The Metabolism of Aromatic Compounds in Higher Plants

VII. THE ORIGIN OF THE NITRILE NITROGEN ATOM OF DHURRIN
(ß-GLUCOPYRANOSYL-ß-HYDROXYMANDELONITRILE)*

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SUMMARY

The data obtained in these experiments show that the nitrile nitrogen of dhurrin is directly derived from the a-amino group of L-tyrosine. This is analogous to the retention of the a-amino nitrogen of valine on its conversion to linamarin (6). The biosynthetic route from L-tyrosine to dhurrin must involve the modification of the amino acid in a manner such that the C₂-C₃-N unit remains intact.

¹⁵N analyses of the amino acids isolated after L-tyrosine-¹⁴C,¹⁵N feeding suggest that the L-tyrosine-L-alanine transamination couple is more active than the L-tyrosine-L-glutamic acid couple.

Several laboratories have established that the amino acids phenylalanine, tyrosine, valine, and isoleucine are effectively converted to the aglycones of specific cyanogenic glucosides in higher plants (1-5). However, efforts to detect significant quantities of free intermediates in these conversions have been unsuccessful. Although a reaction sequence can be postulated in which the amino nitrogen atom is lost as the amino acids are converted to the aglycones (5), Butler and Conn (6) have shown that the nitrogen atom of L-valine is efficiently retained when this amino acid is converted to linamarin by intact shoots of flax seedlings. In these experiments, the nitrogen atom of valine was utilized 60% as effectively as the a carbon atom for the synthesis of the nitrile group of the aglycone. Although these results were taken as evidence that the nitrogen atom of the amino acid is not lost during the conversion, it was desirable to establish this point by an examination of another cyanogenic glucoside, dhurrin, found in Sorghum vulgare.

EXPERIMENTAL PROCEDURE

Three-day-old etiolated seedlings of Sorghum vulgare (var. Honey drip), cultured by the method of Akazawa, Miljanich, and Conn (7), were administered isotopic tyrosine, as described by Koukol, Miljanich, and Conn (3). In Experiment 1 0.016 µmole of L-tyrosine-¹⁴C (Volk) and 18 µmoles of L-tyrosine-¹⁵N were administered to the same number of seedlings. The ¹⁴C:¹⁵N ratios in these two experiments were then arbitrarily taken as 1:1 and 2:1, respectively. The seedlings were placed approximately 1 foot from two 20-watt Gro-Lux (Sylvania F20T12) fluorescent tubes to permit the uptake of tyrosine. After 36 hours, the seedlings were removed from the feeding solution and the roots were rinsed with distilled water. The rinse solution was combined with the feeding solution and the roots were rinsed with distilled water. The uptake was determined by counting aliquots of this solution as well as the original feeding solution in a Packard liquid scintillation counter (model 314 EX), with the counting medium devised by Bray (8).

The seedlings were frozen with liquid nitrogen and ground to a fine powder in a mortar. The powder was suspended in 200 ml of hot 80% ethanol (v/v) and boiled for 5 min. The suspension was then filtered through Whatman No. 1 paper, and the filtrate was extracted three times with equal volumes of petroleum ether. The petroleum ether, which contained plant pigments, was discarded and the ethanolic solution was subjected to column chromatography. The ethanolic extracts were passed at the rate of 3 ml per min through a column (1.5 x 40 cm) containing 19 g of Dowex 50-X8 resin prepared by the method of Plaisted (9). The column was then washed with 30-ml portions of 80% ethanol and the washes were added to the eluent solution. This eluent, which contained the acidic and neutral compounds, was adjusted to pH 7.0 with 0.1 N NaOH and taken to dryness in a vacuum at 35°C. The residue was dissolved in 5 ml of 80% ethanol and assayed for radioactivity as described above. The dhurrin content of this fraction was assayed spectrophotometrically (7).

The accurate determination of the ¹⁵N content of the nitrile group of dhurrin-¹⁴C,¹⁵N required the isolation of the glucoside in a pure state. Paper chromatography was the method employed because of the limited amount of dhurrin-¹⁴C,¹⁵N available and the difficulty of obtaining the glucoside in crystalline form. The specific activity of the glucoside was determined after each chromatographic separation by counting aliquots of the dhurrin-¹⁴C,¹⁵N in the liquid scintillation counter and determining the dhurrin spectrophotometrically. This method of assay was used because of the speed and the ease with which it may be performed. It should be noted, however, that the values for glucoside content obtained by this method, although re-
producing, are 15 to 20% less than those obtained by enzymatic hydrolysis of the glucoside and analysis of the HCN produced.

