THESIS

This is to certify that the

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THE CATABOLISM OF INDOLE-3-ACETIC ACID

IN ZEA MAYS ENDOSPERM

presented by

DENNIS M. REINECKE

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Major professor

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THE CATABOLISM OF INDOLE-3-ACETIC ACID IN *ZEA MAYS* ENDOSPERM

By

Dennis M. Reinecke

A THESIS

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ABSTRACT

THE CATABOLISM OF INDOLE-3-ACETIC ACID
IN ZEA MAYS ENDOSPERM

By

Dennis M. Reinecke

[1-^{14}C]-oxindole-3-acetic acid was identified as a catabolic product of [1-^{14}C]-indole-3-acetic acid metabolism in Zea mays seedlings. IAA catabolic products were purified by the following chromatographic sequence: DEAE-cellulose, DEAE-Sephadex, LH-20 Lipophilic Sephadex, and HPLC. The putative oxindole-3-acetic acid cochromatographed with authentic oxindole-3-acetic acid in all chromatographic systems tested, and gas chromatography-mass spectrometry confirmed the identity of the catabolic product as oxindole-3-acetic acid. Oxindole-3-acetic acid accounted for, at least, 26% of the expected carboxyl retaining IAA catabolism of endosperm tissue without allowance for further metabolism. The amount of oxindole-3-acetic acid was determined to be 73 µg/1000 kernels or 356 µg/kg dry wt. This is the first identification of oxindole-3-acetic acid as a major catabolic product of IAA, and the first quantitation of oxindole-3-acetic acid in plant tissue.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSTFA	Bis-(trimethylsilyl)trifluoroacetamide
DEAE	diethylaminoethyl
GLC	gas-liquid chromatography
GC-MS	gas chromatography-mass spectrometry
HPLC	high pressure liquid chromatography
IAA	indole-3-acetic acid
m/z	mass to charge ratio
OxIAA	oxindole-3-acetic acid
PFB	pentafluorobenzyl
TLC	thin-layer chromatography
TMS	trimethylsilyl
UV	ultraviolet

INTRODUCTION

The development of the growth hormone concept in plants can be traced back over one hundred years to the pioneering work of Ciesielski (8) studying the geotropic response in roots, and Darwin (11) studying the phototropic response in coleoptiles. These workers observed that removal of the tip tissue resulted in loss of tropic sensitivity, suggesting that the tip was a source of a transitory growth stimulus which regulated growth and tropic responses. Subsequently, indole-3-acetic acid was isolated and identified as a plant growth hormone (41, 24).

To understand how the hormone regulates growth, it is essential to understand how metabolism regulates the level of hormone. In detipped Avena coleoptiles, Bonner and Thimann (6) correlated growth with the inactivation of hormone. More recent studies have indicated that the level of hormone may be regulated by catabolism (23), compartmentation (4), conjugation (1), and synthesis (57). In the endosperm of 4 day old dark-grown seedlings, the plant system studied in this work, indole-3-acetic acid (IAA) is not being conjugated, but is rapidly turning over through an unidentified catabolic route. The following discourse will be limited

to the examination of how IAA is catabolized in various plant systems, and to the elucidation of the IAA catabolic pathway in Zea mays.

Kisser et al. (38) and Thimann (65) were the first to observe that crushed leaves and water extracts of leaves, respectively, inactivated the growth promoting substance. The inactivation of the hormone was shown to be enzymatic and to require oxygen by Larsen (43). Galston et al. (21) demonstrated that the IAA oxidizing system of pea extracts, first reported by Tang and Bonner (14), had a peroxidase and a flavin component. Galston's group also showed that crystalline horseradish peroxidase had a similar IAA oxidizing activity.

There are many papers reporting the inactivation of IAA in plant systems, and these are discussed in detail in the review articles of Galston and Hillman (23), Schneider and Wightman (55), Sembdner et al. (57), and Hare (27). These studies measured IAA catabolism colorimetrically, most often using the Ehrlich (15), and Salkowski reagents (52); manometrically, measuring CO_2 evolution or O_2 uptake; with bioassay; or radiologically. With the exception of the radiological studies, none of these methods was sufficiently sensitive to measure the amount of IAA "used up" by the tissue during growth.

Care must be exercised in the comparison of the catabolism of exogenously applied IAA in whole tissues, crude homogenates, and enzyme preparations to the catabolism of

endogenous levels of IAA *in vivo*. Also, one must be careful to exclude nonenzymatic destruction of IAA by UV radiation, strong acids, and oxidizing agents (23, 32, 33). Deverall (13) reported the decarboxylation of IAA in a buffered solution, pH 4.5-4.7, approaching 20% decarboxylation in 1 hour, dependent on the washing treatment of the glassware. Bacterial contamination (2), and the concentration of IAA (32) may change the profile and extent of IAA catabolism also.

Several studies have followed radioactive $^{14}CO_2$ evolution of IAA labeled in the 1, 2, and ring positions. Andreae et al. (2) observed 19% decarboxylation of [1-^{14}C]-labeled IAA with pea root tips over 24 hours with 10^{-4} M IAA. Andreae reported that 17% of the IAA was conjugated to IAA aspartate, and 6% was degraded without carboxyl loss. Only negligible decarboxylation of [2-^{14}C] and [7a-^{14}C]-label was observed in this system, although up to quantitative release of $^{14}CO_2$ was observed from the 7a labeled IAA when the incubation mixture was contaminated with microorganisms. Davies (12) estimated that the amount of decarboxylation of IAA in kidney bean and pea segments was 34% and 29%, respectively, over 6 hours of incubation. Troxler and Hamilton (66) with geranium callus cultures, and Strydom and Hartman (61) with plum stem cuttings observed 32% and 31% of the [2-^{14}C]-labeled IAA as respired $^{14}CO_2$ after 7 and 1 day incubations, respectively. However, Hamilton et al. (26) working with a crown-gall tissue culture of *Parthenocissus*

tricuspidata observed only 1% loss of [2-^{14}C]-label, and 53% loss of [1-^{14}C]-label over a 48 hour incubation period. Epstein and Lavee (18) observed that the age of the culture influences the amount of decarboxylation in apple tissue culture. Three month old nongrowing apple callus cultures quickly decarboxylated 90% of the [1-^{14}C]-labeled IAA during 4 hours, while young, growing callus cultures decarboxylated only 20% of the [1-^{14}C]-labeled IAA during 4 hours. Fang and Butts (19) found that corn seedlings had a light requirement for the decarboxylation of [1-^{14}C]-labeled IAA applied to primary leaves, with negligible degradation of IAA in the dark. The leaves of pea and bean decarboxylated IAA in both the light and the dark, but more slowly in the dark. Excised corn shoots only decarboxylated 12-19% of labeled IAA over 6 hours in the light, and 8-13% in the dark; while corn root tips decarboxylated 82% in the light, and 85% in the dark (20). Pea tissues were more active in degrading IAA, with pea shoots decarboxylating 80-90% of the labeled IAA in the light, and 54-80% in the dark; and pea roots quantitatively decarboxylating labeled IAA in the light or the dark. These experiments demonstrated that there can be qualitative and quantitative differences in the destruction of IAA in different plant species, and there may be qualitative and quantitative differences in the destruction of IAA in different tissues of the same plant.

Many studies of IAA oxidation have indicated that IAA was oxidatively decarboxylated, but it was the work of

Hinman and Lang (32) with an in vitro horseradish peroxidase system that elucidated the major products as oxindole-3-carbinol (hydroxymethyloxindole), and methyleneoxindole (Figure 1a) by means of UV spectroscopy, and chemical analogue studies. Hydroxymethyloxindole and methyleneoxindole were subsequently reported as products of IAA oxidation in pea (68) and corn extracts (5). Other reported decarboxylation products of IAA catabolism include: indolealdehyde (50), methyloxindole (68), indole-3-methanol (45), indole-3-carboxylic acid (45), 3-acetoxyindole, and oxindole-indole polymers (62). Whether these compounds are of physiological significance is uncertain, since many of the studies were performed in vitro with high IAA concentrations.

Kinashi and coworkers (37) have isolated several compounds from rice bran which are possible IAA catabolites that retain the carboxyl group, including: the methyl esters of oxindole-3-acetic acid and dioxindole-3-acetic acid, the 5-hydroxy analogues of the aforementioned compounds, and 5-hydroxy-oxindole-3-acetic acid (Figure 1b). Klämbt (39) has presented colorimetric evidence for the occurrence of oxindole-3-acetic acid (OxIAA) in seeds and seedlings of Zea mays, in germinating seeds of Brassica rapa, and in developing seeds of Ribes rubrum. Klämbt also reported the isolation of the glucoside of OxIAA (40) from feeding [1-^{14}C]-IAA to several plant species, but his use of ammoniacal solvents in the chromatographic separations makes the isolation of a labile glucose ester impossible (34). Siehr (58) has

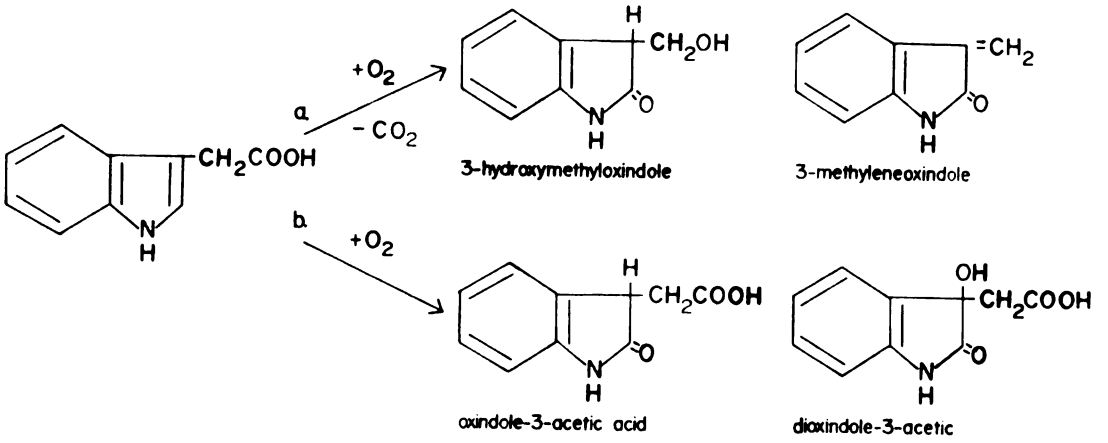

Figure 1.--Oxidative pathways of indole-3-acetic catabolism.

a. Oxidative decarboxylation of IAA to 3-hydroxymethyloxindole and 3-methyleneoxindole.

b. Oxidation of IAA to oxindole-3-acetic acid and dioxindole-3-acetic acid.

shown that the basidiomycete Hygrophorus conicus can metabolize tryptamine and IAA to OxIAA. The quinolinic acids, B-acid (2,6-dihydroxycinchonic acid) in rice bran (37), and zeanic acid (2,8-dihydroxycinchonic acid) in corn (47), are other possible catabolites from IAA which retain the carboxyl group. However, B-acid and possibly zeanic acid are artifacts of isolation since dioxindole-3-acetic acid, and oxindole-3-acetic acid rearrange in the presence of base or acid to similar compounds (35, 67, 39, 63). Tsurumi and Wada (67) have reported dioxindole-3-acetic acid derivatives in Vicia faba from IAA transport studies.

