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OCCURRENCE AND METABOLISM OF 7-HYDROXY-2-INDOLINONE-3-ACETIC ACID IN *ZEA MAYS*

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Key Word Index—*Zea mays*; Gramineae; biosynthesis; IAA; catabolism; 2-indolinone-3-acetic acids; 7-hydroxy-2-indolinone-3-acetic acid.

Abstract—7-Hydroxy-2-indolinone-3-acetic acid was identified as a catabolite of indole-3-acetic acid in germinating kernels of *Zea mays* and found to be present in amounts of *ca* 3.1 nmol/kernel. 7-Hydroxy-2-indolinone-3-acetic acid was shown to be a biosynthetic intermediate between 2-indolinone-3-acetic acid and 7-hydroxy-2-indolinone-3-acetic acid-7′-*O*-glucoside in both kernels and roots of *Zea mays*. Further metabolism of 7-hydroxy-2-[5-^{3}H]-indolinone-3-acetic acid-7′-*O*-glucoside occurred to yield tritiated water plus, as yet, uncharacterized products.

INTRODUCTION

Previous studies demonstrated that IAA is catabolized in *Zea mays* L. without loss of the carboxyl carbon. Incubation of radiolabelled IAA established [1, 2] its conversion to 2-indolinone-3-acetic acid (OxIAA, **1**) and subsequently [3, 4] to 7-hydroxy-2-indolinone-3-acetic acid-7′-*O*-glucoside (7-OH-OxIAA-glu, **3**). These compounds were shown to be endogenous components of the kernels, shoots and roots. However, the postulated intermediate between OxIAA and 7-OH-OxIAA-glu, 7-hydroxy-2-indolinone-3-acetic acid (7-OH-OxIAA, **2**), has not been shown to be an endogenous plant component nor has it been shown that radiolabelled IAA or OxIAA is converted to 7-OH-OxIAA by plants. Our finding [5] that [5-^{3}H]-IAA was converted to [5-^{3}H]-7-OH-OxIAA by *Zea* root segments prompted us to examine the kernels for the presence of this catabolite. This paper reports the characterization and quantitative estimation of 7-OH-OxIAA as an endogenous component of *Zea* kernels. Further, the metabolism of [5-^{3}H]-7-OH-OxIAA and its 7′-*O*-glucoside were investigated and the results are reported.

We have also shown the tissue-catalysed conversion of 7-OH-OxIAA to 7-OH-OxIAA-glu and the hydrolysis of 7-OH-OxIAA-glu to 7-OH-OxIAA. In addition, we demonstrate that the further metabolism of both [5-^{3}H]-7-OH-OxIAA and its glucoside leads to loss of tritium as water and the formation of more polar metabolites, suggesting that the next oxidation step involves ring hydroxylation at the 5-position with only partial tritium retention.

RESULTS AND DISCUSSION

An 80% aqueous methanolic extract from 4-day-old *Z. mays* kernels was purified by chromatography on a DEAE-Sephadex column, using trace amounts of added [5-^{3}H]-7-OH-OxIAA as an internal standard and as an aid in determining retention times. Appropriate fractions from the eluate were then chromatographed to constant specific radioactivity on C_{18}-ODS and PRP-1 HPLC columns.

The 250 MHz ^{1}H NMR spectrum of the HPLC-purified fraction in D_2O was identical with that of an authentic specimen of 7-OH-OxIAA. The spectral data indicated the lability [6] of H-3 in the 2-indolinone-3-acetic acid series. Thus, the spectra showed a pair of doublets at δ2.96 and 2.82 for the 3′-methylene protons in the ^{2}H-3 labelled compound, and a signal at 3.74, much reduced in intensity, due to the residual H-3. In addition, small doublets of doublets at δ2.97 and 2.83 were observed due to the 3′-methylene protons in the 3-protio compound. Exchange of the H-3 proton proceeded to 73% completion upon lyophilizing the sample twice from D_2O and analysis after 15 min, and *ca* 94% after 24 hr as determined by integration of the H-3 and CH_2-3′ NMR signals. As expected, lyophilization of the deuterated sample from water caused full exchange of the H-3 proton. The same exchange behaviour was observed in DMSO-d_6, although not in C_5D_5N or acetone-d_6. These NMR data indicate that determination of the absolute stereochemistry of plant-produced 2-indolinone-3-acetic acids is essentially precluded owing to the rapid racemization at C-3 in aqueous medium.

