ISOLATION OF INDOLE-3-ACETIC ACID FROM CORN KERNELS & ETIOLATED CORN SEEDLINGS [1, 2]

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INTRODUCTION

Indole-3-acetic acid (IAA) has been commonly accepted as an important plant auxin (4, 14, 20). Grain of corn (*Zea mays* L.) is a rich source and milligram quantities have been isolated by alkaline hydrolysis of the mature kernels (2, 16), or by direct extraction from immature kernels (15). A number of reports have appeared concerning occurrence of IAA in vegetative portions of plants, but in many cases diethyl ether has been used as the extracting solvent in a manner which can allow the enzymatic conversion of tryptophan to IAA (40). In other cases, the existence of IAA is presumed from the bioassay of chromatograms run in a single solvent. Thus proof that free IAA is generally distributed in growing plant tissue is not yet available (1).

In a previous study (35), relatively large amounts of IAA have been detected in *Ustilago Zeae* (Beckm.) Ung. tumors and smaller amounts in healthy, early-tassel-stage corn stalks.

The objective of the present study was to isolate free IAA from vegetative plant tissue using sufficiently rigorous isolation and assay procedures to establish its identity. Methods were developed to permit the use of kilogram quantities of plant tissue, and the addition of trace amounts of IAA-2-C^{14} facilitated evaluation of losses during isolation.

METHODS

EXTRACTION, PAPER CHROMATOGRAPHY, & ELECTROPHORESIS. Michigan 350 hybrid corn was soaked in water for 4 hours and germinated on wet absorbent paper in plastic trays at 25° C in the dark and 90 % relative humidity. The 5-day-old shoots were harvested by cutting off the coleoptile plus the first internode. The diethylether used was purified just before use by shaking with a slurry of $FeSO_4$ in water and then distilling.

The plant tissue was homogenized in a blendor with sufficient 95 % ethyl alcohol to make the final concentration 80 % ethanol. After 1 to 4 hours, the ethanol homogenate was filtered and the residue re-extracted once or twice with 80 % ethanol. The combined ethanolic extracts were freed of ethanol in vacuo using a rotating evaporator and a bath temperature of 55° C, or in later experiments with a vacuum spray drier at 35 °C. In experiments where IAA-2-C^{14} was added to permit recovery calculation, it was added to the ethanol filtrate or to the homogenate prior to filtration, with identical results. At this stage one of two methods was employed for extraction of IAA into ether. These alternative methods are designated in the text as acid or alkaline extraction procedures.

For the acid extraction procedure the aqueous concentrate (500–1000 ml) was adjusted to pH 4.0 with 5 N H_2SO_4 and the resultant precipitate removed by filtration through glass wool. The filtrate was then extracted three times with diethylether, and the pooled ether extract was shaken three times with 8 % $NaHCO_3$. After acidification of the combined bicarbonate fractions to pH 4.0 with 5 N H_2SO_4, acidic substances were again extracted into ether.

For the alternative alkaline extraction procedure, the aqueous concentrate was adjusted to pH 8 with $NaHCO_3$ and extracted three times with ether. The combined ether fractions were extracted once with 8 % $NaHCO_3$ and the bicarbonate phase combined with the aqueous concentrate. After acidification to pH 4 with 5 N H_2SO_4 (& filtration) the procedure described above was used.

The ether fractions, obtained by one of the above procedures, were dried over anhydrous Na_2SO_4 at 1° C, decanted from the Na_2SO_4 and the ether removed in vacuo. The residue was washed three times with 5 ml of ether and the combined ether fractions reduced in volume to about three milliliters. This fraction was applied to a buffered ether-water partition column (see below) and eluted with ether. The IAA containing fractions were pooled and dried over anhydrous sodium sulphate at 1° C. The dry ether was evaporated and the residue was taken up in a few drops of ethanol for paper chromatography.