Kopcewicz et al. (42) observed several apparent oxidation products of IAA from a 24 hour incubation of mature corn kernels in a solution of [1-^{14}C]-IAA. The products did not yield IAA or indoleacetamide upon ammonolysis, but further chemical identification was not undertaken. Epstein et al. (17) collected $^{14}CO_2$ evolved from [1-^{14}C]-IAA applied to the cut endosperm of 4 day old dark-grown corn seedlings. Only 12 pmol/h/endosperm of IAA was decarboxylated. Isotope dilution experiments demonstrated that IAA remained relatively constant over 4 days of growth at 308 pmol/endosperm. Turnover studies showed that the specific activity of labeled IAA was diluted by an apparent first order rate over 8 hours. From these data on dilution of specific activity over time, the first order rate constant, k, of the reaction was determined to be 0.22/h and the turnover time was calculated to equal 3.2 h. Since there were 308 pmol/endosperm,

the turnover rate was calculated to be 96 pmol/h/endosperm. Of this rapidly turning over IAA pool, only 12 pmol/h was decarboxylated, so that 83 pmol/h of IAA catabolism was through an unidentified carboxyl retaining catabolic route (Figure 2). In the following experiments the carboxyl retaining IAA catabolites are isolated and identified, as the first step towards understanding the function of the rapid turnover of IAA in endosperm tissue and its role in growth.

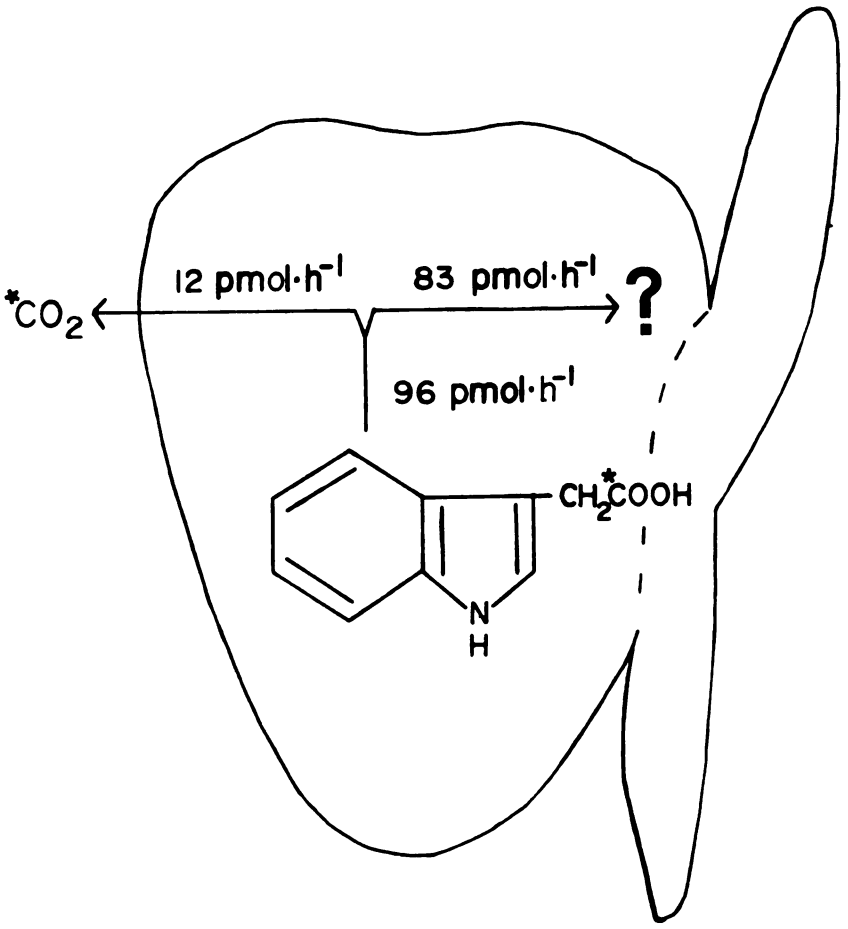

Figure 2.--The catabolic turnover of indole-3-acetic acid in Zea endosperm tissue.

MATERIALS AND METHODS

Plant Material

Seeds of Zea mays cv. Stowell's Evergreen Sweet corn were purchased from Vaughan Jacklin Co., Ovid, Mi., and W. Atlee Burpee Co., Clinton, Iowa.

Chromatographic Material

Thin-layer chromatography was on Silica Gel 60 TL plates without fluorescent indicator (E. Merk, Darmstadt, Germany). DEAE-cellulose coarse mesh, DEAE-Sephadex A-25-120, and lipophilic Sephadex LH-20-100, were purchased from Sigma. A Whatman Partisil 10 ODS 25 x 0.46 cm C_{18} column was employed for high pressure liquid chromatography (HPLC). A Hewlett-Packard model 402 was used for preliminary gas-liquid chromatography (GLC) on a 1.2 m x 2 mm ID 3% OV-1 on Gas Chrom Q (100/120) column (Applied Science Laboratories, State College, Pa.). Subsequent work was performed on a Varian Series 2700 gas chromatograph with a 1.8 m x 2 mm ID OV-1 on Gas Chrom Q (100/120) column, or 3% OV-17 on Gas Chrom Q (60/80) column (Applied Science Laboratories, State College, Pa.). A Hewlett-Packard 5985 gas chromatograph-mass spectrometer (GC-MS) using a 1.8 m x 2 mm ID 3% SP2250

on Supelcoport (80/100) column (Supelco, Inc., Bellefonte, Pa.) was employed for GC-MS analysis.

Spectrophotometric Determinations

A Gilford 240 spectrophotometer, and a Cary 15 spectrophotometer were used for spectrophotometric analysis.

Chemicals

IAA, Sigma Chemical Co., St. Louis, Missouri; gramine, Diazald (N-methyl-N-nitroso-p-toluenesulfonamide), 2-(2-ethoxy)-ethanol, α-bromopentafluorotoluene, and dimethyl sulfate, Aldrich Chemical Co., Milwaukee, Wisconsin; potassium cyanide [^{13}C] 90 atom %, Merk, Sharp, and Dohme, Rahway, NJ; N-ethylpiperidine, Pfaltz and Bauer, Flushing, NY; N-bromosuccinimide, Eastman Organic Chemicals, Rochester, NY; Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% Trimethylchlorosilane, Regis Chemical Co., Chicago, Il.

Radiological Material

[1-^{14}C]-IAA (57 μCi/μmol and 58 μCi/μmol) was purchased from Amersham, Arlington Heights, Il. Radioactivity was measured on a Packard Tri-Carb liquid scintillation spectrometer, and on a Beckman LS 7000 liquid scintillation counter. ACS (Amersham) was used as the scintillation cocktail.

Determination of Specific Activity

Specific activity (μCi/μmol) was determined by measuring radioactivity by liquid scintillation counting, and concentrations by UV absorbance. Concentrations were determined

by measuring the absorbance of the solution of interest on a Cary 15 spectrophotometer. The absorbance and the known molar extinction coefficient may be used in the Beer-Lambert law:

$$A = \varepsilon c l$$

where: A = absorbance

ε = molar extinction coefficient (liters/mole-centimeter)

c = concentration (moles/liter)

l = pathlength (centimeters)

The radioactivity of the solution was measured by liquid scintillation counting and corrected for efficiency by an internal [^{14}C]-toluene standard, or an external [^{137}Cs] standard.

Synthesis of [^{13}C]-Carboxyl Labeled IAA

Stowe (60) described a method for the synthesis of high specific activity [^{14}C]-carboxyl labeled IAA by reacting gramine methosulfate (Schöpf & Thesing) with K^{14}CN. The product, indoleacetonitrile was then hydrolyzed to yield [1-^{14}C]-labeled IAA. By using K^{13}CN, the reaction was modified for the synthesis of [1-^{13}C]-labeled IAA.

The reactive tertiary amine gramine methosulfate was synthesized at 1/10 scale using the method of Schöpf and Thesing (56). Gramine was recrystallized from hot acetone and hexane; tetrahydrofuran was redistilled over potassium and under nitrogen; acetic acid was dried over P_2O_5 and redistilled, and dimethyl sulfate was redistilled under

reduced pressure. For the synthesis, 0.5 mmol acetic acid was mixed with a solution of 14 mmol gramine and 25 ml of peroxide-free tetrahydrofuran. The mixture was added dropwise with stirring to a solution of 0.1 mol of dimethyl sulfate and 0.5 mol of acetic acid in 10 ml of tetrahydrofuran at 16°C in the dark. After 30 minutes the reaction flask containing a white oil was transferred to a dark room at 5°C. Crystallization occurred only after the oil was washed with anhydrous ethyl ether. The crystals melted initially at 135-137°C, and 141-143°C after drying over P_2O_5. Stowe reported a melting point of 146-148°C for the product and yields of 87-98%. I obtained 4.0 grams of gramine methosulfate for a 96% yield based on gramine.

