Indole-3-acetic Acid Catabolism in Zea mays Seedlings

METABOLIC CONVERSION OF OXINDOLE-3-ACETIC ACID TO 7-HYDROXY-2-OXINDOLE-3-ACETIC ACID 7'-O-B-D-GLUCOPYRANOSIDE

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A new metabolite of the plant growth substance indole-3-acetic acid has been extracted from Zea mays seedlings and characterized as the 7'-O-B-D-glucopyranoside of 7-hydroxy-2-oxindole-3-acetic acid. This compound is the major product formed in [5-3H]2-oxindole-3-acetic acid, incubated with intact plants or root and coleoptile sections. Identification was by gas chromatography-mass spectrometry of the trimethylsilyl derivative and by analysis of the hydrolysis products. A synthesis is reported for 7-hydroxy-2-oxindole-3-acetic acid. These results and prior work demonstrate the following catabolic route for indole-3-acetic acid in Zea: indole-3-acetic acid → 2-oxindole-3-acetic acid → 7-hydroxy-2-oxindole-3-acetic acid → 7-hydroxy-2-oxindole-3-acetic acid glucoside.

Indole-3-acetic acid (IAA) is the first plant growth hormone to be discovered and is involved in the control of developmental processes such as stem elongation and phototropism (1). Knowledge of the role and controls of IAA synthesis and breakdown is necessary for understanding how the plant regulates endogenous amounts of the hormone.

Previous studies (2-4) established that 2-oxindole-3-acetic acid (OxIAA) is the major catabolite of IAA in Zea mays seedlings. OxIAA is the most abundant metabolite formed from labeled IAA supplied to roots, coleoptiles, and endosperms (2, 4, 5). Endogenous OxIAA is present in the shoots and kernels of maize seedlings at levels similar to those of IAA (360 nmol/kernel, 50 pmol/shoot (3)).

In the present work, [5-3H]OxIAA was synthesized and fed to intact plants by application to the endosperm and to root and coleoptile segments. The major radiolabeled metabolite after 24 h of incubation was extracted, purified, and shown to be 7-hydroxy-2-oxindole-3-acetic acid 7'-O-B-D-glucopyranoside (7-OH-OxIAA-Glc). This is a new compound not previously found in nature and represents the second step in catabolism of a major plant hormone.

There is precedence for ring hydroxylation of oxindoles by plants. For example, 5-hydroxyoxindole-3-acetic acid and 5-hydroxydihydroindole-3-acetic acid have been isolated and characterized (6). In addition, zeaxanthin, a quinolone formed by ring expansion of 7-hydroxydihydroindole-3-acetic acid, was found in corn steep liquor (7).

MATERIALS AND METHODS

Plant Material—Z. mays seedlings (c.v. Stowell’s Evergreen, W. Atlee Burpee Co.) were grown in darkness at 25 °C as previously described (8). About 0.5-1 µmol of oxidation product could be prepared by incubating 1000 root segments with 2.1 × 10^6 Bq OxIAA (2.1 × 10^6 Bq Bq-nmol⁻¹) for 24 h. A similar amount of the identical product was obtained from 1250 kernels of 5-day-old seedlings, 100 of which had each been injected with [3H]OxIAA (830 Bq, 1 × 10^6 Bq-nmol⁻¹), and an identical product could be obtained by application of [5-3H]OxIAA to coleoptile sections.

Synthesis of Unlabeled and [5-3H]OxIAA—Unlabeled OxIAA was synthesized using the method of Hinman and Bauman (9) in which IAA, dissolved in dry t-butyl alcohol, is oxidized by equimolar amount of N-bromosuccinimide. The product was purified by partitioning into ethyl acetate from an aqueous solution and recrystallized from acetone/benzene. mp 141-143 °C; UV λmax in nanometers (i) 280 shoulder (1251), 249 (7686), 206 (2237) in 85% ethanol; the 70-eV mass spectrum of the methyl ester, m/z (relative intensity, percent) molecular ion 205 (23), 172 (48), 146 (40), 145 (100), 144 (72), 132 (16), 117 (55), 116 (47), in agreement with the mass spectrum previously published (6).