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Whatman 3MM or Whatman 1 paper was used for ascending chromatography using isopropanol, ammonium hydroxide, water solvent (8:1:1, v/v) in most cases. The sample was usually applied as a band with known IAA spotted at one end so as to overlap the unknown slightly. Chromatography was conducted in the dark at 1° C for 20 to 30 hours, after equilibration for 8 to 12 hours. IAA was located on the paper by a light-blue fluorescence under shortwave ultra-violet radiation (2537 A) or by spraying (with Ehrlich's reagent) a strip cut from the edge of the band containing the co-spotted IAA. The Ehrlich's reagent contained 1 g *p*-dimethylaminobenzaldehyde dissolved in 91.2 ml of 95 % ethanol and 8.8 ml of concentrated HCl. Chromatograms were eluted with 50 % ethanol and the eluant concentrated to 1 ml in vacuo. Sometimes rechromatography in the same solvent or electrophoresis was required for further purification.

Paper electrophoresis was conducted in a closed strip, solid support, plexiglass chamber using Whatman 3MM paper, 0.1 M citrate buffer, pH 5.25, 200 or 250 volts, and a temperature of 1° C. Under these conditions, the mobility was 2.94×10^{-5} cm²/v-sec.

In some instances, continuous flow electrophoresis proved to be valuable. The continuous flow electrophoresis unit was that of Durrum (10). Whatman 3MM paper was washed successively with ethylenediaminetetraacetate at pH 8.0, 0.1 N HCl, and distilled water. Electrophoresis at pH 6.55 with 0.025 M phosphate buffer at room temperature (in the dark) was found to be satisfactory. Under these conditions, the IAA migrated half way toward the positive electrode when the wick was 1 inch from the negative edge, while the pigments did not migrate.

Usually, 4 ml of Salkowski reagent (32) were added to 1 ml of eluted concentrate for the detection of IAA. The red color complex was measured with a colorimeter using a wide band, 540 mμ filter. Standard concentrations of IAA were included in each determination. The modified Avena section straight growth test of McRae et al (23) was used for bioassays.

The IAA-2-C^{14} used in these studies had a specific activity of 9.18×10^4 d/m/μg (31). Due to possibility of radio-decomposition, the sample was purified by column chromatography and paper chromatography twice during the course of these investigations. The stock solution was kept as a very dilute solution in ethanol and stored at −20° C. After the initial purification, no decomposition products were noted when the IAA-2-C^{14} was chromatographed.

COLUMN SEPARATION. Corn seedlings contain high concentrations of yellow-brown phenol-like pigments.[4] Chromatograms of corn seedling extracts were colored and it was impossible to detect μg amounts of IAA by fluorescence or with Ehrlich and Salkowski reagents. Column chromatography has been used in the isolation of IAA (11, 21, 22, 24). However, none of those columns appeared to be entirely satisfactory under our particular conditions.

The column chosen for use was a true partition column employing a buffered aqueous phase adsorbed on analytical grade diatomaceous silica (Celite) using ether as the mobile phase. This partition was similar to that used by Holley et al (18) except these workers used a Craig apparatus. Analytical grade Celite was thoroughly ground in a mortar with 0.8 times its weight of phosphate buffer. Portions of the damp Celite were slurried in the column with buffer saturated ether and the slurry packed with a glass rod. A flow rate of 0.5 to 2.0 ml per minute was obtained with 3 to 5 pounds pressure per square inch of nitrogen. The column was eluted with ether saturated