For the synthesis of labeled IAA, 70 mg of gramine methosulfate plus 15 mg of $K^{13}CN$ were added to a 5 ml round bottomed flask. Next, 2 ml of 0.2 M K_2HPO_4 was purged with nitrogen, and then added to the flask. The mixture was agitated with a nitrogen stream through a glass capillary while the reaction vessel was heated to 65°C for 5 hours. One pellet of KOH and boiling chips were added and the mixture refluxed for 1 hour. The contents were filtered through a sintered glass funnel into a 10 ml beaker, and the flask and filter washed with two 0.5 ml aliquots of H_2O. The filtrate was chilled to ice temperature and acidified to pH 3.5 with concentrated phosphoric acid, whereupon thick white crystals formed. The crystals were washed twice with 0.5 ml of distilled H_2O, and then recrystallized from

95% ethanol. Melting occurred at 164-166°C with the reported value for the melting of IAA being 165-166°C. The yield of [^{13}C]-carboxyl labeled IAA was 33% based on [^{13}C]-cyanide. Stowe reports a yield of between 20-80% under similar conditions. A summary of the synthesis of gramine methosulfate and [^{13}C]-labeled IAA is given in Figure 3.

The UV spectrum of the putative IAA had a peak at 282 nm and 220 nm as did authentic IAA. The ratio of the molar extinction coefficients of IAA at 282 nm to 222 nm, 6060/33,200 (3), equals 0.183 which is identical to the ratio of the absorbances of the putative IAA at 282 nm to 220 nm, with 0.268/1.465 equaling 0.183. The putative IAA cochromatographed with authentic IAA on TLC in the solvent system chloroform:methanol:water 85:14:1, and the putative IAA was Ehmann positive as was authentic IAA (14). The mass spectrum of the methyl ester of the putative IAA had a molecular ion at 190, and characteristic fragment ions at 130, 103, and 77 while authentic IAA had a molecular ion 189, and characteristic fragment ions at 130, 103, and 77. Figure 4 shows the fragmentation pattern of the methyl ester of the [^{13}C]-labeled IAA.

[1-^{13}C]-IAA Labeling Experiments

Initial feeding experiments were conducted using [1-^{13}C]-labeled IAA at a ratio of 1:1 with the endogenous free IAA. The [1-^{13}C]-labeled IAA plus a small amount of [1-^{14}C]-IAA, as an easily followed tracer, was applied to

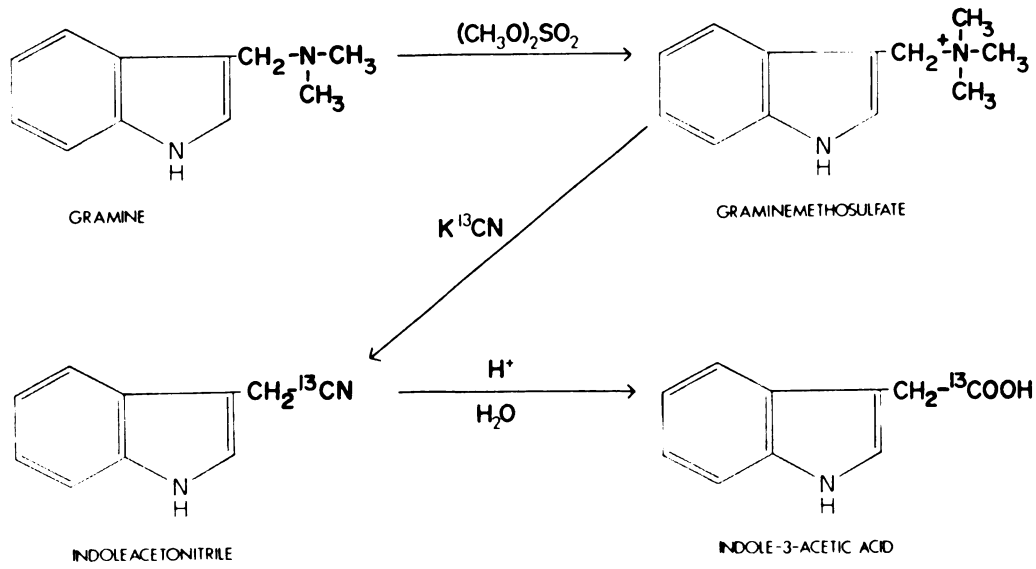

Figure 3.--The synthesis of [^{13}C]-carboxyl labeled indole-3-acetic acid from gramine.

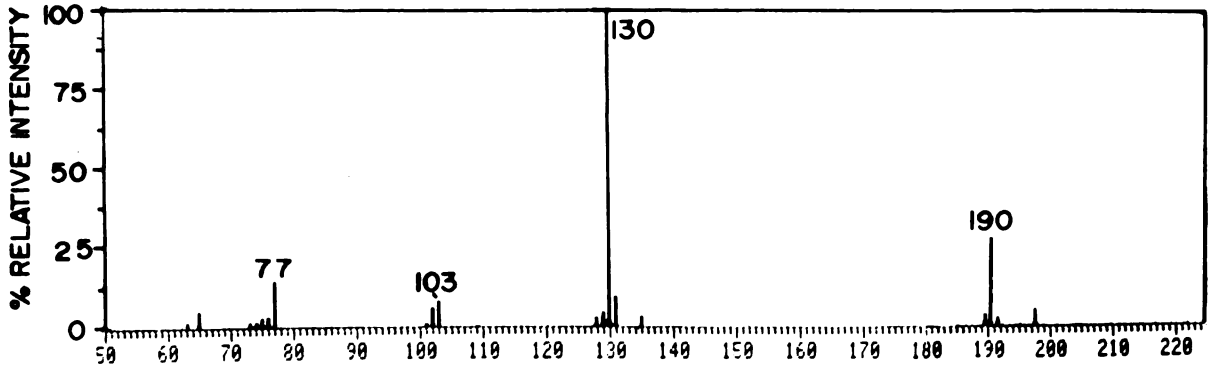

Figure 4.--The 70 eV mass spectrum of the methyl ester of [^{13}C]-carboxyl labeled indole-3-acetic acid.

The methyl ester of [^{13}C]-IAA was chromatrographed on a Hewlett-Packard 5985 GC-MS using a 1.8 m x 2 mm ID SP2250 column.

the cut endosperm of 4 day old dark-grown Zea mays seedlings. All manipulations were at 25°C, 80% relative humidity, using a phototropically inactive green safe light (17). After a 4 hour incubation period the corn kernels were excised and homogenized in enough acetone to make the final concentration 70% allowing for the water content of the kernels. The carboxyl retaining IAA catabolites were purified by a sequence of partitionings with 1-butanol, and chloroform; and by column chromatography with DEAE-cellulose, DEAE-Sephadex, and LH-20 lipophilic Sephadex. IAA catabolites which retained the carboxyl group were to be identified by gas chromatography-mass spectrometry. Since [1-^{13}C]-labeled IAA was added to the tissue in a 1:1 ratio to the endogenous free IAA, carboxyl retaining catabolites would have distinctive double-peaked molecular ions of similar intensity by gas chromatography-mass spectrometry.

The method of identifying carboxyl retaining IAA catabolites by the double-peaked mass spectral marker proved unsuccessful owing to the occurrence at 1000 fold greater concentration of the plant phenolic compounds (3). Typically, IAA and IAA carboxyl retaining catabolites lose the carboxyl label at the first fragmentation in GC-MS. The double label would only be detected in a clean sample where the doubled-peaked molecular ion could be clearly distinguished from contaminating fragments. To circumvent this problem the [1-^{13}C]-IAA experiments were suspended, and labeling experiments with higher specific activity

[1-^{14}C]-IAA were begun. The [1-^{14}C]-experiments were initiated to develop better purification procedures for the isolation of IAA catabolites for GC-MS identification.

Isolation of OxIAA from Zea mays Endosperm Tissue

[1-^{14}C]-labeled IAA was applied to the cut endosperm of 4 day old dark-grown seedlings. After a 4 hour incubation period, the kernels were excised and extracted with 70% acetone at -78°C. Figure 5 gives a summary of the initial extractions and partitionings used to isolate the IAA catabolites from the endosperm tissue. The acetone extraction recovered up to 90% of the initial radioactivity added to the tissue, indicating that decarboxylation of the IAA by the tissue was very low. The recovery yield through the butanol partitioning and the chloroform extraction ranged between 60% and 70%.

The crude chloroform extract was further purified with DEAE-cellulose, and DEAE-Sephadex anion exchange chromatography, LH-20 lipophilic Sephadex chromatography, and C_{18} reverse phase HPLC as summarized in Figure 6. Two peaks of radioactivity were resolved by LH-20 chromatography. The purified, derivatized samples were analyzed on gas chromatography-mass spectrometry.

Quantitation of Oxindole-3-acetic Acid

The previous experiments indentified [1-^{14}C]-OxIAA as a carboxyl retaining catabolic product of [1-^{14}C]-IAA metabolism in Zea mays. The next questions to answer were

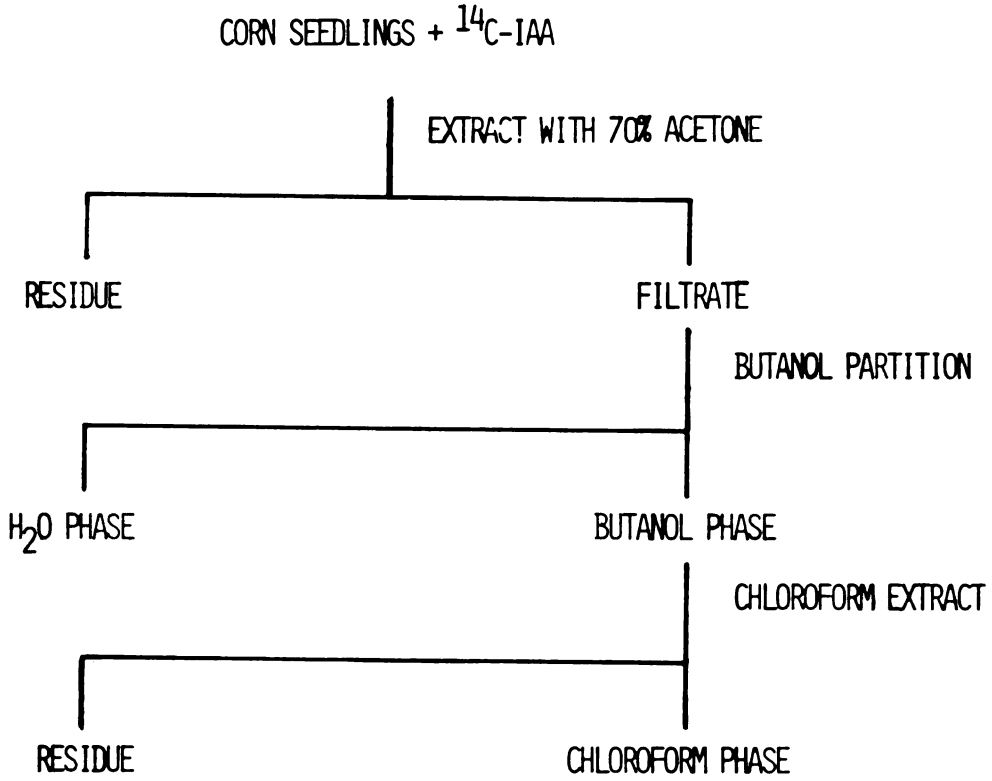

Figure 5.--The extraction procedure for the isolation of carboxl retaining indole-3-acetic acid catabolites.