The HPLC-purified extract was further analysed as its trimethysilyl derivative by capillary GC/MS. In common with the behaviour of 7-OH-OxIAA-glu [4], the derivatization of plant-produced 7-OH-OxIAA yielded several products. One of the products was identical in retention time and mass spectral fragmentation pattern to the major product from the derivatization of authentic 7-OH-OxIAA. A more useful derivative could be obtained from the purified plant material by acid-catalysed rearrangement [3, 4, 7] to 8-hydroxy-1,2,3,4-tetrahydro-2-quinolone-4-carboxylic acid (8-OH-THQCA, **8**). This compound trisilylated readily using the same derivatization conditions as for the non-rearranged compound (**2**). Both silylated compounds had very similar mass spectra,

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with major ions at m/z 423 $[M]^+$, 408 $[M-Me]^+$, 306 $[M-CO_2TMSi]^+$, 290 $[M-HCO_2TMSi-Me]^+$ and 233 $[M-CO_2TMSi-TMSi]^+$, although having different GC retention times (8-OH-THQCA: 6.3 min; 7-OH-OxIAA: 7.0 min).

The amount of 7-OH-OxIAA in the kernels was estimated by two methods: (1) by using the 254 nm absorbance of the HPLC peaks; and (2) by GC/SIM/MS analysis using [3',3''-$^{13}C_2$]-7-OH-OxIAA as an internal standard. By UV absorbance an estimate of 4.8 nmol/kernel was made whereas by GC/SIM/MS and measuring the ratio of ions counted at $[M]^+$ to those at $[M+2]^+$ a value of 3.1 nmol/kernel was obtained. The GC/SIM/MS estimate is most reliable but, in any event, it is clear that the amount of 7-OH-OxIAA in the kernel is about equal to that of 7-OH-OxIAA-glu (see Table 1).

That the 7-OH-OxIAA was not produced from the glucoside during the extraction procedure was demonstrated by the isolation of 7-OH-OxIAA from a kernel homogenate to which [5-^{3}H]-7-OH-OxIAA-glu had been added. Less than 1% of the radioactivity added eluted in the 7-OH-OxIAA HPLC fraction.

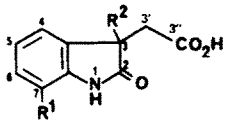
Having established 7-OH-OxIAA to be an endogenous IAA-catabolite in 4-day-old *Zea* kernels, the metabolism of this compound and its 7'-*O*-glucoside was investigated. Initial experiments using whole plants showed that after 24 hr *ca* 65% of the applied ^{3}H could be recovered by methanol extraction of a kernel homogenate (with less than 1% transported into the shoots plus roots), compared with a control experiment using boiled plants. However, in the control experiments, typically only 45–70% of the applied radioactivity could be accounted for. Further, the kernel extracts required purification before HPLC analysis was possible. During this time, both the [^{3}H]-7-OH-OxIAA and its glucoside were partially converted non-enzymatically to previously reported [5] but unidentified more polar compounds. For example, incubating 45 kernels with 4.1×10^6 dpm of [^{3}H]-7-OH-OxIAA over a 24 hr period gave, upon isolation, 1.6×10^6 dpm of a mixture containing 25% substrate, 14% 7'-*O*-glucoside, plus 56% 7-OH-OxIAA-decomposition product (by HPLC analysis). By analogy with the decomposition of [^{3}H]-OxIAA, where the major product and its acid-rearrangement product behave on HPLC like 3-OH-OxIAA (**4**) and 2-quinolone-4-carboxylic acid (**7**), respectively, the decomposition products of 7-OH-OxIAA and 7-OH-OxIAA-glu are probably the corresponding 3-hydroxylated compounds **5** and **6**. The ease of oxidation of OxIAAs at the 3-position possibly accounts for the reported isolation of zeanic acid from sulphite-treated corn steep liquor [8].