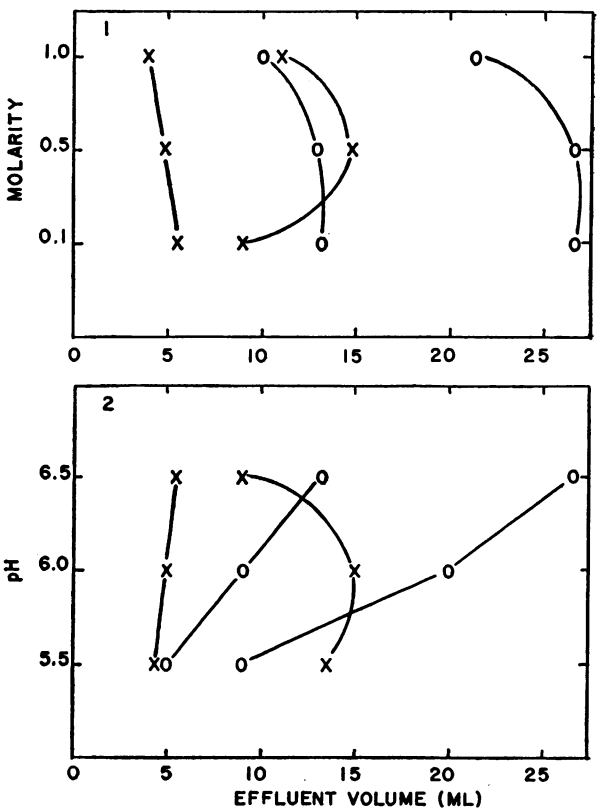

FIG. 1 (*top*). The effect of molarity of the potassium phosphate buffer (pH 6.5) on the migration of IAA and corn shoot pigments on a 10 g Celite column 25 mm (O.D.) in diameter. The boundary lines indicate qualitatively where elution of IAA (O–O), and pigments (×–×) begins and ends.

FIG. 2 (*bottom*). The effect of pH on the migration of IAA and corn shoot pigments on a 10 g Celite column 25 mm (O.D.) in diameter. The boundary lines indicate qualitatively where elution of IAA (O–O), and pigments (×–×) begins and ends.

[4] These are breakdown products of the cyclic hydroxamate 1,3-dihydroxy-2-keto-6-methoxybenzoxazine (17, 38).

with the same buffer used on the column. Figures 1 and 2 indicate the effect of molarity and pH of the phosphate buffer on the elution of IAA from a 2.5 cm column containing 10 g of Celite. High buffer concentrations depressed the solubility of IAA in the aqueous phase. Over the small range of hydrogen ion concentrations tested, there was an almost linear relationship between pH and rate of movement of IAA through the column.

Maximum separation of IAA and the corn pigments was obtained with 0.1 M phosphate buffer at pH 6.5[5]. Under these conditions, with a 1.8 cm diameter, 20 g column, IAA came off in the 40 to 60 ml fractions. (The IAA containing fractions from a 2.5 cm, 20 g column or a 5 cm, 100 g column were, respectively, 50–70 ml & 200–500 ml.) The highest concentration of IAA was found just after the first tube or two indicating that the IAA band trailed upward on the column. Usually 2 to 3 ml of ether were collected per tube, and the IAA located by adding 4 ml of Salkowski reagent and shaking. The color development was somewhat slower than in the regular Salkowski assay.

Results

Isolation & Recovery Experiments Employing Unlabeled Carrier IAA. One hundred μg of IAA were added to 1 liter of 95 % ethanol and carried through concentration, ether extraction, column and paper chromatography. The IAA was located on the paper chromatogram by UV fluorescence and eluted with 50 % ethanol. The ethanol was removed in vacuo at 55° C, 2 ml of water were added, and the amount of IAA estimated by Salkowski assay. In two experiments, recoveries were 50 and 56 %. Other similar experiments, where losses for individual steps were evaluated, indicated small cumulative losses for each operation.

Eight experiments without added IAA, using the acid ether extraction procedure, were conducted using from 178 to 1,000 g of 5-day-old etiolated corn shoots. Since no IAA was detected, recovery experiments were necessary. In the first experiment, 100 μg of IAA were added to the 80 % ethanol extract of 1,000 g of corn shoots. Another 100 μg of IAA were mixed with the residue and the residue re-washed with 2 liters of 80 % ethanol. Recovery was 25 % in both cases. In the second experiment, 120 μg of IAA added to 316 g of 5-day-old corn shoots before homogenization resulted in 27 % recovery. In the third experiment, 200 μg of IAA added to 700 g of corn shoots resulted in 30 % recovery. It appeared that recovery for the overall isolation procedure using 100 to 200 μg IAA per kilogram of corn shoots is 25 to 30 %.