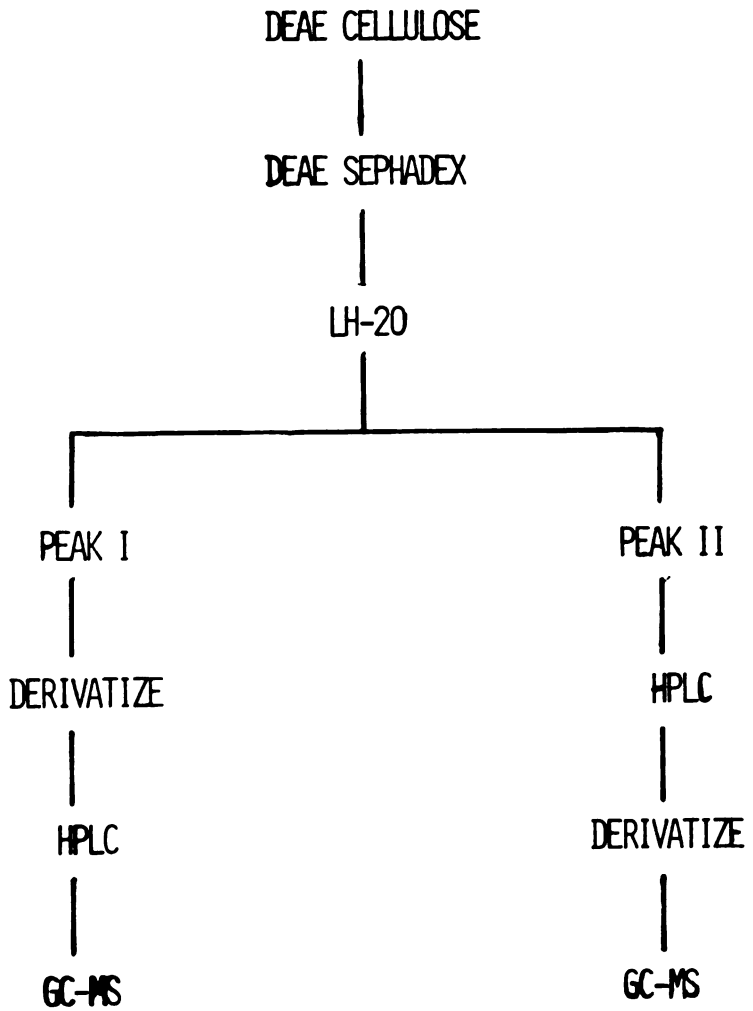

Figure 6.--The isolation and identification of carboxyl retaining indole-3-acetic catabolites.

whether OxIAA is a naturally occurring compound in Zea endosperm tissue, and if so, at what concentration does it occur. An external standard method, and the double standard isotope dilution assay were employed to quantitate the endogenous levels of OxIAA.

The external standard method compares the GLC peak area of an external standard of known amount, to the GLC peak area of a plant sample of unknown amount:

$$\frac{\mu\text{mol external standard}}{\text{peak area external standard}} \times \text{peak area plant sample} = \mu\text{mol plant sample eluting from GLC}$$

The GLC peak areas of both compounds are quantitated by taking the average weight of three photocopies of each peak tracing. The amount in μmoles of external standard eluting from the GLC effluent is calculated by collecting the radioactivity eluting from the extinguished FID at the standard's known retention time. The radioactivity collected, times the known specific activity for the external standard, equals the amount in μmoles of standard eluting from the GLC.

With this information, the preceding equation can be solved for the amount in μmoles of a plant sample required to give the observed GLC peak response. The amount of OxIAA initially in the tissue can be calculated from the GLC value, by correcting for the recovery yield for the isolation of OxIAA.

The main weakness of the external standard method is its dependence on an accurate determination of recovery

yield, which may vary depending on the concentration of OxIAA in the tissue.

The double standard isotope dilution assay was employed for a more exact determination of levels of OxIAA in endosperm tissue (10). This method can quantitate labile compounds in small amounts such as plant hormones and their catabolites. The double standard isotope dilution assay circumvents the difficulties of the external standard method, accurate measurement of yield recovery and accurate quantitative gas chromatography, by employing two internal standards (10). The first internal standard is a radioactively labeled standard of the compound to be quantitated in the tissue. The initial specific activity of the standard is determined, and the specific activity of the standard after its addition and reisolation from the tissue is determined. Any OxIAA present in the tissue will dilute the specific activity of an OxIAA standard added to the tissue. The initial specific activity, the final diluted specific activity, and the amount of standard added to the tissue can be measured to determine the endogenous levels of OxIAA, as in the following isotope dilution equation (51):

$$Y = \left(\frac{C_o}{C_f} - 1\right) X$$

where: C_o = specific activity of ^{14}C-OxIAA added

C_f = specific activity of ^{14}C-OxIAA recovered

X = amount of ^{14}C-OxIAA added

Y = amount of endogenous plant OxIAA

To determine the final specific activity of the compound to be quantitated, a second internal standard is added to the purified sample prior to gas-liquid chromatography. In the present study IAA is used as the second internal standard. By measuring the radioactivity, and peak areas (as described in the external standard section), the final diluted specific activity of OxIAA may be calculated (10):

$$\frac{\text{collected OxIAA (DPM)}}{\text{OxIAA peak area}} \times \frac{\text{IAA peak area}}{\text{collected IAA (DPM)}} \times \text{specific activity of IAA} = \text{final specific activity OxIAA}$$

The final specific activity of the OxIAA thus determined, the known initial specific activity, C_o, and the known amount of OxIAA added, X, can be used to solve the isotope dilution equation for the endogenous level of OxIAA:

$$\text{OxIAA in endosperm tissue} = \left(\frac{C_o}{C_f} - 1 \right) X$$

Labeled OxIAA, 10 μCi/μmol, was synthesized for the isotope dilution assay using the method of Hinman and Bauman (30), and further purified on an LH-20 column and eluted with 50% 2-propanol. The radiological purity of the synthetic OxIAA was shown to be at least 95% by HPLC eluted with 20% ethanol plus 1% acetic acid, and 94% by TLC developed with G solvent.

Both the external standard method and the double standard isotope dilution assay depend on clean GLC peaks free from cochromatographing contaminants. The identity of the peak was further verified by GC-MS in the present study.

EXPERIMENTAL

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.

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/e=371, and the expected major fragment ions at m/z=181, 146, and 145 (Figure 7a).

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/e=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 7b) had a molecular ion at m/e=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/e=261 and characteristic fragment ions at m/z=202, 130, and 77, identifying it as IAA.

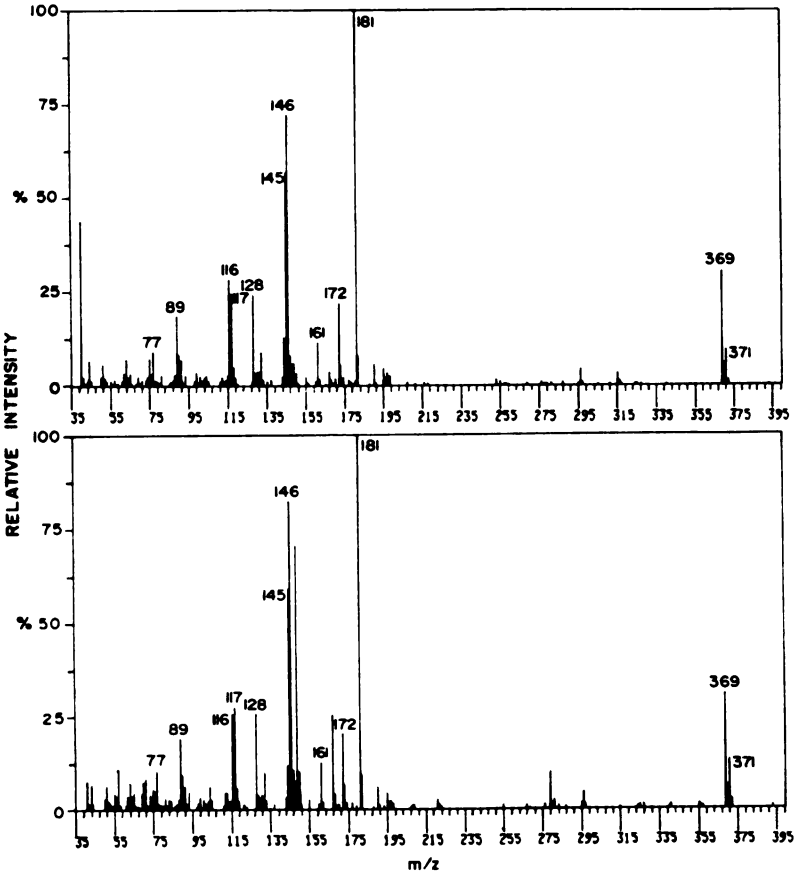

Figure 7.--The 70 eV mass spectra of the pentafluorobenzyl ester of authentic oxindole-3-acetic acid (a), and that of the putative oxindole-3-acetic acid isolated from Zea endosperm (b).