The formation of the decomposition products was reduced by manipulating the extracts under nitrogen. To reduce oxidation further and to increase recoveries, subsequent experiments were performed using root segments, since root extracts required less purification. A 24 hr incubation of either [5-^{3}H]-7-OH-OxIAA (**2**) or its 7'-*O*-glucoside (**3**) gave the results shown in Table 2. Following incubation of 7-OH-OxIAA, less than 1% was recovered unchanged, while almost 30% was recovered as the glucoside (**3**). Upon incubation of the 7'-*O*-glucoside (**3**), *ca* 24% remained unchanged, while 2% was recovered as 7-OH-OxIAA (**2**). Moreover, incubations of the glucoside (**3**) yielded *ca* 15% of the initial radioactivity in the THO fraction. This indicates hydroxylation at C-5 with only partial tritium retention, possibly by an 'NIH-shift' reaction, as has been previously observed for aromatic hydroxylations in plants (e.g. ref. [9]).

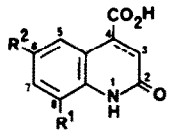
The major radioactive fraction obtained from incubation of 7-OH-OxIAA or 7-OH-OxIAA-glu was the

Table 1. Levels of IAA and its catabolites in kernels of *Z. mays*

Compound	pmol/plant	Ref.
IAA	308	11
OxIAA (1)	357	1
7-OH-OxIAA (2)	3100	
7-OH-OxIAA-glu (3)	4800	4



(1) R^1 = H R^2 = H
(2) R^1 = OH R^2 = H
(3) R^1 = O-B-D-glucose R^2 = H
(4) R^1 = H R^2 = OH
(5) R^1 = OH R^2 = OH
(6) R^1 = O-B-D-glucose R^2 = OH



(7) R^1 = H,Δ3,4 R^2 = H(T)
(8) R^1 = OH R^2 = H(T)
(9) R^1 = OH R^2 = OH

Table 2. Distribution of radioactivity following 24 hr incubation of [5-n-^{3}H]-7-OH-OxIAA (**2**) or [5-n-^{3}H]-7-OH-OxIAA-glu (**3**) with *Z. mays* root segments

Fraction	% Radioactivity incubated			
	2	[control*]	3	[control*]
THO	n.d.	n.d.	15.3	[0.5]
MeOH	28.5†	[94.5]	26.5‡	[92.1]
OH^-	44.0	[n.d.]	39.3	[13.0]
Total	72.5	[94.5]	81.1	[105.6]

n.d., not determined.

*HPLC analysis indicated each control fraction to contain only unchanged substrate plus 5–10% of the respective decomposition product (see text).

†2:3:6 :: 3:60:37 (by HPLC).

‡2:3 :: 7:93 (by HPLC).

fraction soluble in aqueous alkali. This fraction (i) eluted immediately after the column void volume on reverse-phase HPLC analysis using 0.5% aqueous acetic acid, and (ii) was unchanged by treatment with β-glucosidase. (iii) Treatment with 2 M HCl at 100° for 6 hr resulted in an approximately 50% ^3H loss. Reverse-phase HPLC analysis of the remaining radioactivity yielded two new radioactive fractions, the least polar of which co-eluted with authentic 8-OH-THQCA (**8**). The more polar fraction eluted in the region expected for 6,8-dihydroxy-1,2,3,4-tetrahydro-2-quinolone-4-carboxylic acid (**9**).

The behaviour of the major radioactive fraction in base and acid suggests that its greater polarity compared with that of the glucoside (**3**) may be due to additional ether-linked polar groups such as saccharides.

CONCLUSIONS

The following conclusions may be drawn:

(i) 7-OH-OxIAA is a major IAA catabolite in *Z. mays* kernels;

(ii) 7-OH-OxIAA is converted to its glucoside in both kernels and root segments. The conversion shown in Table 2 may be an underestimate owing to subsequent metabolism of the glucoside (**3**); and

(iii) 7-OH-OxIAA-glu is converted to more polar compounds by 5-hydroxylation and possibly by additional conjugation reactions.

These conclusions are summarized in Figs 1 and 2.