One attempt was made to isolate IAA from 11.3 kilogram of 5-day-old etiolated corn shoots using the acid ether extraction procedure. Five lots of corn shoots were grown over a 30-day interval. Each individual lot was harvested, extracted with 80 % ethanol, and worked up to the partition column stage. This acid ether fraction was concentrated to dryness in vacuo and stored at −20° C. The pooled lots were dissolved in ether and the ether concentrate divided into three portions. Each portion was partitioned on a 5 cm diameter, 100 g column, and the IAA fractions pooled. The pooled eluant was electrophoresed in two runs using the continuous flow unit. No IAA could be detected by Salkowski assay. When 26 μg of IAA were placed on the 5 cm diameter partition column, recovery was 77 %; and when 10 μg of IAA were run on continuous flow electrophoresis, recovery was 70 %.

Isolation Experiments Using IAA–2–C^{14}. The above recovery experiments might not be valid owing to the addition of relatively large amounts of IAA. Therefore, low level experiments with corn shoots, using IAA–2–C^{14}, were carried out. The use of trace amounts of high activity IAA–2–C^{14} was valuable in several ways. Recovery data thus could be obtained during the actual isolation experiments. Even if losses were 90 % during isolation, addition of 1 to 3 μg of radioactive IAA was sufficient for recovery evaluation. The results of these isolation experiments are summarized in table I.

In experiment 1, no C^{14}-labeled IAA was recovered for unknown reasons. In experiments 2 through 5, employing the acid extraction procedure, recoveries of C^{14}-IAA averaged 14 %. About two μg of IAA could have been detected and accurately measured by the Salkowski assay procedure used. Thus one may calculate upper limits for the amount of free IAA which could have been present and escaped detection. For experiment 2, this would be 2 μg/0.145 minus the 1.89 μg IAA–C^{14} added or 11.9 μg IAA/Kg of fresh tissue. Since no trace of color was observed, one must conclude that substantially less than this amount of free IAA exists in corn as estimated by the acid

Table I

Recovery of IAA–2–C^{14} From Etiolated Corn Shoots & Detection of IAA in Corn Shoots[1]

	Fr wt	μg IAA–2–C^{14} added	% Recovery of IAA–2–C^{14}	μg IAA/kg by Salkowski assay corrected for recovery
1.	1,000	1.89	0	0
2.	1,000	1.89	14.5	0
3.	1,000	2.84	12.4	0
4.	1,000	1.89	12.0	0
5.	1,000	2.84	16.1	0
6.	3,000	1.65	28.1	13.3

[1] In experiments 1 through 5 the acid extraction procedure, and in experiment 6 the alkaline extraction procedure, was employed.

[5] The partition column sometimes failed when alkaline extraction was used.

extraction procedure. The comparable values for experiments 3 to 5 would be 13.2, 14.7, and 9.6 μg/kg. In experiment 6 where the preliminary alkaline extraction procedure was employed, an IAA concentration corresponding to 13.3 μg/kg was found.

FREE & BOUND IAA IN CORN GRAIN. Corn grain was a rich source of free and bound IAA in confirmation of numerous reports of the occurrence of IAA in the grain (2, 7, 15, 29, 30, 37, 41). The soaked grain was allowed to germinate in the dark for 0, 12, or 24 hours. The samples were ground and then extracted three times with 80 % ethanol. A gummy, yellow-orange IAA liberating precipitate was obtained upon concentration of the ethanol extracts. Following washing with water, the precipitate was dissolved in 150 ml of 1 N KOH and heated 1 hour at 100° C. Upon acidification, the re-precipitated material was removed by filtration through glass wool. The filtrate was carried through the IAA isolation procedure and the residue was again heated with alkali.

The free IAA and IAA released by hydrolysis (table II) were characterized by electrophoresis, and by paper chromatography in four solvents. The estimate of concentration, obtained by the Salkowski assay, was verified by direct spectrophotometric assay of chromatograms sprayed with Ehrlich's reagent and by Avena section bioassay.