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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REFERENCES

1. Ciesielski, T. (1872) Beitraege Biol. Pflanzen. 1, 1-30.
2. Darwin, C., and Darwin, F. (1880) The Power of Movement in Plants, Appleton, London.
3. Heslop-Harrison, J. (1980) Plant Growth Substances 1979, F. Skoog editor. Springer-Verlag. New York. pp. 3-13.
4. Bonner, J., and Thimann, K.V. (1935) J. General Physiol. 18, 649-658.
5. Kögl, F. A., Haagen-Smit, A. J., and Erxleben, H. (1934) Z. Physiol. Chem. 228:90-103.
6. Hinman, R. L., and Lang, J. (1965) Biochemistry 4, 144-157.
7. Epstein, E., Cohen, J. D., Bandurski, R. S. (1980) Plant Physiol. 65, 415-421.
8. Davies, P. J. (1973) Physiol. Plant. 28, 95-100.
9. Kinashi, H., Suzuki, Y., Takeuchi, S., and Kawarada, A. (1976) Agr. Biol. Chem. 40, 2465-2470.
10. Siehr, D. J. (1961) J. Am. Chem. Soc. 83, 2401-2402.
11. Klämbt, H. D. (1959) Naturwiss. 46, 649.
12. Hall, P. L., and Bandurski, R. S. (1978) Plant Physiol. 61, 425-429.
13. Schlenk H., and Gellerman, J. L. (1960) Anal. Chem. 32, 1412-1414.
14. Epstein, E., and Cohen, J. D. (1981) J. Chromatogr. 209, 413-420.
15. Martin, R. H. (1951) Nature 168, 32.
16. Hinman, R. L., and Bauman, C. P. (1964) J. Org. Chem. 29, 1206-1215.
17. Ehmann, A. (1977) J. Chromatogr. 132, 267-276.
18. Julian, P.L., Printy, H.D., Ketcham, R., and Doone, R. (1953) J. Am. Chem. Soc. 75, 5305-5309.

RESULTS

Identification of Oxindole-3-acetic Acid

The chromatographic similarities between authentic OxIAA and the isolated plant unknown I are summarized in Table 1. The putative plant OxIAA and the authentic OxIAA had similar chromatographic properties on LH-20 lipophilic Sephadex, and TLC developed with G solvent (chloroform:methanol:water 85:14:1) and A solvent (methyl ethyl ketone:ethyl acetate: ethanol:water 3:5:1:1). However, when either sample was reacted with diazomethane, a potent methylating agent, two zones of radioactivity were observed. Lengthening the reaction time of methylation had no effect on the relative amounts of the compounds. When the mixture was chromatographed on GLC only one peak was observed. GC-MS identified the compound as the methyl ester of OxIAA with a molecular ion at 205, and characteristic major fragment ions at 146, 145, 128, and 117. The mass spectral fragmentation pattern was identical to published spectra for the methyl ester of OxIAA (37), with the addition of an M-2 peak at 203. The two compounds could be separated by HPLC with 30% ethanol as eluant, after which the UV spectrum of the major compound with TLC R_f of 0.78, was found to be the same as OxIAA. The

Table 1.--Chromatographic similarities between authentic and putative OxIAA.

CHROMATOGRAPHY		PLANT UNKNOWN I	OXINDOLE-3-ACETIC ACID
LH-20		86-110 ML.	92-102 ML.
TLC	G SOLVENT	0.22	0.22
	" " (DIAZOMETHANE)	0.6, 0.78	0.6, 0.78
	A SOLVENT	0.83	0.8
HPLC	FREE ACID	6-7 ML.	7 ML.
	PFB ESTER	8 ML.	8 ML.
GLC	PFB ESTER	9.3 MIN.	9.3 MIN.

compound with R_f of 0.6 did not give a detectable GC peak under the conditions employed, which precluded its identification by GC-MS. The unidentified compound is most likely the methyl ester of the product of lactam ring opening (hydrolysis between the nitrogen and the carbonyl) which has been reported to occur under acidic (35, 44) and basic conditions (67), or the lactone ring formation (reaction of the side chain acid with the carbonyl) as suggested by Klämbt to occur under basic conditions (39).

On C_{18} HPLC the putative plant OxIAA and the authentic OxIAA cochromatographed both as free acids and as pentafluorobenzyl (PFB) esters. Finally, by GLC both the PFB esters of the putative OxIAA and the authentic OxIAA had identical retention times.

Final identification of the plant sample as OxIAA was accomplished by GC-MS. The PFB esters of both the putative OxIAA and the authentic OxIAA had identical 70 eV mass spectral patterns as are shown in Figure 7. As expected, the molecular ion of the PFB ester of OxIAA was at 371. An M-2 peak at 369 was also observed, which appears to result from dehydrogenation and rearrangement of the molecular ion to the stable quinolinium fragment. The methyl ester of OxIAA gave a similar M-2 peak, while the bis-trimethylsilyl (TMS) derivative of OxIAA had no M-2 peak. Since derivatization with the silylating agent bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) derivatizes the side chain acid group and the nitrogen, a free N-H group appears to be

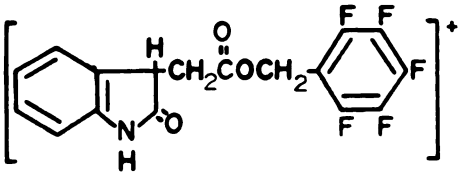
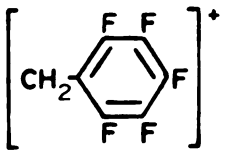
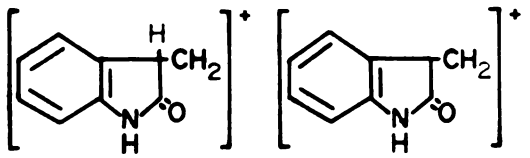
necessary for the M-2 rearrangement fragment. Other major fragments from the PFB esters were at mass 181, the pentafluorobenzyl fragment, and at mass 146, and 145, the oxindole fragments. A summary of the major fragments of the PFB ester of the plant OxIAA and the authentic OxIAA is listed on Table 2. The percentage abundances between the plant OxIAA and the authentic OxIAA fragments agree very well. The relative percentage abundances are, of course, dependent on the concentration of the sample analyzed, and the conditions of analysis, but the authentic and the putative samples varied similarly.

Turnover of Labeled IAA

In this study, 13% of the labeled IAA metabolized was converted to OxIAA after a 4 hour incubation period with endosperm tissue. The following discussion examines the data of Epstein et al. (17) to determine whether the turnover observed in the present study approaches their reported value.

Epstein et al. determined the first order rate constant for the dilution of specific activity of labeled IAA, when labeled IAA was added to the endosperm of 4 day old dark-grown Zea seedlings. The first order rate constant and the following rate equation can be used to calculate how much turnover of labeled free IAA would occur over a 4 hour incubation period:

Table 2.--Major GC-MS fragments and the percentage relative abundances for authentic and putative OxIAA.

m/z	FRAGMENT	% ABUNDANCE	
		OxIAA	PLANT SAMPLE
371		10	13
369	M-2	30	31
181		100	100
146, 145		72,57	83,59

$$\log \frac{C_o}{C_t} = \frac{kt}{2.303}$$

where: k = first order rate constant

t = time

C_o = specific activity at time 0

C_t = specific activity at time t

Epstein and coworkers calculated the first order rate constant to be 0.22/h. The specific activity of IAA at time 0 in the present experiments can be expressed as 0.32, for the ratio between labeled free IAA to total free IAA.

Using this information and the rate equation, one may calculate C_t, the specific activity of labeled free IAA after a 4 hour incubation period in the endosperm tissue. C_t was determined to be equal to 0.13; that is, 32% of the IAA is initially labeled at time 0, but only 13% of the IAA remains labeled after the 4 hour incubation owing to dilution by newly produced IAA.

The total free IAA has been determined to remain relatively constant over these time intervals, so that the ratio of C_t to C_o will equal the amount of labeled free IAA which remains unmetabolized after 4 hours:

$$\frac{C_t}{C_o} = \frac{\text{IAA labeled at 4 h}}{\text{total IAA}} \div \frac{\text{IAA labeled at 0 h}}{\text{total IAA}}$$

$$= \frac{\text{IAA labeled at 4 h}}{\text{IAA labeled at 0 h}}$$

$$= \frac{0.13}{0.32} \text{ or } 41\% \text{ of the labeled IAA remains unmetabolized.}$$

If 41% of the labeled IAA was not turned over, then 59% of the labeled IAA would have been catabolized to other products. Transport of labeled free IAA to other tissues and conjugation of IAA are not significant at this stage of development. The data of Epstein and coworkers, also found that 13% of IAA catabolized proceeds through decarboxylation, so that:

59% turnover - (59% turnover x 13% decarboxylation)
= 51% turnover through carboxyl retaining catabolites

Experimentally, the present study observed 13% turnover to carboxyl retaining IAA catabolites after 4 hours, or 26% of the expected rate predicted by the Epstein paper. The difference between the observed 13% turnover and the calculated 51% turnover may be explained by differences in seed lot, isolation procedure, etc. The work of Epstein and coworkers predicted a rate of IAA turnover which approaches the level observed in this study, and they predicted a carboxyl retaining IAA catabolite which the present study confirmed by isolation and identification.

Quantitation of Oxindole-3-acetic Acid in Zea mays Endosperm Tissue

OxIAA (21.5 µg) with a specific activity of 10 µCi/µmol was added to 1000 excised Zea kernels. After purification, the diluted final specific activity was determined to be 2.3 µCi/µmol. This value was the average of two determinations, where the specific activities were measured to be

2.27 μCi/μmol and 2.23 μCi/μmol. With these data and the isotope dilution equation, it was determined that there is 73 μg OxIAA/1000 kernels. Since there was 0.21 g dry wt/kernel, there was 356 μg OxIAA/kg dry wt. The amount of OxIAA determined from the feeding experiment, calculated using the external standard method, was 387 μg OxIAA/kg dry wt, and this agrees well with the isotope dilution value.

The isotope dilution assay demonstrates that OxIAA is a natural component of Zea endosperm at a level near that of free IAA (17), 234 μg/kg dry wt.

DISCUSSION

The present work reports the first identification of OxIAA as a major carboxyl retaining IAA catabolite in higher plants. The amount of OxIAA present in 4 day old dark-grown endosperm tissue is 356 µg/kg dry wt.

