast, less than 10% of this value was found in the mesophyll protoplast preparations. Since these two fractions were not completely homogeneous, simultaneous equations were used to correct for the approximately 10% mesophyll contamination of the epidermal population and for the 5 to 10% epidermal contamination of the mesophyll protoplast population. Such calculations suggest that the epidermal protoplasts should possess about 47 times more dhurrin than the mesophyll protoplasts. A ratio of 35:1 was indeed observed experimentally with the purest fractions obtained; even in this case, the dhurrin content of the almost homogeneous mesophyll preparation could be accounted for by the epidermal protoplast contamination. The dhurrin content of the bundle sheath strands was only 0.02% of that of the epidermal cells (expressed here as μmol/mg protein). We therefore concluded that the dhurrin within the 6-day-old leaf blade is localized almost exclusively in the epidermal cells.

Distribution of UDPG:Aldehyde Cyanohydrin β-Glucosyltransferase. The activity of this enzyme was monitored as a possible marker of dhurrin synthesis. Table III shows that no glucosyltransferase activity was detectable in the bundle sheath extracts. About eight times greater activity (expressed as μmol dhurrin synthesized/h·10⁶ protoplasts) was found in the epidermal protoplast extracts than in the mesophyll extracts. Simultaneous equations were used as described above to correct for the cross-contamination of the protoplast preparations, yielding an activity ratio (epidermal to mesophyll) closer to 17:1. By observation of thin transverse and longitudinal sections of Sorghum leaves, the estimation was made that the ratio of mesophyll to epidermal cells in the whole leaf blade is approximately 8:1. At least 68% of the total glucosyltransferase activity of the leaf blade is localized in the epidermal layer.

Dhurrin β-Glucosidase Activity. High dhurrin β-glucosidase activity was detectable in extracts from mesophyll protoplasts, whereas epidermal extracts exhibited much lower activities (Table III). After correction for the 5 to 10% contamination of the latter extracts by mesophyll protoplasts, epidermal protoplasts possessed only approximately 6.5% of the activity (expressed as enzyme units/protoplast) of the mesophyll protoplasts. Assuming that the ratio of mesophyll to epidermal cells in the leaf blade is 8:1, one may conclude that the epidermis possesses less than 1% of the total β-glucosidase activity present in the mesophyll tissue. The activity of the bundle sheath preparation could not be expressed in terms of cell number. Kinetic analysis showed that the bundle sheath strand fraction contained only 6% and 7% of the dhurrin β-glucosidase activity of the mesophyll fraction, when Chl and protein contents, respectively, were used as basis for comparison.

Hydroxynitrile Lyase Activity. Extracts from mesophyll proto-

Table III. The distribution of dhurrin and enzymes involved in its metabolism in different fractions from six-day-old sorghum leaf blades.

<table>
<thead>
<tr>
<th>Component</th>
<th>Epidermal protoplasts</th>
<th>Mesophyll protoplasts</th>
<th>Bundle sheath strands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per 10⁶ protoplasts</td>
<td>per 10⁶ protoplasts</td>
<td>per mg chlorophyll</td>
</tr>
<tr>
<td>dhurrin (μmol)</td>
<td>5.3</td>
<td>0.47</td>
<td>10.4</td>
</tr>
<tr>
<td>chlorophyll (mg)</td>
<td>26.5</td>
<td>1.27</td>
<td>0.006</td>
</tr>
<tr>
<td>protein (μmol)</td>
<td>0.200</td>
<td>0.370</td>
<td>8.2</td>
</tr>
<tr>
<td>UDPG:aldehyde cyanohydrin β-glucosyltransferase (μmol/h)</td>
<td>0.420</td>
<td>0.055</td>
<td>1.22</td>
</tr>
<tr>
<td>dhurrin β-glucosidase (μmol/h)</td>
<td>0.55</td>
<td>8.5</td>
<td>191</td>
</tr>
<tr>
<td>hydroxynitrile lyase (μmol/h)</td>
<td>0.99</td>
<td>19.7</td>
<td>441</td>
</tr>
</tbody>
</table>

1 N.D., not determined.
plants exhibited high hydroxynitrile lyase activity (Table III). In contrast, identical experiments using extracts from epidermal protoplasts demonstrated that they possessed relatively low activities. After correction had been made for 5 to 10% mesophyll contamination, the epidermal protoplasts exhibited only 5% of the hydroxynitrile lyase activity (expressed as units/protoplast) shown by the mesophyll protoplasts. Because of the 8:1 ratio of mesophyll to epidermal cells in the leaf blade, one may conclude that the epidermis possesses less than 1% of the total lyase activity present in the mesophyll tissue. The bundle sheath strand fraction contained only approximately 3 and 4% of the lyase activity of the mesophyll fraction, when activities were compared using Chl and protein content as basis, respectively.