The solution of neutral and acidic compounds was applied as a streak to sheets of Whatman No. 3MM paper. Authentic dhurrin was placed as a marker on separate spots adjacent to the streak. The paper was developed in the descending direction with the L-butanol-propionic acid-water system described by Bassham and Calvin (10). The chromatograms were dried and the marker areas were cut off, sprayed with a solution containing 5 mg of emulsin per ml (Calbiochem), and placed in a chamber saturated with water vapor for 2 hours to permit the enzymatic hydrolysis of dhurrin. After hydrolysis, the papers were dried and sprayed with a solution of 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl. The intense orange phenylhydrazone of p-hydroxybenzaldehyde revealed the location of dhurrin on the chromatograms. Material in the areas containing the biosynthesized dhurrin was then eluted quantitatively with water and assayed for glucoside and radioactivity. In Experiment 1, 63.0 μmoles (428,000 cpm) of dhurrin-14C,15N were recovered, while 69.0 μmoles (892,000 cpm) were recovered in Experiment 2. These values gave a specific activity for the glucoside of 6,630 cpm per μmole in Experiment 1 and 12,900 cpm per μmole in Experiment 2. The solutions containing the dhurrin were then concentrated under reduced pressure and again applied as a streak to sheets of Whatman No. 3MM paper adjacent to authentic markers of dhurrin. The papers were developed in the descending direction with the L-butanol-propionic acid-water system described by Bassham and Calvin (10). The dhurrin was again located, as described previously, eluted with water, and assayed for radioactivity and glucoside. Analysis of the eluate revealed that 64.0 μmoles (424,000 cpm) of dhurrin-14C,15N had been recovered in Experiment 1, while 68 μmoles (880,000 cpm) of dhurrin-14C,15N were recovered in Experiment 2. The specific activities of the dhurrin-14C,15N recovered after this second chromatography step, therefore, were 6,630 cpm per μmole in Experiment 1 and 12,900 cpm per μmole in Experiment 2. The insignificant change in the specific activities after the second chromatographic separation indicated that the dhurrin-14C,15N was radiochemically pure at this stage.

The specific activity of the nitrile carbon atom of the purified glucoside was determined by recovery of the nitrile atoms as H14CO2N. Aliquots of purified dhurrin solution containing approximately 2.5 μmoles of glucoside were hydrolyzed with emulsion in H14CO2N, p-hydroxybenzaldehyde, and glucose. The H14CO2N was aerated into traps containing 10 ml of 0.1 N NaOH. These were changed at 2-hour intervals during aerating, and the alkaline trapping solutions from each aeration period were assayed for radioactivity by counting 1-ml aliquots in 10 ml of Bray’s solution. Cyanide was determined by the method of Aldridge (11), and these data were used to calculate the specific activity of the Na14CO3N present in each trap. The specific activity of the Na14CO3N recovered in the first two aeration periods was found to be the same.

Since it was possible that the purified dhurrin-14C,15N might contain a nitrogenous impurity, it seemed desirable to obtain ammonia from the cyanogenic glucoside by more than one method in order to determine its 15N content.

 Approximately 10 μmoles of dhurrin were hydrolyzed to yield ammonia by refluxing the solution in 5 N NaOH in a Kjeldahl distillation apparatus for 40 min. During the hydrolysis, the outlet of the apparatus was submerged in 10 ml of 2% boric acid. Although dhurrin is known to be decomposed by dilute alkali to ammonia and HCN, it was necessary to employ a steam distillation apparatus for 4 hours. The ammonia was recovered from this digest by steam distillation into the 2% boric acid and determined colorimetrically by nesslerization of samples containing 1 to 2 μmoles.

Dhurrin (10 μmoles) was also hydrolyzed by boiling with 1.5 ml of a Kjeldahl digestion solution (13) in a micro-Kjeldahl apparatus for 3 hours. The ammonia was recovered from this digest by making its alkaline with concentrated base and steam-distilling the ammonia into 10 ml of 2% boric acid. This method of hydrolysis of the glucoside gave quantitative recovery of the nitrate nitrogen as ammonia, as shown on analysis of aliquots of the boric acid trapping solution by nesslerization. The ammonia recovered by the two methods of hydrolysis was prepared for mass spectrometric analysis by adding 1 ml of 1 N H2SO4 and 45 μmoles of (NH4)2SO4 to the boric acid trapping solution and evaporation to dryness in a vacuum. Subsequent analysis showed that the ammonia recovered by either hydrolytic