[5-3H]OxIAA was synthesized in the same manner on a smaller scale. [5-3H]IAA (1.4 mg, specific activity 1 × 10^8 Bq-nmol⁻¹) was dissolved in 30 µl of dry t-butyl alcohol. To this was added, in 6, µl aliquots with mixing, N-bromosuccinimide (1.42 mg) dissolved in 20 µl of t-butyl alcohol. After 2 h at ambient temperature with intermittent mixing, the reaction mixture was diluted with 1 ml of 50% aqueous propan-2-ol. This was loaded onto a 170 column of Sephadex LH-20, eluted with 50% aqueous propan-2-ol. OxIAA eluting after 8-9 ml was obtained in 34% yield. Half of the [3H]OxIAA was diluted with unlabeled OxIAA to a specific activity of 5.7 × 10^5 Bq-nmol⁻¹; the remainder had a specific activity of 1 × 10^6 Bq-nmol⁻¹.

The identity of the labeled product was confirmed by co-chromatography with authentic material on two high-performance liquid chromatography (HPLC) columns: Partisil-10 ODS (Whatman) and PRP-1 (Hamilton), eluted with 20% aqueous ethanol plus 1% acetic acid (a). This was followed by 20% aqueous acetonitrile plus 1% acetic acid (b).

7-Hydroxy-2-oxindole-3-acetic acid, 12685, was prepared in 88% overall yield from 2-nitro-3-methylphenol by the method used to prepare 4-benzoyloxindole (10). Reaction of this indole with 1.15 eq formaldehyde in aqueous acetic acid (11) produced 7-benzyloxindole, m.p. 170-172 °C; NMR (CDCl3) δ 2.3 (s, 3H), 3.7 (s, 2H), 5.2 (s, 2H), 6.8-7.5 (br m, 9H total). Conversion of 7-benzyloxindole to 7-benzyloxindole-3-acetonitrile was effected in 75% yield by treatment with potassium cyanide (4 eq) and iodomethane (4.8 eq) in N,N-dimethylformamide followed by extraction and recrystallization from ethyl acetate/hexane: mp 116-117 °C.
NMR (CDCl₃) δ 3.8 (s, 2H), 5.3 (s, 2H), 6.8-7.5 (br m, 9H total). Hydrolysis of the indoleacetonitrile with excess NaOH in aqueous ethanol at reflux gave 7-benzyloxyindole-3-acetic acid in 56% yield after acidification and recrystallization from propan-2-ol/water: m.p. 163-165 °C; NMR (CDCl₃-dimethyl sulfoxide-d₄) δ 3.7 (s, 2H), 5.3 (s, 2H), 6.7-7.6 (br m, 9H total). Oxidation of 7-benzyloxyindole-3-acetic acid with 1 eq N-bromosuccinimide in moist t-butyl alcohol and treatment of the crude oxidation product with phenyldiazomethane gave (after chromatography on silica gel with 2:1 hexane/ethyl acetate and recrystallization from ethyl acetate/hexane) a 30% yield of benzyl-7-benzyloxy-2-oxindole-3-acetate: m.p. 144-146 °C; NMR (acetone-d₆) δ 2.82 and 3.08 (AB of ABX, 2H, J_AB = 16 Hz, J_AA = 9 Hz, J_BB = 3 Hz), 3.8 (m, X of ABX, 1H), 6.5 (s, 1H), 7.0-7.6 (br m, 13H total). Hydrogenolysis of benzyl-7-benzyloxy-2-oxindole-3-acetate over 10% palladium on charcoal in tetrahydrofuran at 50 psi for 72 h provided 7-hydroxy-2-oxindole-3-acetic acid in 61% yield after chromatography on silica gel using 2% acetic acid in ethyl acetate as eluant and recrystallization from acetone/benzene: m.p. 189-191 °C; IR (Nujol mull) 3600, 3600-2900, 1710, 1680, 1650 cm⁻¹; NMR (acetone-d₆) δ 2.8 (d of q, AB of ABX, 2H, J_AB = 16 Hz, J_AA = 9 Hz, J_BB = 7 Hz), 3.8 (m, X of ABX, 1H), 6.9 (s, 3H); field-desorption high-resolution mass spectrum; exact mass; calculated 207.053; found 207.054.