Ribulose-DP Carboxylase Activity. The observed carboxylase activities of the bundle sheath strands (about 170 pmol CO₂ fixed/hr-mg Chl) are comparable with those reported elsewhere (10) and, in addition to measured shikimate oxidoreductase activities (data not shown), provide an indication that the isolated strands are still biochemically functional.

**ADDITIONAL CONTROLS AND CONFIRMATORY EVIDENCE**

**Confirmation of Localization of Dhurrin and Degradative Enzymes in Different Tissues.** The release of HCN from mixtures of epidermal and mesophyll protoplasts was also undertaken in the absence of exogenous emulsin. Table IV demonstrates that the production of HCN was much smaller when the two protoplast populations were incubated separately than when incubated together. These data may be easily explained by the foregoing sections, which demonstrated the specific localization of dhurrin in the epidermal protoplasts and of the degradative enzymes in the mesophyll protoplasts.

**Investigation of Possibility of Enzyme Inhibitors.** Control experiments were undertaken in which extracts from the three tissue fractions were mixed and subsequently analyzed for enzymic activity. Such “mixing” experiments failed to reveal the presence of any inhibitors in the bundle sheath strands, which could inhibit the hydroxynitrile lyase of β-glucosidase activities of the mesophyll protoplasts or the UDPG-cyanohydrin glucosyltransferase activity of the epidermal protoplasts.

Table IV. The release of HCN from epidermal and mesophyll protoplasts in the absence of exogenous emulsin.

<table>
<thead>
<tr>
<th>Epidermal protoplasts</th>
<th>Mesophyll protoplasts</th>
<th>HCN released (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(number added)</td>
<td>(number added)</td>
<td></td>
</tr>
<tr>
<td>7.5 x 10³</td>
<td>--</td>
<td>0.4</td>
</tr>
<tr>
<td>7.5 x 10³</td>
<td>7.5 x 10³</td>
<td>3.0</td>
</tr>
<tr>
<td>7.5 x 10³</td>
<td>7.5 x 10⁴</td>
<td>15.9</td>
</tr>
<tr>
<td>--</td>
<td>7.5 x 10³</td>
<td>0.4</td>
</tr>
<tr>
<td>--</td>
<td>7.5 x 10⁴</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**Effect of Gel Filtration on Activities of Degradative Enzymes.** The possibility that the enzyme extracts possessed an endogenous inhibitor was further investigated using gel filtration chromatography. Passage of a mesophyll extract (in buffer C) through a Sephadex G-25 column (preequilibrated and eluted with buffer C) resulted in the recovery of 113 and 105% of the hydroxynitrile lyase and β-glucosidase activities, respectively. In identical experiments employing bundle sheath strand extracts, 100% of both activities was recovered in the column eluate. These data indicate the absence of an inhibitor in the bundle sheath strands, but suggest that the enzymic activities given in Table III for the mesophyll extracts may be 5 to 15% lower than the real values.

**DISCUSSION**

In recent years, extensive use has been made of digestive enzymes in isolating protoplasts and bundle sheath strands from plant tissues (6, 9, 10). Biochemical analysis of such preparations has elucidated how various cell types cooperate in certain aspects of primary metabolism. For example, this approach has established the complementary roles of mesophyll and bundle sheath cells during photosynthesis in C₄ plants (12). In this paper, the isolation of epidermal and mesophyll protoplasts from leaves of 6-day-old *Sorghum* seedlings has been described. These fractions, in addition to bundle sheath strands isolated from the same source, were used to investigate the location and metabolism of the cyanogenic glucoside dhurrin, which is found in high concentrations in these leaves.