The most thorough prior identification of oxindole-3-acetic acid and dioxindole-3-acetic acid derivatives in plant tissues is from the work of Kinashi et al. (37). Using rice bran extracts, they identified the methyl esters of oxindole-3-acetic acid and dioxindole-3-acetic acid, their 5-hydroxy analogues, and the free 5-hydroxy-dioxindole-3-acetic acid. Although Kinashi et al. proposed a route of IAA catabolism through oxindole-3-acetic acid and dioxindole-3-acetic acid, the precursor-product relationship between IAA and these compounds was not demonstrated. Siehr (58) was the first to demonstrate that IAA or tryptamine may be metabolized to OxIAA by the basidiomycete Hygrophorus conicus. The present radiological studies unambiguously demonstrate that OxIAA is a catabolic product of IAA metabolism in Zea endosperm tissue.

Oxindole-3-acetic Acid as a Major Route of IAA Catabolism

There are several lines of evidence that indicate OxIAA formation is a major route of IAA catabolism in Zea endosperm tissue. After a 4 hour incubation period, the extract contained only two peaks of radioactivity: unmetabolized IAA and OxIAA as isolated and identified by chromatographic and mass spectral analysis. IAA catabolic products which lost their labeled carboxyl group would not be identified by this isolation procedure. However, after the 4 hour incubation period, up to 90% of the initial radioactivity could be recovered by the 70% acetone extractions. Most of the remaining radioactivity could be accounted for in the endosperm tissue residue, so that catabolism through decarboxylation played only a minor role in IAA metabolism during the incubation period. This data confirms the work of Epstein and coworkers (17), who predicted that the majority of IAA catabolism proceeds through a carboxyl retaining route, since IAA was observed to turnover rapidly with low levels of IAA decarboxylation.

Figure 2 indicates that there are two routes of catabolism in Zea endosperm tissue: a route with retention of the carboxyl moiety, and a route through oxidative decarboxylation; nevertheless, there may be only a single route of catabolism of IAA, through oxidation of IAA to OxIAA with subsequent OxIAA decarboxylation. The metabolism of OxIAA must be further studied to resolve whether there are one or two pathways regulating IAA levels in Zea.

More evidence that OxIAA is a major catabolite of IAA metabolism is that the present study accounts for at least 26% of the predicted turnover of IAA to carboxyl retaining catabolites from the data of Epstein et al. (17). The exact proportions of IAA catabolism accounted for by OxIAA formation awaits complete turnover studies.

Indole-3-acetic Acid Turnover

Free IAA is turning over rapidly in the endosperm of 4 day old Zea endosperm tissue, but its relationship to the growth of the seedling remains unknown. Free IAA does not appear to be an important transport form of IAA to the shoot or the root (25); but IAA conjugates, as IAA inositol, do appear to be IAA precursors for vegetative growth (49). When [^{3}H]-IAA is applied to the cut endosperm of Zea seedlings (25), 98% of the radioactivity observed in the shoot after 8 hours of incubation is no longer IAA or IAA esters. The identity of the IAA metabolized during transport is unknown.

At this stage of development, IAA is not being conjugated in the endosperm, so the purpose of the high turnover rate of IAA is unknown. The turnover of free IAA may be a way of regulating the levels of IAA in endosperm tissue, since the majority of free IAA is not being transported to actively growing vegetative tissue. Corn endosperm tissue is generally considered to be storage tissue surrounded by the living aleurone layer, scutellum, and embryo (36). The presence of free IAA and its catabolism in endosperm tissue

may indicate a role of IAA in seedling development during germination.

Biological Activity of Oxindole-3-acetic Acid

After Hinman and Bauman (30) developed a new synthesis for OxIAA, several groups tested the biological activity of OxIAA. Galston and Chen (22) reported the OxIAA had biological activity with etiolated, and green pea stem sections. The greatest increase in growth occurred between the concentrations 1.6-5.2 x 10^{-4}M for green pea sections, and between the concentrations 1.6 x 10^{-4}-1.6 x 10^{-3}M for etiolated pea sections. The growth promotion induced by 1.6 x 10^{-4}M OxIAA was reported to be similar to the growth promotion with 2 x 10^{-4}M IAA in green pea sections. All subsequent researchers have shown that OxIAA is inactive in growth promotion in the bioassays tested. Weis (69) observed that OxIAA did not promote growth of tobacco tissue cultures, and inhibited fresh weight increases at 10^{-4}-10^{-7}M. OxIAA was inactive in the *Avena* first internode test, and the *Avena* coleoptile curvature test in the concentration range 5 x 10^{-8} to 5 x 10^{-4}M (28). Kinashi et al. (37) found no increased growth in *Avena* coleoptile sections when treated with 0.1 to 100 µg/ml for the methyl esters of OxIAA, 5-hydroxy-OxIAA, dioxindole-3-acetic acid, or 5-hydroxy-dioxindole-3-acetic acid. The same group (63) showed that 5-hydroxy-dioxindole-3-acetic acid, and 5-hydroxy-OxIAA were synergistic in IAA promotion of ethylene in mung bean hypocotyl segments, but

OxIAA and dioxindole-3-acetic acid were not, in the concentration range 0.1-100 μg/ml or 5.2×10^{-7}-5.2×10^{-5}M OxIAA.

The general concensus is that OxIAA does not promote growth in the bioassays tested. The work of Galston and Chen should be repeated to show whether OxIAA is growth promoting for pea stem sections, is metabolized to other growth promoting substances, or was contaminated by a product of OxIAA synthesis from IAA. Galston and Chen used TLC and melting point analysis as an indicator of OxIAA purity. Assuming that OxIAA is inactive as a growth regulator in corn, it is interesting to speculate that the catabolism of IAA to OxIAA occurs when IAA is "used up" during growth.

Other possible carboxyl retaining IAA catabolites: zeanic acid, 2,8 dihydroxycinchonic acid, isolated from corn steep liquor (47), and B-acid, 2,6 dihydroxycinchonic acid, isolated from rice bran (37), (Figure 8), have been reported to have biological activity. Zeanic acid was reported to increase grape fruit set, and to promote dwarf rice seedling growth. It was also reported to increase proliferation of rice callus growth, and radish cotyledon growth in the absence of kinetin and auxin (48). B-acid was observed to supress dwarf rice seedling growth (37). However, when OxIAA and dioxindole-3-acetic acid are isolated under similar acidic and basic conditions, ring expansion to similar oxo-quinoline-4-carboxylic acids are observed (35, 67), (Figure 9). Until zeanic acid and B-acid are isolated under

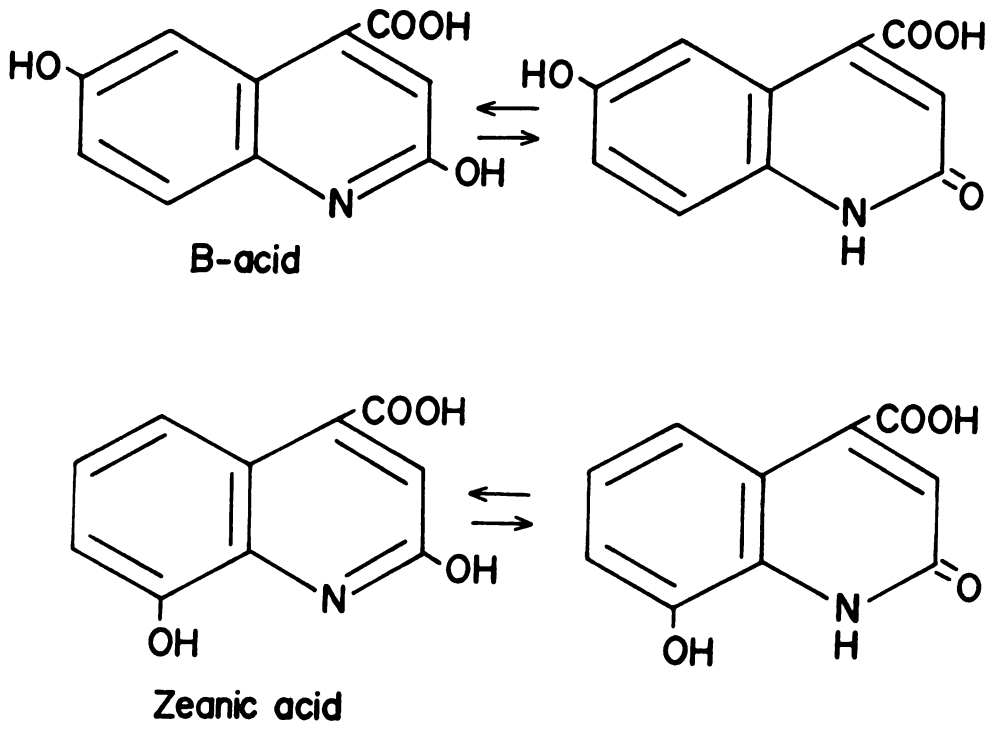


Figure 8.--The keto and enol forms of zeanic acid and B-acid.

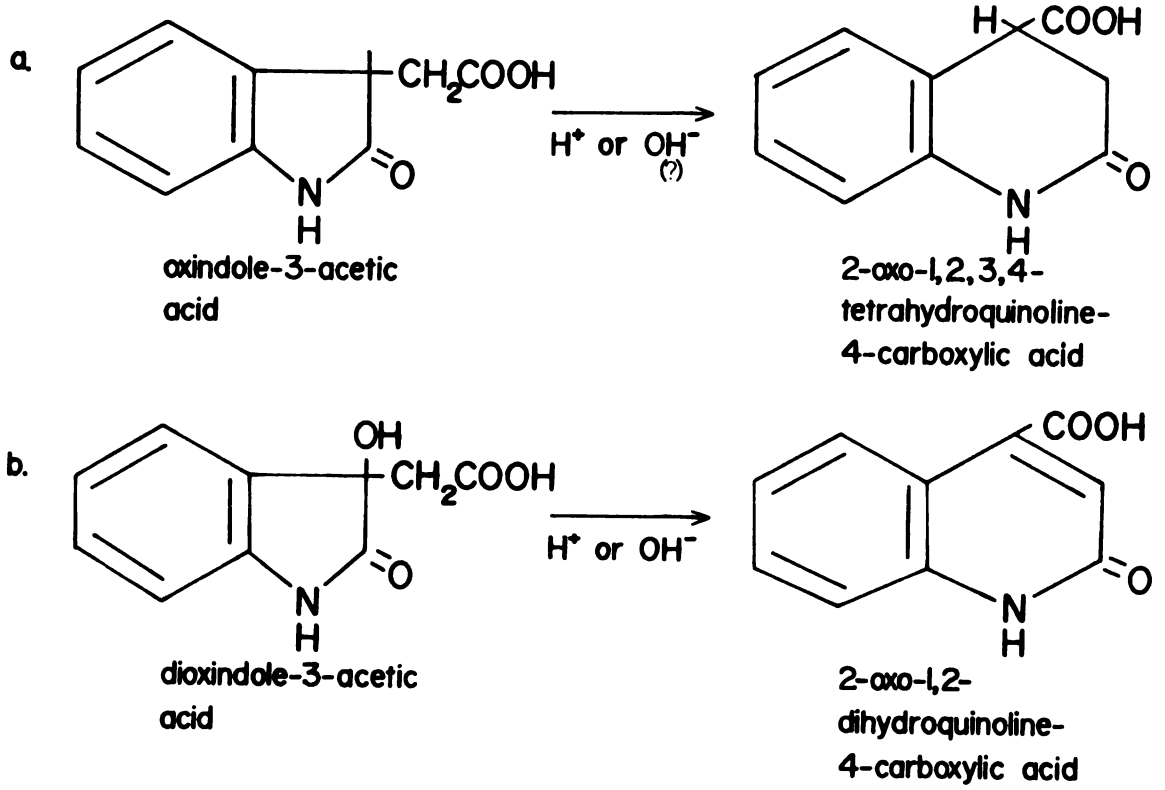


Figure 9.--The acid and base rearrangement products of OxIAA and dioxindole-3-acetic acid. (The base catalyzed rearrangement product of OxIAA awaits further characterization.)

