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METABOLIC CONVERSION OF ^{14}C-INDOLE-3-ACETIC ACID TO ^{14}C-OXINDOLE-3-ACETIC ACID

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SUMMARY: We have identified [1-^{14}C]-oxindole-3-acetic acid as a catabolic product of [1-^{14}C]-indole-3-acetic acid metabolism in *Zea mays* seedlings. The isolation, and chemical and mass spectral characterization of oxindole-3-acetic acid from corn kernel tissue is described together with data suggesting oxindole-3-acetic acid to be a major catabolic product of indole-3-acetic acid.

Studies of the geotropic response of roots by Ciesielski (1) and of the phototropic response of shoots by Darwin and Darwin (2) led to the conclusion that a growth stimulus was transmitted backwards from the tip (cf.3). Removal or damage to the tip resulted in loss of tropic sensitivity indicating that the stimulus must be "used up" when the tip of the plant is removed(4).

Subsequent research led to the identification of indole-3-acetic acid (IAA) (5) as a plant growth hormone, and a pathway for catabolism of IAA was discovered which involved decarboxylation of the IAA, typically as catalyzed by horseradish peroxidase *in vitro* (6). However, among the few plants studied, *Zea mays* (7) and, possibly, *Pisum* (8) destroy only a minor portion of the IAA peroxidatively with carboxyl loss. Several IAA-related compounds that retain the carboxyl have been reported in rice bran, including methyl-oxindole-3-acetic acid, methyl-dioxindole-3-acetic acid, the 5-hydroxyl analogs of both the aforementioned compounds, and 5-hydroxydioxindole-3-acetic acid (9).

In addition, Siehr (10) found that a basidiomycete *Hygrophorus conicus* metabolizes tryptamine and IAA to oxindole-3-acetic acid (OxIAA), and Klämbt (11) has presented colorimetric evidence for the occurrence of OxIAA in 3 plant species including *Zea* seedlings. The present work is the first demonstration of the metabolic production of [1-^{14}C]-OxIAA from [1-^{14}C]-IAA in plants.

MATERIALS AND METHODS

Reagents:

DEAE-Sephadex A-25, DEAE-cellulose coarse mesh, lipophilic Sephadex LH-20, and indole-3-acetic acid, Sigma Chemical Co.; α-bromopentafluorotoluene, Diazald (N-methyl-N-nitroso-p-toluenesulfonamide), Aldrich; N-ethylpiperidine, Pflatz and Bauer; Silica Gel 60 thin-layer plates without fluorescent dye, E. Merck; 3% OV-17 on Gas Chrom Q (60/80), Applied Science Laboratories; 3% SP-2250 on Supelcoport (80/100), Supelco, Inc.; [1-^{14}C]-indole-3-acetic acid, 57 mCi/mmol, Amersham; and N-bromosuccinimide, Eastman Organic Chemicals.

Abbreviations: IAA, indole-3-acetic acid; OxIAA, oxindole-3-acetic acid.

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Incubation and Crude Extract Preparation:

Corn kernels, *Zea mays* cv. Stowell's Evergreen Sweet corn (W. Atlee Burpee Co.), were germinated for four days in darkness at 25°C and 80% relative humidity using a phototropically inactive green safe light for necessary manipulations (7). About 30% of the endosperm was cut from the end of the kernel and 5 μl of 50% ethanol containing 25 ng of [1-^{14}C]-indole-3-acetic acid (57 mCi/mmol) was applied. This amounts to 46% of the endogenous free IAA contained in a kernel (7). After a four hour incubation of the 1000 treated seedlings, the kernels were excised and dropped into sufficient acetone at −78°C to make the final acetone concentration 70% allowing for the water in the kernels. This labeled material was used to develop purification and derivatization techniques, whereas a large incubation mixture prepared from 5000 seedlings to which was added approximately 10% of the labeled extract was used for the final chemical characterization of the unknown compounds.

In a control experiment, [1-^{14}C]-IAA was added to the cut endosperm of seedlings, the excised kernels were immediately dropped into acetone at −78°C, and the homogenate extracted as described. Only unchanged labeled IAA could be reisolated from the control unincubated seedlings.

The incubated kernels were homogenized for two minutes in a four liter Waring blendor and extracted at 4°C for 12 hours. The homogenate was filtered and the residue reextracted two times with 70% acetone using 12 hour extraction periods. Acetone was removed from the filtrates under reduced pressure, and the aqueous phase partitioned three times with 1-butanol. The butanol phase was reduced to a yellow paste under reduced pressure, and the yellow paste extracted overnight in chloroform. After filtration, the yellow $CHCl_3$ filtrate was evaporated to about 20 ml for further purification using column chromatography.