Synthesis of 1,2,3,4-Tetrahydro-8-hydroxy-2-oxoquinoline-4-carboxylic acid-7'-O-D-glucoside

Acid was converted to the quinolone derivative by dissolving in 6% HC₁ and heating in a boiling water bath for 1.5 h (Fig. 1). The reaction was quantitative. The identity of the product was confirmed by mass spectrometry of the trimethylsilyl (Me₃Si derivative; m/z (relative intensity, per cent) molecular ion 423 (6.8), 408 (87), 351 (16), 306 (65), 290 (64), 218 (13), 202 (10); UV absorbance; λ_max = 212, 250 and 294.

Extraction and Purification Procedure—After incubation, plant material was washed with water, kernels were homogenized, and all tissues were extracted at 4 °C in methanol. Extracts were filtered, evaporated to the aqueous phase, and diluted to five times the volume with distilled water. The aqueous extracts were partitioned three times against equal volumes of freshly distilled diethyl ether. The aqueous phase was retained and loaded onto Sep-Pak CN cartridges (Waters Associates), and the radioactive material was eluted with 50% ethanol. Further purification was carried out using a DEAE-Sephadex column (acetate form) eluted sequentially with 25 ml of aqueous ethanol, 25 ml of 1% (v/v) acetic acid in 50% aqueous ethanol, and finally 100 ml of 10% (v/v) acetic acid in 50% aqueous ethanol. OxIAA and its metabolites elute in the last solvent and were further purified using a Sephadex LH-20 column eluted with 50% propan-2-ol.

Samples were purified on two HPLC columns: first an ion exchange Partisil-10 SAX (Whatman) column eluted with a gradient of 0-10% acetic acid in 50% aqueous ethanol over 20 min and secondly a reverse-phase Partisil-10 ODS (Whatman) column eluted with a gradient of 10-60% ethanol in 1% aqueous acetic acid over 30 min. In each case, the extracts were injected in 200 µl of the initial eluting solvent. Radioactive metabolites were located by counting aliquots using a Beckman LS 7000 liquid scintillation spectrometer.

Spectrometry—UV spectra were obtained using a Cary model 15 scanning spectrophotometer. NMR spectra were obtained using a Bruker WM-360 360 spectrometer. Mass spectra were obtained at 70 eV using a Hewlett-Packard 5985 quadrupole combined gas chromatograph-mass spectrometer (GC-MS) with a 1.8 m x 3 mm inner diameter 3% SP2250 column. For Me₃Si derivatives of the free oxindoles and quinolones, the column temperature was programmed from 200 to 280 °C at 10 °C min⁻¹. Chromatography of the Me₃Si glucoside was isothermal at 300 °C. The high resolution exact mass mass spectrum was obtained using a Varian MAT CH5 double focusing...
mass spectrometer. Trimethylsilyl derivatives were prepared by dissolving the dried compounds in 20 μl of dry pyridine and heating with 30 μl of bis(trimethylsilyl)trifluoroacetamide at 60 °C for 2 h.

α- and β-Glucosidase—α-Glucosidase from yeast and β-glucosidase from almonds were obtained from Sigma. Both were made up in 0.05 M Na2HPO4/citrate buffer (0.5 mg·ml⁻¹) with α-glucosidase at pH 6.8 and β-glucosidase at pH 5.0. Enzyme solutions (0.5 ml) were added to dried samples and incubated for 30 min at 30 °C. The protein was precipitated by addition of 1 ml of ethanol followed by centrifugation. The products were analyzed by C18 HPLC.

Enzymatic Glucose Determination—Colorimetric enzymatic glucose determination was carried out using Sigma kit 115. The assay is based on hexokinase and glucose-6-phosphate dehydrogenase coupled to the reduction of p-iodonitrotetrazolium chloride to the formazan.
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7-Hydroxy-2-oxindole-3-acetic Acid 7'-O-β-D-Glucoside

(14). This compound has a molar extinction coefficient of 14,600 at 520 nm.