less severe conditions their occurrence may well be an artifact.

Identification of OxIAA

The lability of OxIAA and their derivatives in acid and base is one reason why these compounds may not have been reported more often in biological materials. Oxindole-3-acetic acids were first successfully synthesized in 1953 by Julian and coworkers (35). Previous attempts to synthesize OxIAA dating back to 1926 were unsuccessful since hydrolytic conditions were used in the syntheses causing rearrangement to 2-oxo-1,2,3,4-tetra hydroquinoline-4-carboxylic acid (Figure 9). The first reports of OxIAA in plant tissue by Klämbt (39) in 1959, and Siehr (58) in 1961 followed the successful synthesis of OxIAA by Julian. Lawson and Witkop (44) were the first to show that N-bromosuccinimide could oxidize indoles to oxindoles. Subsequently, Hinman and Bauman (30, 31) developed the relatively simple synthesis of OxIAA from IAA, by N-bromosuccinimide oxidation; the OxIAA biological studies followed.

Even with OxIAA standards available, a sensitive assay for identifying IAA catabolites was also necessary. The typical methods for following IAA catabolism, manometric measurements, bioassay, or colorimetric assays would have been little value in identifying IAA catabolites as OxIAA or dioxindole acetic acid. Under normal conditions, OxIAA produces no color with Ehrlich (39), Salkowski (28), or

Ehmann (see Experimental) reagents. OxIAA does, however, produce a green color reaction with Ehrlich reagent under basic conditions (39). Plant hormones and their metabolic products are present in tissues in low concentrations, so that a sensitive radiological assay is needed to isolate these products. Even with radiological tracing techniques, the present study found purification and characterization of the carboxyl retaining IAA catabolic products difficult due to interfering compounds.

Another obstacle in identifying oxindole-3-acetic acid compounds may be the level of peroxidase activity in crude homogenates. Peroxidases are ubiquitous in plant tissues, occurring in cell walls, and in the cytoplasm (59). Peroxidase activity oxidatively decarboxylates IAA to methyleneoxindole and hydroxymethyloxindole, as in the well-characterized horseradish peroxidase system (32). However, Zea, Pisum, and horseradish peroxidases do not degrade IAA conjugates (9). Peroxidases are believed by many to be the "IAA oxidase" of biological importance, although much of the characterization of peroxidative IAA degradation has been studied in vitro. An IAA catabolic route through OxIAA might be outcompeted for substrate in a tissue homogenate with peroxidase activity present. Briggs et al. (7) gave evidence that peroxidase activity was a cut surface phenomenon in Osmunda cinnamonea, which was active in tissue homogenates in degrading IAA. When peroxidase activity is isolated from plant tissues, the activity can be resolved

into many isozymes, with 7-20 isozymes in horseradish peroxidase depending on extraction procedure, storage, and tissue age, and 10 isozymes in corn depending on tissue age (c.f. 53). Whether all peroxidase isozymes have "IAA oxidase" activity, whether there are IAA oxidizing systems unique from peroxidases, and whether peroxidative IAA destruction regulates IAA levels *in vivo* remains unresolved in the literature (57).

Methods which facilitated the isolation of OxIAA from *Zea* endosperm tissue, were the use of radioactive feeding studies, and the mild conditions of isolation. Only 1% and 5% acetic acid were used to elute compounds from DEAE-cellulose and DEAE-Sephadex anionic exchange columns, respectively, and 1% acetic acid was used in the elution of compounds from HPLC. Even with low concentrations of acid, compounds with low R_f values on TLC (G solvent) accumulated in storage at 5°C over time. The formation of the compounds which remained at the origin after TLC, were virtually eliminated by rapid isolation through the isolation procedure up to LH-20 chromatography. LH-20 chromatography was an ideal purification step since no acid or base was involved, and it separated unknown I from unknown II. Once the carboxyl retaining compounds were separated and peak unknown II was identified as unmetabolized IAA, the problem became the identification of peak I. Gas chromatography of peak I proved difficult due to interfering compounds and to the variable results with diazomethane derivatization. Synthesis

of OxIAA for use as a model compound was required to develop a purification procedure, synthesize a stable derivative for GLC, and develop GC-MS analysis applicable to unknown I identification, (that is, Zea OxIAA identification).

Presently, only Siehr with the basidiomycete Hygrophorus conicus, and the present work with Zea mays have shown that IAA may be catabolized through a carboxyl retaining route to OxIAA. Hinman and Lang (32) gave UV spectroscopic evidence that OxIAA is not oxidized by the horseradish peroxidase system, so that the preliminary data indicates that OxIAA is not an intermediate of peroxidative decarboxylation of IAA. Whether OxIAA is a naturally occurring component of vegetative tissue other than Zea kernels is also unknown. Klämbt (39) gave colorimetric evidence for OxIAA in vegetative tissue and kernels of Zea. Interesting questions which the corn system may answer are whether OxIAA can be identified in vegetative tissue, and if OxIAA formation can be correlated with IAA induced growth. If IAA is to remain growth limiting at the active site of growth in vegetative tissues, the level of hormone must be carefully regulated. It is interesting to speculate that once the hormone does its "growth promoting action" it will be in some way altered to keep the hormone limiting, so that the site of action of IAA may be physically, or temporally close to the site of catabolism of IAA.

SUMMARY

A method for the isolation and identification of carboxyl retaining indole-3-acetic acid catabolites has been developed. The carboxyl retaining catabolite of indole-3-acetic acid metabolism in Zea endosperm, predicted by the work of Epstein et al. (17), has been identified as oxindole-3-acetic acid by chemical and mass spectral characterization. This is the first demonstration that oxindole-3-acetic acid is a product of indole-3-acetic acid catabolism in higher plants as evidenced by [1-^{14}C]-indole-3-acetic acid feeding studies.

An isotope dilution assay has shown that oxindole-3-acetic acid is a naturally occurring component of 4 day old Zea endosperm tissue at 356 µg/kg dry wt, or an amount similar to the levels of endogenous free indole-3-acetic acid, 234 µg/kg dry wt (17). Oxindole-3-acetic acid is a major catabolite of indole-3-acetic acid since nearly all of the label could be accounted for as either oxindole-3-acetic acid or unmetabolized indole-3-acetic acid. Also, oxindole-3-acetic acid accounted for at least 26% of the expected amount of carboxyl retaining indole-3-acetic acid catabolism after a 4 hour incubation (17).

Now that oxindole-3-acetic acid has been identified as a catabolite of the plant hormone indole-3-acetic acid, the function of oxindole-3-acetic acid in the regulation of indole-3-acetic acid levels in Zea endosperm, its presence in vegetative tissue, and its role in indole-3-acetic acid mediated growth may be explored.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Andreae, W. A., and N. E. Good. 1955. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30:380-382.

2. Andreae, W. A., J. R. Robinson, and M. W. H. van Ysselstein. 1961. Studies on 3-indoleacetic acid metabolism. VII. Metabolism of radioactive 3-indoleacetic acid by pea roots. Plant Physiol. 36:783-787.

3. Bandurski, R. S., and A. Schulze. 1974. Concentration of indole-3-acetic acid and its esters in Avena and Zea. Plant Physiol. 54:257-262.

4. Bandurski, R. S., A. Schulze, and P. H. Hall. 1980. Localization of free and esterified indole-3-acetic acid in mesocotyls of Zea seedlings. Plant Physiol. Supplement 65:157.

5. BeMiller, J. N., and W. Colilla. 1972. Mechanism of corn indole-3-acetic acid oxidase in vitro. Phytochem. 11:3393-3402.

6. Bonner, J., and K. V. Thimann. 1935. Studies on the growth hormone of plants. VII. The fate of growth substance in the plant and the nature of the growth process. J. General Physiol. 18:649-658.

7. Briggs, W. R., G. Morel, T. A. Steeves, I. M. Sussex, and R. H. Wetmore. 1955. Enzymatic auxin inactivation by extracts of the fern, Osmunda cinnamomea L. Plant Physiol. 30:148-154.

8. Ciesielski, T. 1872. Untersuchungen über die abwärtskrümmung der wurzel. Beitr. Biol. Pflanzen. 1:1.

9. Cohen, J. D., and R. S. Bandurski. 1978. The bound auxins: protection of indole-3-acetic acid from peroxidase-catalyzed oxidation. Planta 139:203-208.

10. Cohen, J. D., and A. Schulze, 1981. Double standard isotope dilution assay. I. Quantitative assay of indole-3-acetic acid. Anal. Biochem. 112:249-257.

11. Darwin, C., and F. Darwin. 1880. The power of movement in plants. Appleton, London.

12. Davies, P. J. 1973. The uptake and fractional distribution of differentially labeled indoleacetic acid in light grown stems. Physiol. Plant. 28:95-100.