Chromatography:

The extract was chromatographed on a 2.5 × 20 cm DEAE-cellulose column to remove lipoidal material (12), and yielded a single peak of radioactivity eluted with $CHCl_3/CH_3OH/CH_3COOH$ 70:30:1. The components of this peak could be resolved into two zones by thin-layer chromatography ($CHCl_3$:CH_3OH:H_2O 85:14:1) with R_f values of 0.20 and 0.40 with standard IAA at R_f=0.42. The DEAE-cellulose peak was pooled and chromatographed on a 2.5 ml DEAE-Sephadex column, washed with 50% ethanol, and eluted with a linear gradient from 50% ethanol to 50% ethanol containing 5% acetic acid. The radioactive peak was pooled and chromatographed on a 2.3 × 20 cm LH-20 column and eluted with 50% 2-propanol. Two peaks of radioactivity eluted from the LH-20 column and the R_f values of these peaks on TLC using the above solvent system were: peak I R_f=0.19, peak II R_f=0.42, and standard IAA at R_f=0.47.

The material in peak II from LH-20 chromatography was methylated with diazomethane (13), and the resultant ester reacted with bis-(trimethylsilyl)-trifluoroacetamide at 45°C for 15 minutes to derivatize the imine nitrogen. The pentafluorobenzyl ester of the material in peak I was synthesized using α-bromopentafluorotoluene in the presence of N-ethylpiperidine (14) and purified by HPLC on a C_{18} reverse phase column using 50% ethanol as eluant. The 70 eV mass spectral fragmentation pattern of these derivatives was analyzed with a Hewlett-Packard 5985 GC-MS using a 1.8m × 2mm ID SP2250 column programmed from 220-280°C.

RESULTS

Synthesis of oxindole-3-acetic acid:

OxIAA was synthesized by oxidation of IAA to OxIAA with 1 mole equivalent of N-bromosuccinimide [caution-explosion hazard (15)] according to the method of Hinman and Bauman (16) at 1/100 scale with addition of 2 μCi of [1-^{14}C]-IAA. The resultant OxIAA was purified by LH-20 chromatography with 50% 2-propanol as eluant, and by HPLC using 20% ethanol plus 1% acetic acid as eluant. The following criteria indicated the product to be OxIAA: the UV spectrum of the product in 95% ethanol evidenced a peak at 248 nm, and a shoulder at 280 nm; the product was unreactive with Ehmann reagent (17); a green color was produced with Ehrlich reagent after TLC of the product using 2-propanol:NH_4OH:H_2O 80:15:5 as solvent (12); and the product retained the labeled carboxyl group. The 70 eV mass spectrum of the pentafluorobenzyl ester proved the product to be oxindole-3-acetic acid with a molecular ion at m/z=371, and the expected major fragment ions at m/z=181, 146, and 145 (Figure 1a).