RESULTS

Following a 24-h incubation of the plant tissues with [3H] OxIAA, HPLC analysis of crude methanolic extracts disclosed one major metabolic peak with a retention time on a C8 column of 8.3 min compared to that of 13.5 min for OxIAA. Thus, the compound was more polar than OxIAA. We presumed that the metabolite might be 7-hydroxylated since Matsushima et al. (7) described the isolation of zeanic acid (1,2-dihydro-8-hydroxy-2-oxoquinoline-4-carboxylic acid) from corn steep liquor. Zeanic acid could have resulted from cyclization of 7-hydroxydioxindole-3-acetic acid since ring expansion is a common reaction of oxindoles upon heating with acid or base (13). The UV absorption spectrum of the metabolite in 50% ethanol showed λmax at 210, 249 and 286 nm, appropriate for an oxindole hydroxylated in the 7-position. On heating the plant metabolite in 6% HCl at 100 °C for 1 h, the radioactive product co-chromatographed with synthetic 1,2,3,4-tetrahydro-8-hydroxy-2-oxoquinoline-4-carboxylic acid on both the ion exchange and reverse-phase HPLC columns having retention times of 7.2 and 14.3 min, respectively. The Me3Si derivatives of the synthetic quinoline and the hydrolyzed plant metabolite were analyzed by GC-MS. The spectra obtained were identical, with the molecular ion at m/z 423 and major fragment ions at m/z 409 (M – Me), 351 (M – Me3Si), 306 (M – COO-Me3Si), 290, 218, and 204.

Despite the formation of identical products upon acid treatment of authentic 7-hydroxydioxindole-3-acetic acid or the plant metabolite, it was found that the plant metabolite, before acid treatment, differed in its HPLC retention time from authentic 7-hydroxydioxindole-3-acetic acid. To attempt to explain this difference, the Me3Si derivative was prepared from the untreated metabolite and analyzed by GC-MS. Ions were observed at m/z (relative intensity, per cent) 801 (0.97), 450 (2.6), 423 (11), 362 (31), 351 (37), 271 (7.9), 234 (8.1), 217 (19), 204 (2.9), 191 (3.1) as can be seen in Fig. 2. The mass spectrum of the intact metabolite was consistent with it being a conjugate of 7-hydroxydioxindole-3-acetic acid with a hexose sugar, and thus the acid treatment had caused ring expansion and also removed a conjugating hexose moiety. Ions at m/z 450, 361, 271, 204, and 191 are indicative of a 1-0-glucose conjugate (16). A molecular ion at m/z 873, for the fully derivatized compound with seven Me3Si groups, was not observed, probably owing to the facile loss of Me3Si from the enolized 2-hydroxyl group. However, M – Me3Si=801 was observed, as was m/z 423, resulting from cleavage of the tetrasilylated glucose from the trisilylated 7-OH-OxIAA nucleus. Ions at m/z 351 and 234 would be expected from the oxindole fragment.

The compound could also be hydrolyzed quantitatively by β-glucosidase but not by α-glucosidase. The radioactive product from enzymatic hydrolysis co-eluted with synthetic 7-hydroxyoxindole-3-acetic acid, both having retention times of 5.1 and 7 min, respectively, on the ion exchange and the C8 HPLC columns.

These data prove that the metabolite is a hexose conjugate of a hydroxylated derivative of oxindole-3-acetic acid. However, the mass spectrum does not establish the location of the hydroxyl group. The UV absorbance spectra of 4-, 5-, 6-, and 7-hydroxyoxindoles are distinct (15). The UV spectra of 7-hydroxyoxindole-3-acetic acid and the product of enzymatic hydrolysis showed identical λmax at 212, 248, and 291 nm. The spectra of the acid hydrolysis product and that of the synthetic quinolone derivative were also identical (λmax 212, 250, and 294), confirming that the hydroxyl group was at the 7-position of the oxindole.

Acid hydrolysis of the OxIAA metabolite followed by silylation and GC-MS analysis revealed two peaks with retention times and spectra identical to the Me3Si derivatives of the α and β anomers of D-glucose. Fragment ions were found at m/z 435, 393, 361, 345, 305, 217, 204, and 191. The molecular ion was not present. The identity of the conjugating moiety...
was further confirmed by a colorimetric enzymatic method. This method is based on the enzymes hexokinase and glucose- 
6-phosphatase from liver and, in the absence of hexose isomerases (14). A sample of the plant metabolite was hydrolyzed for 30 min with β-
glucosidase. The enzyme-color reagent was then added and 
cultivated for a further 10 min. After terminating the reaction by the addition of HCl, the absorbance at 520 nm was mea-
sured and the amount of glucose was calculated by comparison to 
a salinum standard. The ratio of glucose to 7-OH-OxaIAA was 1.15:1.