EXPERIMENTAL

[5-n-^3H]-7-OH-OxIAA and its 7′-*O*-glucoside, of specific activity *ca* 1 Ci/mmol, were prepared by incubation of [5-n-^3H]-IAA with *Zea mays* root segments as described in ref. [5]. [3′,3″-$^{13}C_2$]-7-OH-OxIAA (99 atom% ^{13}C at each labelled position) was synthesized [5] by a two-step procedure involving the condensation of dibenzyl [$^{13}C_2$]oxalate with 7-benzyloxy-2-indolinone followed by reductive dehydration. *Zea mays* cv Silver Queen was germinated and grown as previously reported [5].

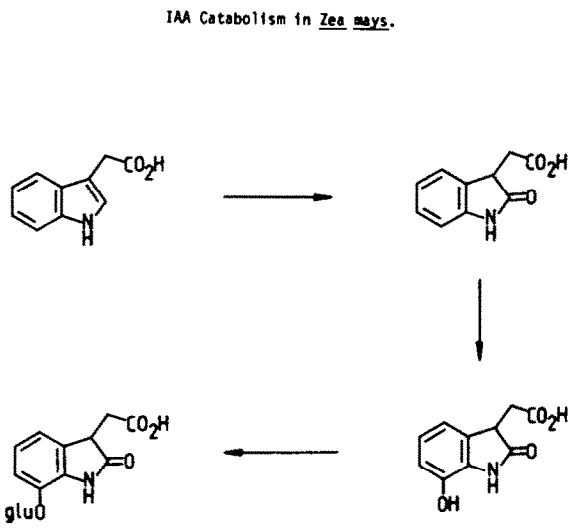


Fig. 1. A summary of the catabolism of IAA in *Z. mays*. The oxidation of IAA to OxIAA was established by Reinecke and Bandurski [1, 2] and the oxidation of IAA or OxIAA to 7-OH-OxIAA-glucoside was established by Nonhebel *et al.* [3, 4]. The present work established the natural occurrence of 7-OH-OxIAA, its interconvertability with 7-OH-OxIAA-glucoside, and its formation from IAA.

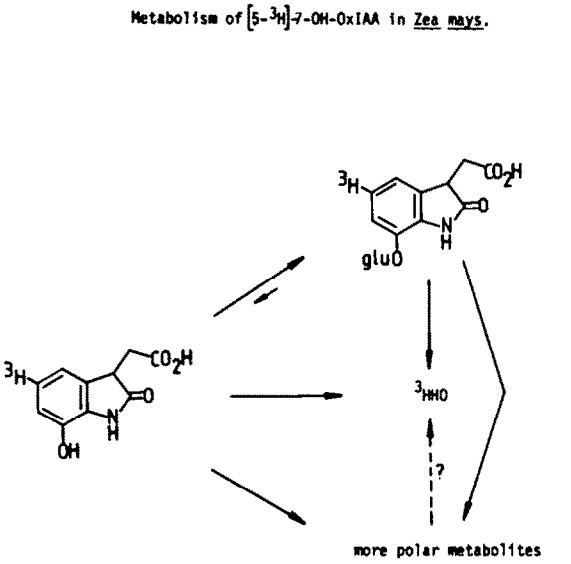


Fig. 2. The metabolism of [5-^3H]-7-OH-OxIAA by root segments from *Z. mays* seedlings. The segments will also oxidize [5-^3H]IAA to [5-^3H]-7-OH-OxIAA.

GC/MS conditions. Samples were dissolved in dry C_5H_5N (20 μl) and heated with BSTFA [bis(trimethylsilyl)-trifluoroacetamide] (20 μl) for 1.5 hr at 75°. After cooling, aliquots (2 μl) were analysed using a modified [10] Hewlett–Packard 5992A GC/MS. Injections were made into a (11 m × 0.32 mm i.d.) fused silica WCOT column (Chrompack CP Sil 8 CB, 0.11 μm) in the splitless mode. The column temp. was increased from 100° to 280° at 16° min, with He as the carrier gas at a flow rate of 1 ml/min. The injector and source temps. were 225°.