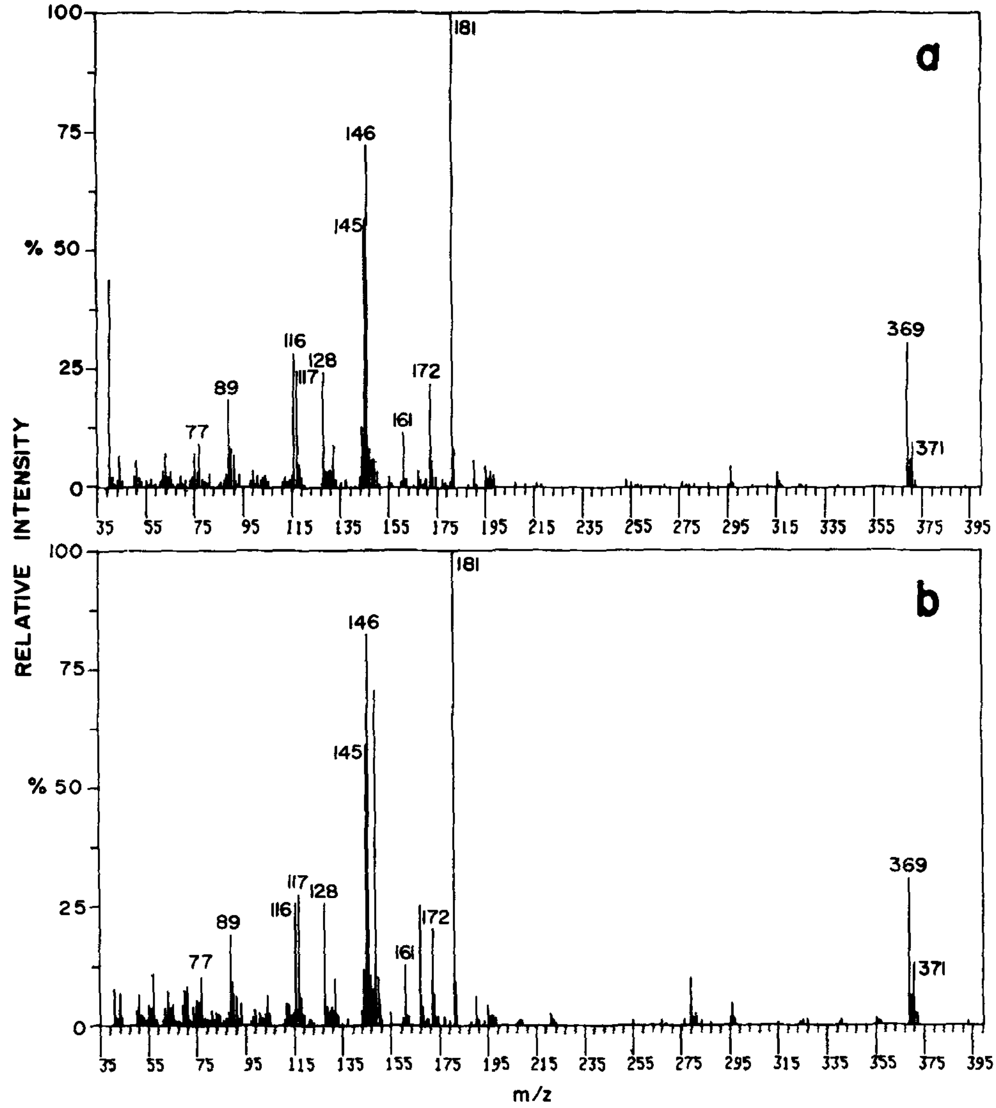

FIGURE 1. The 70 eV mass spectra of the pentafluorobenzyl ester of authentic oxindole-3-acetic acid (a), and that of the putative oxindole-3-acetic acid isolated from *Zea* endosperm (b).

Product characterization:

Peak I and peak II from LH-20 chromatography were shown to be OxIAA and IAA, respectively, by the following procedures: peak I cochromatographed with authentic OxIAA on TLC with an R_f of 0.2 using $CHCl_3$:CH_3OH:H_2O 85:14:1 as solvent, and had a retention volume on a 20 x 2.3 cm LH-20 column of 92-110 ml identical to that of synthetic OxIAA; the HPLC retention volume on a Partisil 10 ODS 25 x 0.46 cm C_{18} column was 6-7 ml using 20% ethanol plus 1% acetic acid as solvent as was that of authentic OxIAA; and GLC of the pentafluorobenzyl ester gave a retention time of 9.3 minutes on a 1.8m × 2mm ID OV-17 column programmed from 200-250°C, which again was identical to the derivative of the authentic OxIAA. TLC of the diazomethane derivatized peak I, developed in $CHCl_3$:MeOH:H_2O 85:14:1, yielded the same compounds at R_f 0.6 and 0.8 as did authentic OxIAA. GC-MS identified the compound at R_f 0.8 as the methyl ester of OxIAA with a molecular ion at m/z=205 and major fragment ions at m/z=146, 145, 128, and 117 (9). The compound at R_f 0.6 was unstable to the normal conditions of GC-MS probably owing to lactam ring opening (18). Both the authentic and putative OxIAA yielded the same products and in similar proportions when treated with diazomethane. The putative OxIAA of Peak I was Ehmann negative (17) and the mass spectrum of the pentafluorobenzyl ester of peak I (Figure 1b) had a molecular ion at m/z=371 and characteristic fragment ions at m/z=181, 146, and 145 identical to those of authentic OxIAA (Figure 1a). Peak II cochromatographed with authentic IAA on TLC with an R_f of 0.42 using $CHCl_3$:CH_3OH:H_2O 85:14:1 as solvent, and was identical to IAA on HPLC using 20% ethanol plus 1% acetic acid as eluant with a retention volume of 13 ml. Upon LH-20 chromatography, the putative IAA retention volume was 130-146 ml as was that of authentic IAA. Peak II was Ehmann positive (17) and the GC-MS mass spectrum of the N-trimethylsilyl methyl ester had a molecular ion at m/z=261 and characteristic fragment ions at m/z=202, 130, and 77, identifying it as IAA.

Further characterization of plant OxIAA:

^{14}C-carboxyl labeled OxIAA pentafluorbenzyl ester (10 μCi/μmol) was added to the putative plant OxIAA pentafluorobenzyl ester. The mixture chromatographed to a constant specific activity of 0.68 ± 0.02 μCi/μmol on Partisil 10 silica gel and C_{18} HPLC columns eluted with 20% acetonitrile and 50% ethanol, respectively. This data along with the chromatographic and mass spectral data identifies peak I as OxIAA.

DISCUSSION

By comparing the GLC peak area of the plant OxIAA to the peak area of an OxIAA standard of known concentration, it was estimated that 12.2 μg of plant OxIAA was obtained from the above purification procedure. Since the yield from the purification procedure was about 6% with labeled authentic OxIAA, it could be calculated that 203 μg of OxIAA was present in 2500 corn kernels. This amounts to 387 μg per kg dry weight of kernel tissue, and would be about 170% as much as the free IAA in 4-day-germinated corn kernel tissue.

Two findings suggest that OxIAA is a major catabolite of IAA. First, there is about as much OxIAA in the kernels as IAA, 387 $\mu g \cdot kg^{-1}$ (this paper) and 234 $\mu g \cdot kg^{-1}$ (7) respectively. Second, from the studies of Epstein et al. (7) on the turnover of IAA in the kernels, it can be calculated that in 4 h about one-half of the IAA of the kernel would be catabolized. We find that the labeled OxIAA isolated accounts for 26% of the expected IAA catabolized during the incubation period—and this does not include OxIAA that was further metabolized. Thus, this is the first demonstration that a major route of IAA catabolism in corn kernels is oxidation of IAA to OxIAA.

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