13. Deverall, B. J. 1965. Apparently spontaneous decarboxylation of indole-3-acetic acid. 207:207-208.

14. Ehmann, A. 1977. The Van Urk-Salkowski reagent--a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. J. Chrom. 132:267-276.

15. Ehrlich, F. 1901. Zur kenntniss des m-cyanbenzylchorids. Chem. Ber. 34:3366.

16. Epstein, E., and J. D. Cohen. 1981. Microscale preparation of pentafluorobenzyl esters: Electron capture gas chromatographic detection of indole-3-acetic acid from plants. J. Chrom. 209:413-420.

17. Epstein, E., J. D. Cohen, and R. S. Bandurski. 1980. Concentration and metabolic turnover of indoles in germinating kernels of Zea mays L. Plant Physiol. 65:415-421.

18. Epstein, E., and S. Lavee. 1975. Uptake and fate of IAA in apple callus tissue using IAA-1^{14}C. Plant and Cell Physiol. 16:553-561.

19. Fang, S. C., and J. S. Butts. 1957. Studies of carboxyl-^{14}C-labeled 3-indoleacetic acid in plants. Plant Physiol. 32:253-259.

20. Fang, S. C., P. Theisen, and J. S. Butts. 1959. Metabolic studies of applied indoleacetic acid-1-^{14}C in plant tissues as affected by light and 2,4-D treatment. Plant Physiol. 34:26-32.

21. Galston, A. W., J. Bonner, and R. S. Baker. 1953. Flavoprotein and peroxidase as components of the indole-3-acetic acid oxidase system of peas. Arch. Biochem. 42:456-470.

22. Galston, A. W., and H. R. Chen. 1965. Auxin activity of isatin and oxindole-3-acetic acid. Plant Physiol. 40:699-705.

23. Galson, A. W., and W. S. Hillman. 1961. The degradation of auxin. In Handbuch der Pflanzenphysiologie, pp. 647-670, Ruhland, W., ed. Springer-Verlag, Berlin.

24. Haagen-Smit, A. J., W. D. Leech, and W. R. Bergren. 1942. The estimation, isolation, and identification of auxins in plant material. Am. J. of Bot. 29:500-506.

25. Hall, P. L., and R. S. Bandurski. 1978. Movement of indole-3-acetic acid and tryptophan-derived indole-3-acetic acid from the endosperm to the shoot of Zea mays L. Plant Physiol. 61:425-429.

26. Hamilton, R. H., H. E. Meyer, R. E. Burke, C. S. Feung, and R. O. Mumma. 1976. Metabolism of indole-3-acetic acid. II. Oxindole pathway in Parthenocissus tricuspidata crown-gall tissue cultures. Plant Physiol. 58:77-81.

27. Hare, R. C. 1964. Indoleacetic acid oxidase. Bot Rev. 30:129-165.

28. Henderson, J. H. M., and C. S. Patel. 1972. Oxindole-3-acetic acid: Physical properties and lack of influence on growth. Physiol. Plant. 27:441-442.

29. Heslop-Harrison, J. 1980. Plant growth substances 1979, F. Skoog Ed., Springer-Verlag, New York, pp. 3-13.

30. Hinman, R. L., and C. P. Bauman. 1964. Reactions of N-bromosuccinimide and indoles. A simple synthesis of 3-bromooxindoles. J. Org. Chem. 29:1206-1215.

31. Hinman, R. L., and C. P. Bauman. 1964. Reactions of 3-bromooxindoles. The synthesis of 3-methyleneoxindole. J. Org. Chem. 29:2431-2437.

32. Hinman, R. L., and J. Lang. 1965. Peroxidase catalyzed oxidation of indole-3-acetic acid. Biochem. 4: 144-157.

33. Horng, A. J., and S. F. Yang. 1975. Aerobic oxidation of indole-3-acetic acid with bisulfite. Phytochem. 14:1425-1428.

34. Jepson, J. B. 1958. Indolyacetamide--a chromatographic artifact from the natural indoles, indolylacetyl-glucosiduronic acid and indolylpyruvic acid. Biochem. J. 69:22.

35. Julian, P. L., H. C. Printy, R. Ketcham, and R. Doone. 1953. Studies in the indole series. XIV. Oxindole-3-acetic acid. J. Am. Chem. Soc. 75: 5305-5309.

36. Kiesselbach, T. A., and E. R. Walker. 1952. Structure of certain specialized tissues in kernel of corn. Amer. J. Bot. 39:561-569.

37. Kinashi, H., Y. Suzuki, S. Takeuchi, and A. Kawarada. 1976. Possible metabolic intermediates from IAA to B-acid in rice bran. Agr. Biol. Chem. 40: 2465-2470.

38. Kisser, J. R., R. Stasser, E. Kiffe, and S. Göllner. 1931. Anx. Akad. Wiss. Wien 68:275.

39. Klämbt, H. D. 1959. Die 2-hydroxy-indol-3-essigsäure, ein pflanzliches indolderivat. Naturwiss. 46: 649.

40. Klämbt, H. D. 1964. 2-hydroxyindole-3-acetic acid and similar compounds in seeds and other plant parts. In Régulateurs Naturels de la Croissance Végétale, pp. 235-239, ed. J. P. Nitsch, C. N. R. S., Paris.

41. Kögl, F. A., J. Haagen-Smit, and H. Erxleben. 1934. Über ein neues auxin (hetero-auxin) aus harn. Zeit. Physiol. Chem. 228:104-112.

42. Kopcewicz, J., A. Ehmann, and R. S. Bandurski. 1974. Enzymatic esterification of indole-3-acetic acid to myo-inositol and glucose. Plant Physiol. 54:846-851.

43. Larsen, P. 1936. Untersuchungen über aus Phaseolus-Keimpflanzen. Planta 25:311-314.

44. Lawson, W. B., and B. Witkop. 1961. A simple method for the preparation of oxindoleacetic and propionic acids from the parent indoles. J. Org. Chem. 26:263-264.

45. Magnus, V., S. Iskric, and S. Kueder. 1971. Indole-3-methanol--a metabolite of indole-3-acetic acid in pea seedlings. Planta 97:116-125.

46. Martin, R. H. 1951. Dangers attendant on the use of N-halogenimides in organic reactions. Nature 168:32.

47. Matsushima, H., H. Fukumi, and K. Arima. 1973. Isolation of zeanic acid, a natural plant growth-regulator from corn steep liquor and its chemical structure. Agr. Biol. Chem. 37:1865-1871.

48. Matsushima, H., and K. Arima. 1973. Physiological activities of zeanic acid, a new plant-growth promotor from corn steep liquor. Agr. Biol. Chem. 37:1873-1880.

49. Nowacki, J., and R. S. Bandurski. 1980. Myo-inositol esters of indole-3-acetic acid as seed auxin precursors of Zea mays L. Plant Physiol. 65: 422-427.

50. Racusen, D. 1955. Formation of indole-3-aldehyde by indoleacetic acid oxidase. Arch. Biochem Biophys. 58:508-509.

51. Rittenberg, D., and G. L. Foster. 1940. A new procedure for quantitative analysis by isotope dilution with application to the determination of amino acids and fatty acids. J. Biol. Chem. 133: 737-744.

52. Salkowski, E. 1885. Über das verhalten der skatolcarbonsäure im organismus. Z. Physiol. Chem. 9:23.

53. Scandalios, J. G. 1974. Isozymes in development and differentiation. Ann. Rev. Plant Physiol. 25: 250-251.

54. Schlenk, H., and J. L. Gellerman, 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412-1414.

55. Schneider, E. A., and F. Wightman. 1974. Metabolism of auxins in higher plants. Ann. Rev. Plant Physiol. 25:496-504.

56. Schöpf, C., and J. Thesing. 1951. Die umsetzung quartärer salze von Mannich-Basen mit tertiären aminen. (Zur reindarstellung quartärer salze des gramins). Angew. Chem. 63:377-378.

57. Sembdner. G., D. Gross, H.-W. Liebisch, and G. Schneider. 1980. Biosynthesis and metabolism of plant hormones. In Encyclopedia of Plant Physiology, New Series, vol. 9, Hormonal regulation of development. I. Molecular aspects of plant hormones, pp. 336-347, ed. J. MacMillan, Springer-Verlag, Heildelberg.

58. Siehr, D. J. 1961. The formation of oxindole acetic acid from indoles by a basidiomycete. J. Am. Chem. Soc. 83:2401-2402.

59. Stafform, H. A. 1974. The metabolism of aromatic compounds. Ann. Rev. Plant Physiol. 25:459-486.

60. Stowe, B. B. 1963. Synthesis of high specific activity ^{14}C-carboxyl indoleacetic acid and of ^{14}C-nitrile indoleacetonitrile. Anal. Biochem. 5:107-115.

61. Strydom, D. K., and H. T. Hartmann. 1960. Absorbtion, distribution, and destruction of indoleacetic acid in plum stem cuttings. Plant Physiol. 35:435-442.

62. Suzuki, Y., and A. Kawarada. 1977. Products of peroxidase catalyzed oxidation of indolyl-3-acetic acid. Agric. Biol. Chem. 42:1315-1321.

63. Suzuki, Y., H. Kinashi, S. Takeuchi, and A. Kawarada. (+)-5-Hydroxy-dioxindole-3-acetic acid, a synergist from rice bran of auxin-induced ethylene production in plant tissue. Phytochem. 16:635-637.

64. Tang, Y. W., and J. Bonner. 1947. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13:11-25.

65. Thimann, K. V. 1934. Studies on the growth hormone in plants. VI. The destruction of the growth substance in plant tissues. J. Gen. Phys. 18:23-34.

66. Troxler, R. F., and R. H. Hamilton. 1965. The metabolism of indole-3-acetic acid by geranium stem callus cultures. Plant Physiol. 40:400-405.

67. Tsurumi, S., and S. Wada. 1980. Metabolism of indole-3-acetic acid and natural occurrence of dioxindole-3-acetic acid derivatives in Vicia roots. Plant and Cell Physiol. 21:1515-1525.

68. Tuli, V., and H. S. Moyed. 1967. Inhibitory oxidation products of indole-3-acetic acid: 3-hydroxymethyloxindole and 3-methyleneoxindole as plant metabolites. Plant Physiol. 42:425-430.

69. Weis, J. S. 1966. Effect of oxindoles on the growth of tobacco tissue cultures. Nature 211:1216-1217.