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific activity of a carbon of L-tyrosine-14C fed*</th>
<th>14N content of L-tyrosine-14N fed</th>
<th>Ratio, 14C:15N, as L-tyrosine-14C,15N</th>
<th>Specific activity of H14CO2N from dhurrin isolated</th>
<th>15N content of H14CO2N from dhurrin</th>
<th>Dilution of 14C</th>
<th>Dilution of 15N</th>
<th>Ratio, 14C:15N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.800</td>
<td>50.03</td>
<td>1:1</td>
<td>010</td>
<td>1.17</td>
<td>34</td>
<td>43</td>
<td>1.26:1</td>
</tr>
<tr>
<td>2</td>
<td>42.300</td>
<td>50.03</td>
<td>2:1</td>
<td>1220</td>
<td>1.18</td>
<td>35</td>
<td>42</td>
<td>2.40:1</td>
</tr>
</tbody>
</table>

* L-Tyrosine administered: in Experiment 1, 18.3 μmoles (0.03 atom % excess) of L-tyrosine-14C; in Experiment 2, 18.3 μmoles of L-tyrosine-14C (0.03 atom % excess) and 6 μC (0.99 x 10⁶ cpm; 0.032 μmole) of L-tyrosine-14C.

† Dilution of 14C is the ratio of the specific activity of L-tyrosine-14C,15N fed to that of the H14CO2N isolated.

‡ Dilution of 15N is the ratio of the 15N content of L-tyrosine-14C,15N fed to that of the nitrogen recovered as 15NH3 from the nitrate group of dhurrin-14C,15N.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acid nitrogen recovered</th>
<th>15N content</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tyrosine</td>
<td>6.6</td>
<td>1.083</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>5.9</td>
<td>0.130</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>2.5</td>
<td>0.513</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>1.8</td>
<td>0.092</td>
</tr>
</tbody>
</table>
method had the same $^{15}$N enrichment, indicating that no nitro-
genous impurity was present in the purified dhurrin.

The amino acids, which had been initially removed on Dowex 50-X8, were eluted from the resin with four 50-ml charges of 0.4 M NH$_4$OH in 80% ethanol. The eluates were combined and

evaporated to dryness in a vacuum, and the residues were

repeatedly dissolved in water and evaporated until no odor of

ammonia remained. The eluted amino acids were then chromo-

tographed in two dimensions on Whatman No. 3MM paper

in the solvent systems 1-butanol-acetic acid-water (630:100:270,

v/v) and 80% phenol (14). Standard chromatograms contain-

ing n-tyrosine, n-aspartic acid, and n-asparagine were run con-

comitantly. The amino acids were located by preparing radio-

autograms of the developed papers and comparing these with the

standard maps.

Areas corresponding to specific groups of amino acids were cut

from the two-dimensional chromatograms and the amino acids

were eluted with water. Further purification of amino acids was

 effected by chromatography in appropriate solvents (15). Se-

lected purified amino acids were digested in a micro-Kjeldahl

apparatus with 1 ml of the digest solution, and the ammonia was

transferred into 10 ml of 2% boric acid, as described above.

The ammonia recovered was assayed and prepared for $^{15}$N analy-

sis, as described for the ammonia recovered from dhurrin.

RESULTS AND DISCUSSION

Table I shows that the specific activity of the H$_2^{15}$N isolated

in Experiment 2 is exactly twice that isolated in Experiment 1.

The $^{15}$N content of the nitrile nitrogen recovered in both experi-

ments is approximately the same. That the nitrogen atom of

L-tyrosine is extensively retained during conversion of the amino

acid to the aglycone is best seen by calculating the dilution factors

for the carbon and nitrogen isotopes which were administered.

Since the dilution factor of 43 for $^{15}$N was only 25% greater than

the factor of 34 for the carbon isotope, the nitrogen atom was

retained approximately 75% as effectively as the carbon atom.

The exact figures for the two experiments were 74 and 80%,

respectively. Such data indicate that the bond between the

$\alpha$-carbon and the nitrogen atoms of tyrosine is not severed during

the formation of dhurrin from the amino acid. Thus the bio-

synthetic route involving C—N cleavage (5) is improbable.

Although the $^{14}$N of the tyrosine fed could have been diluted

by transamination reactions in the intact plant, the retention of

75% of the $^{15}$N isotope indicates very efficient conversion. That

such transamination did occur is shown in Table II, where the

$^{15}$N content of three amino acids known to undergo transamina-

tion readily is shown. The L-tyrosine which was isolated from

the plant at the end of the experiment was also analyzed. It is

interesting to compare the relatively high $^{15}$N content of alanine

with that of glutamic acid, an amino acid known to be active in

transamination.

As reported previously (3), tyrosine administered to sorghum

seedlings is extensively converted to dhurrin under the experi-

mental conditions employed here; 14% of the L-tyrosine-14C, $^{15}$N

administered in Experiment 1 was incorporated, while 12.7 per-

cent was incorporated in Experiment 2.

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dhurrin sample from Dr. Laurens Anderson, and the kindness

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erous gift of L-tyrosine-$^{15}$N used in this work.

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