It has been suggested (12, 27, 37), that IAA may arise from proteins containing tryptophan. Though zein is low in tryptophan, it is the major alcohol soluble protein. As reported (3), alkaline treatment of zein yielded no IAA and similar alkaline treatment of 100 mg of tryptophan yielded no IAA. To further determine whether or not the precursor was protein, about 500 g of corn meal was extracted three times with 1 liter portions of 0.01 M $MgCl_2$. The combined extracts were cooled to 1° C and the protein precipitated by saturation with ammonium sulfate. The protein was collected by filtration and washed with 80 % ethanol. The ethanol was removed in vacuo at 60° C. The ethanol-soluble and ethanol-insoluble proteins were heated for 1 hour in 1 N KOH at 100° C. IAA was isolated from only the alkaline treated ethanol-soluble protein.

Sugar beet leaves and roots, green peas, cucumbers, potatoes, bananas, and etiolated pea shoots were examined qualitatively for the presence of IAA and other Ehrlich reactive substances by the described methods. Vegetative sugar beet roots and leaves and possibly pea shoots contained IAA in sufficient quantities to be detected.

TABLE II

ISOLATION OF FREE & BOUND IAA FROM CORN GRAIN [1]

EXPT.	μg OF IAA/kg		
	FREE	1ST HYDROLYSATE [2]	2ND HYDROLYSATE [2]
1	82	186	98
2	115	509	63
3	171	...	...
4	330	...	...
5 [3]	16,800	...	...

[1] In experiments 1 to 4, Michigan 350 grain was soaked for 4 hours. In experiment 1, it was ground after soaking; in 2 and 3, it was germinated 12 hours at 25° C, and in 4, germinated 24 hours at 25° C.

[2] IAA obtained following hydrolysis of the 80 % ethanol-soluble, water-insoluble residue for two successive 1 hour periods with 1 N KOH at 100° C.

[3] Market stage yellow sweet corn kernels purchased locally.

DISCUSSION

Housley, Booth, and Phillips (19) reported failure to find IAA in corn seedlings, and our preliminary studies confirmed this finding. Reinert and Forstman (25) obtained similar results, and postulated some sort of binding of IAA in a manner perhaps similar to that investigated by Tegethoff (33) in corn scutellum. In the present case using acid extraction, recovery of 1 to 3 μg of IAA was only the order of 12 to 14 %; while recovery of 100 μg was about 25 %. The increased recovery when the aqueous concentrate was made alkaline may suggest some reversible binding. It is doubtful if actual destruction of IAA at pH 4.0 is a factor. Brian (5) has found binding of 2-methyl-4-chlorophenoxy acetic acid is greater at low pH and that crude corn extracts bind comparatively large amounts. One may question whether or not any free IAA exists in vivo. From the present work, the occurrence of *appreciable* free IAA in ethanolic extracts of corn shoots must be considered doubtful, since added IAA (1–3 μg) is largely lost. One could conclude that endogenous and exogenous free IAA is bound to some ethanol soluble component upon grinding the tissue. An alternative is that all the IAA in vivo occurs in the form of a very labile complex. The binding of exogenous IAA may or may not be related to this complex. Adjustment of the pH of the aqueous concentrate to 8.0 for preliminary removal of neutral ether soluble substances, led to an estimate of 13.3 μg of IAA per kilogram. IAA should have been detected, at least qualitatively, in the experiments using acid ether extraction. Since this was not the case, either endogenous IAA was liberated from a complex by alkaline pH adjustment or was produced from tryptophan or other precursors.

In the present work, IAA could actually be detected in corn shoots or roots by wet ether extraction for 3 hours at 4° C. Furthermore, subsequent 70 % ethanol extraction of the residue resulted in detection of IAA. However, no IAA could be detected in ethanol or subsequent ether extracts when the initial extraction was with 80 % ethanol. The greater ease with which IAA is detected by wet ether extraction may be due to its ability to dissociate bound IAA. In this respect, it is interesting to note that ether can prevent polar auxin transport (36). However, Wildman and Muir (40) have demonstrated IAA produc-

tion from trytophan during wet ether extraction with a low temperature optimum.

Though IAA has been detected in Avena coleoptiles (28, 34) experiments with radioactive IAA indicate no redistribution under geo- or phototropic stimulation (6, 8, 13, 26). A complex such as here discussed might explain the experiments on tropisms demonstrating a lack of redistribution of radioactive IAA. Thus exogenous IAA might not be in equilibrium with the endogenous IAA complex.