It is generally assumed that dhurrin is physically separated within *Sorghum* seedlings from the degradative enzymes (β-glucosidase and hydroxynitrile lyase), which catalyze its decomposition. Several modes of compartmentation have been described in the Introductory section, proposing how this might be achieved. Analysis of the three tissue fractions from *Sorghum* leaves clearly demonstrated that dhurrin and the degradative enzymes are localized in different tissues. Essentially all of the dhurrin contained in leaf-blades of 6-day-old *Sorghum* is located in the epidermis. This localization pattern resembles that of several other secondary products derived from L-phenylalanine and L-tyrosine (25-28). In contrast, the β-glucosidase and hydroxynitrile lyase are found in the mesophyll tissue. This mode of compartmentation provides a mechanism under normal physiological conditions, whereby dhurrin may be protected from large scale enzymic breakdown. The rapid release of HCN, which is noted on crushing *Sorghum* leaves, would proceed when the contents of the ruptured epidermal and mesophyll cells were allowed to mix.

The synthesis of dhurrin in etiolated *Sorghum* seedlings is carried out by a microsomal fraction, which catalyzes the conversion of L-tyrosine to p-hydroxy-(S)-mandelonitrile via p-hydroxyphenylacetaldimine (18). A soluble glucosyltransferase then catalyzes the glucosylation of p-hydroxy-(S)-mandelonitrile using UDP-glucose as a glucosyl donor (18, 20). The demonstration of the multistep conversion of L-tyrosine to p-hydroxymandelonitrile by microsomes isolated from light-grown seedlings has so far been unsuccessful (Kojima, unpublished). It was therefore not possible to determine which cell types catalyze this complex reaction sequence. However, it was demonstrated that 68 and 32% of the total UDPG-aldehyde cyanohydrin glucosyltransferase activity of the leaf blade were found in the epidermal and mesophyll tissues, respectively. If these data indicate that both tissues are participating in dhurrin synthesis, one must conclude that dhurrin synthesized in the mesophyll layer must subsequently be translocated into the epidermal cells.

Bough and Gander (4) reported that dhurrin undergoes metabolic turnover in etiolated *Sorghum* seedlings with rates of approximately 0.05 μmol/h. Since the β-glucosidase and hydroxynitrile lyase are presumed to take part in such turnover, dhurrin...
would have to be translocated into the mesophyll cells to accomplish this degradation. Alternatively, the data presented here indicate the presence of low levels of the degradative enzymes within epidermal cells, which might be sufficient to account for the turnover rates observed within etiolated tissue.

Recently in this laboratory, Saunders and Conn (21) compared the dhurrin content of protoplast and vacuole preparations isolated from light-grown Sorghum leaves. In that study, protoplasts obtained primarily from mesophyll cells, as evidenced by their chloroplasts (Fig. 1 of ref. 21), were lysed in hypotonic solutions and the release of vacuoles from such protoplasts was observed. When the vacuoles were subsequently collected and analyzed for dhurrin, it was concluded that all of the dhurrin in the original protoplast preparation was located in the vacuolar space of those protoplasts.

The demonstration in this paper that only epidermal cells contain dhurrin means that the protoplast preparations used by Saunders and Conn contained protoplasts of epidermal origin as well as mesophyll. Structures similar to the epidermal protoplasts described in the present work (Fig. 2B) were indeed present (Fig. 1 of ref. 21) and presumably are the source of dhurrin in those preparations. In our experience, it is often difficult to distinguish between a mesophyll vacuole contaminated by adhering cytoplasm and a small epidermal protoplast, which contains a vacuole comprising over 90% of its volume. It is therefore possible that a low level of contaminating epidermal protoplasts, or even epidermal vacuoles, could account for the dhurrin content of the vacuolar preparations in that study. Saunders and Conn did note cytoplasmic contamination in their vacuoles and were able to reduce but not eliminate it by including 1% BSA in their resuspension medium.

One can estimate (Fig. 2A) that epidermal cells constitute approximately 40% of the Sorghum leaf blades used in these experiments. From the dhurrin concentration (Table I) of such tissue, one can calculate that the concentration of dhurrin in the epidermis is approximately 0.2 μM. (This same value is obtained if one assumes that epidermal protoplasts have a dhurrin concentration of 5 μmol/10^6 protoplasts and an average spherical diameter of 35 μm.) If the cytoplasm occupies 5% of the epidermal cell and the dhurrin were confined to that volume, the dhurrin content would have to be 4 m. Such figures obviously support the earlier conclusion (21) that dhurrin is a vacuolar constituent.

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