Kernels from 960 4-day-old plants were excised and homogenized in three batches in a Waring blender at *ca* −25° in 80% aq. MeOH (3 × 150 ml). [5-^3H]-7-OH-OxIAA (4.0×10^6 dpm) was added to the homogenate, and the mixture was purged with N_2 and stirred overnight at 2°. The tritiated compound amounted to about 300 ng and served as a tracer to monitor the elution of the compound during chromatography and constituted only 0.05% of the 7-OH-OxIAA isolated from the plant. The homogenate was centrifuged (4000 *g*, 15 min) and the pellet washed (× 3) with 50 ml 80% MeOH and resedimented. The supernatant solns were filtered and the filtrate was concentrated to *ca* 100 ml under red. pres. EtOH (250 ml) was added, the mixture was purged with N_2 and left to stand overnight at 2°. The precipitated protein was removed by filtration, washed with 70% aq. EtOH (100 ml) and the combined filtrates were concentrated to *ca* 25 ml. The residue was suspended in H_2O (100 ml), filtered, acidified to pH 2 with 2 M HCl, and extracted with EtOAc (4 × 150 ml). The EtOAc extract was washed with H_2O (100 ml) and concentrated to near dryness. The H_2O contained 0.28×10^6 dpm and the EtOAc contained 2.2×10^6 dpm (63% recovery).

The EtOAc extract was applied in 50% aq. EtOH to a 9 ml column of DEAE-Sephadex-acetate, and eluted with (i) 50% aq. EtOH (20 ml) and (ii) a gradient of 0–10% HOAc in 50% aq.

EtOH (100 × 1 ml). Radioactive material, a total of 2.5 × 10^6 dpm, was recovered from the HOAc gradient in fractions 40–50. This material was purified by successive HPLC steps on (i) Partisil-10 C_{18}-ODS and (ii) Hamilton 5μ PRP-1 columns (both 250 × 4.6 mm) using elution conditions as in ref. [5]. Recovery was 1.95 × 10^6 dpm (49% of that added to the kernel homogenate), corresponding to *ca* 300 μg **2**.

For ^{1}H NMR of the resultant extract, the sample was lyophilized twice from D_2O (99.8% D), then dissolved in D_2O (99.96% D); ^{1}H NMR (250 MHz): δ6.91 (*dd*, $J_1 = J_2 =$ 7.7 Hz), 6.82 (*dd*, $J_1 = 7.4$, $J_2 = 1.3$ Hz), 6.78 (*dd*, $J_1 = 7.9$, $J_2 = 1.3$ Hz), 3.74 (*dd*, $J_1 = J_2 = 6.2$ Hz, $\underline{CH}$–CH_2–CO_2D), 2.97 (*dd*, $J_1 = 16.8$, $J_2 = 3.5$ Hz, CH–$C\underline{H}_2$–CO_2D), 2.96 (*br d*, J = 16.8 Hz, CD–$C\underline{H}_2$–CO_2D), 2.83 (*dd*, $J_1 = 16.8$, $J_2 = 3.5$ Hz, CH–$C\underline{H}_2$–CO_2D), 2.82 (*br d*, $J = 16.8$ Hz, CD–$C\underline{H}_2$–CO_2D).

An aliquot of the 7-OH-OxIAA extracted from the plant was rearranged to the quinolone derivative (**8**) and purified by HPLC on a (250 × 4.6 mm) Partisil-10 SAX column (with a 50 × 4.6 mm guard column of CoPell-ODS). Using a 1 ml/min gradient of 0–10% HOAc in 50% aq. EtOH over 20 min, the elution times of the compounds were 7-OH-OxIAA 13.3 min and 8-OH-THQCA 16.6 min, corresponding exactly to those of authentic standards.

Root segment incubations. Twenty segments, 2 cm long, were cut from 2–3 mm behind the tips of 4-day-old *Zea* roots and incubated in 100 ml round-bottomed flasks fitted with gas-tight stoppers with 2 × 10^6 dpm of [5-^{3}H]-substrate. Following a 24 hr period of agitation at 30° in darkness, the mixture was freeze-dried and the condensate analysed for ^{3}H. The dried roots were extracted with MeOH at 2° overnight in an N_2 gas phase. The MeOH extract was centrifuged, concentrated and analysed by HPLC on a PRP-1 column. The root tissue after the MeOH extraction was extracted with 1 ml 2 M NaOH at 100° for 6 hr. The extract was then neutralized with 2 M HCl, concentrated, centrifuged, and the supernatant solution was examined by HPLC using 7-OH-OxIAA as an internal marker.

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