IAA can be derived from tryptophan in vitro by preparations from various tissues (9, 14, 30, 39, 41) and production of IAA from tryptophan by a particulate fraction from corn seedlings has been observed (17). Tryptophan–2–C^{14} is converted directly to carboxyl labeled IAA (17) and the other acid ether soluble products were similar to those reported by Dannenburg and Liverman (9). However, in contrast, little labeled IAA could be isolated when excised corn shoots were fed tryptophan–2–C^{14}.

In contrast to the growing shoot, corn kernels are a rich source of free IAA (2, 7, 15, 29, 30, 37, 41) and an IAA releasing fraction, presumably identical to that of Berger and Avery (2, 3). This substance might be a protein or polypeptide since it is precipitated with ammonium sulfate. However, Stehsel (29) found the alkali labile IAA complex in immature sweet corn seed was dialyzable.

Summary

Methods found useful in the isolation of IAA from kilogram quantities of plant tissue are described. A buffered ether-water partition column technique was developed. The use of trace amounts of IAA–2–C^{14} was especially valuable during the isolation, and in evaluating the recovery. IAA was detected in 80 % ethanol extracts of corn kernels and shoots, as well as vegetative sugar beet roots and leaves. The difficulties of detection of IAA in corn shoots are discussed in detail and it is suggested IAA may occur as a labile complex in ethanol extracts of this tissue. An 80 % ethanol-soluble, aqueous-insoluble fraction from corn kernels was found to release IAA upon alkaline hydrolysis. It appears this fraction may be protein in nature.

Acknowledgments

We should like to take this opportunity to thank Dr. S. A. Gordon who supplied the IAA–2–C^{14} and Dr. H. Sell for the use of the vacuum spray dry apparatus. It is a pleasure to acknowledge the assistance of Dr. H. Fukui in the ether extraction experiments.

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NEWS & NOTES

REPORT ON 1961 MEETING OF SOUTHERN SECTION OF AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS. On February 6 to 8 the Southern Section of the American Society of Plant Physiologists met with the Association of Southern Agricultural Workers at Jackson, Miss. All the sessions were well attended. The program included 28 contributed papers and a symposium on photosynthesis.

The symposium was chaired by Dr. Hans Gaffron, Florida State University. Papers were presented by Dr. André Jagendorf, McCollum-Pratt Institute, Johns Hopkins University; by Dr. Birgit Vennesland, University of Chicago, and by Dr. Roderick K. Clayton, Biology Division, Oak Ridge National Laboratory. The symposium will be printed and copies will be available from Secretary-treasurer Joseph C. O'Kelley, Biology Dept., University of Alabama, Tuscaloosa, at the cost of $1.00.

The Annual Plant Physiologists' Breakfast was held on the morning of February 8. A first prize award of $25.00 for the best paper given by a graduate student was awarded to Mr. Theodore Holmsen, University of Florida, for a paper, "Geotropism in *Zea mays*". Another award of a copy of the Annual Review of Plant Physiology, volume 11, 1960, was made to Mr. Coleman Ward, Virginia Polytechnic Institute, for his paper, "Effects of Potassium Levels on Orchard Grass and Red Clover in Sand Culture". The annual breakfast address was given by Dr. Harold Evans, North Carolina State College, on "The Essentiality of Cobalt for Leguminous Plants Grown Under Symbiotic Conditions".

Officers elected for 1961 were chairman, Dr. Howard E. Joham, Texas Agricultural Experiment Station; vice-chairman, Dr. Robert D. Powell, University of Florida; secretary-treasurer, Dr. Joseph C. O'Kelley, University of Alabama. Elected members of the Executive Committee are Dr. Howard Teas, University of Puerto Rico; Dr. Wayne C. Hall, Texas Agricultural Experiment Station, and Dr. Robert Burns, U.S.D.A., Experiment, Georgia. Dr. Teas is the Southern Section representative to the American Society of Plant Physiologists.

—JOSEPH C. O'KELLEY, *Secretary-Treasurer*, Southern Section.