Spectroscopic data allow an unambiguous assignment of the 
attachment of the conjugating sugar to 7-OH-OxaIAA among the four possible sites: the 7-hydroxyl group, the 
carboxyl group, the oxindole nitrogen, or the 2-oxygen in the 
2-hydroxyindole tautomeric form. Conjugation through the 2-
oxygen in the 2-hydroxyindole tautomeric form was excluded by the 360-MHz NMR spectra of the natural product (Fig. 3, a and b). Had conjugation occurred through the 2-oxygen in the 2-hydroxyindole tautomeric form, no proton would be attached to the oxindole 3-position and, consequently, no 
spin-spin coupling of this proton to the acetic acid side chain methylene protons would be observed. The signal resulting 
from the oxindole 3-hydrogen is obscured by carbohydrate 
signals in the 3.5-4.0 range of the NMR spectrum (Fig. 3a) and cannot be observed directly. However, the coupling be-
tween the 3-hydrogen and one of the methylene protons and the AB splitting pattern of these protons (Fig. 3b) can be 
seen and establish the presence of the 3-hydrogen and exclude glucosylation through the 2-oxygen of a 2-hydroxyin-
dole tautomer. Carboxyl conjugation could be excluded since 
the plant metabolite eluted from a SAX column with a reten-
tion time of 5.1 min which is similar to that of the acetyl 
derivative of 7-OH-OxaIAA (5.4 min), thus indicating a free 
carboxyl group. This conclusion is supported by the mass 
spectral data since the silylated natural product shows an oxindole fragment at \( m/z \) 351 which loses CO₂-Me₃Si, yielding a fragment ion at \( m/z \) 234. NMR data exclude conjugation to the 
2-oxygen of the tautomized 2-hydroxyindole form, and chromatographic and mass spectral data show that con-
jugation does not occur through the carboxylic acid side chain. 
The UV spectrum affords proof for the site of sugar attach-
ment. The UV spectra of phenolic compounds are dependent 
upon pH and exhibit a characteristic bathochromic shift in 
alkaline solution resulting from an increased concentration of 
the phenoxide ion. At pH 10, the phenolic group in free 7-
OH-OxaIAA is ionized, causing the expected shift in the UV 
spectrum (Fig. 4a). The spectrum of the plant metabolite showed no such change (Fig. 4b), nor did the spectrum of 
oxindole-3-acetic acid (Fig. 4c). Thus, these experiments dem-
onstrate that glucose conjugation occurs through the 7-hy-
droxy of 7-OH-OxaIAA since the unconjugated 7-OH-OxaIAA 
shows the expected bathochromic shift arising from a free 
phenolic group (Fig. 4a), while the conjugated natural product 
does not (Fig. 4b). The other functional groups, carboxyl and 2-oxindole, are unaffected by a change in pH (Fig. 4c).

CONCLUSION

From the above data, we conclude that OxaIAA is metabo-
lized to 7-hydroxy-2-oxindole-3-acetic acid 7'-O-β-D-glucoso-
side (Fig. 5). This compound has not previously been identi-
ﬁed in plant tissue nor have there been any prior reports of 
its occurrence in any biological system. 7-OH-OxaIAA-Glc was 
produced in the roots, shoots, and kernels of Z. mays seedlings 
following application of amounts of [3H]OxaIAA equal to less 
than 1% of the endogenous level. In a separate paper (8), we 
show that 7-OH-OxaIAA-Glc was also produced from [5-3H] 
I AA supplied to Z. mays seedlings and have measured its 
endogenous levels. We therefore conclude that 7-OH-OxaIAA-
Glc is a natural metabolite of IAA in Zea seedlings and that 
IAA is catabolized in Z. mays seedlings by the following route: 
IAA → OxaIAA → 7-hydroxy-2-oxindole-3-acetic acid → 7-
hydroxy-2-oxindole-3-acetic acid 7'-O-β-D-glucoside.

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