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John R. Chisnell

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THE PRESENCE AND TRANSLOCATION OF INDOLE-3-ACETYL-*MYO*-INOSITOL IN SHOOTS OF *ZEA MAYS* L.

By

John Richardson Chisnell

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ABSTRACT

THE PRESENCE AND TRANSLOCATION OF
INDOLE-3-ACETYL-*MYO*-INOSITOL
IN SHOOTS OF *ZEA MAYS* L.

BY

John Richardson Chisnell

I. Enzymatic Synthesis of [5-^{3}H]Indole-3-Acetic Acid and [5-^{3}H]Indole-3-Acetyl-myo-Inositol from L-[5-^{3}H]Tryptophan. High specific actibity L-[5-^{3}H]tryptophan is converted to [5-^{3}H]indole-3-acetic acid by enzymes from *Pseudomonas savastanoi*. [5-^{3}H]indole-3-acetic acid can then be converted to [5-^{3}H]indole-3-acetyl-1-O-β-D-glucose and subsequently to [5-^{3}H]indole-3-acetyl-*myo*-inositol by enzymes from kernels of *Zea mays* sweet corn. Synthesis is on a nano-molar scale with little or no dilution of the initial specific activity. Yields, based on L-tryptophan, were 68% when [5-^{3}H]indole-3-acetic acid was the end product and 40% when [5-^{3}H]indole-3-acetyl-*myo*-inositol was the end product.

II. Myo-Inositol Esters of Indole-3-Acetic Acid are Endogenous Components of Zea mays L. Shoot Tissue. Indole-3-acetyl-*myo*-inositol esters have been demonstrated to be endogenous components of etiolated *Zea mays* shoot tissue. This was accomplished by comparison of the putative compounds with authentic, synthetic esters. The properties compared were liquid and gas-liquid chromatographic retention times and

the 70 eV mass spectral fragmentation pattern of the pentaacetyl derivative. The amount of indole-3-acetyl-myo-inositol esters in the shoots was determined to be 74 nmol·kg^{-1} fresh weight as measured by isotope dilution, accounting for 19% of the ester indole-3-acetic acid of the shoot. This work is the first characterization of an ester conjugate of indole-3-acetic acid from vegetative shoot tissue using multiple chromatographic properties and mass spectral identification. It is of interest that the kernel and the seedling shoot both contain indole-3-acetyl-myo-inositol esters, and that these esters comprise approximately the same percentage of the total ester content of the kernel and of the shoot.

III. Translocation of Radiolabeled Indole-3-Acetic Acid and Indole-3-Acetyl-myo-Inositol from Endosperm to Shoot of Zea mays L. The distributions of total radioactivity, radiolabeled indole-3-acetic acid and radiolabeled ester conjugated indole-3-acetic acid in the shoots of dark-grown *Zea mays* after the application of either [5-^{3}H]indole-3-acetic acid or [5-^{3}H]indole-3-acetyl-myo-inositol to the endosperm have been determinded. Sharp differences were found in the distribution and chemical form of the radiolabeled indole-3-acetic acid in the shoot depending whether [5-^{3}H]indole-3-acetic acid or [5-^{3}H]indole-3-acetyl-myo-inositol was applied to the endosperm. We show that indole-3-acetyl-myo-inositol applied to the endosperm provides both free and ester conjugated indole-3-acetic acid to both the mesocotyl and coleoptile. We calculate that indole-3-acetic acid in the endosperm may supply some of the indole-3-acetic acid in the mesocotyl but that it supplies essentially none to the coleoptile or primary leaves.

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INTRODUCTION

It is well documented that the tip of a growing shoot plays a special role in regulating the growth of the lower portion of the shoot (Boysen-Jensen, 1936, Went and Thimann, 1937, Van Overbeek, 1939 Thimann, 1977). The studies of Went, Cholodny, Snow and others indicated that the shoot tip supplied the growth hormone auxin (presumably indole-3-acetic acid (IAA)) to the lower portion of the shoot. However, the exact chemistry of IAA production by the tip is unclear. It has not been established whether the tip is synthesizing IAA *de novo*, or from tryptophan, or by hydrolysis of IAA conjugates. This work is addressed to that question by studying one of the possible precursors of IAA (IAA-*myo*-inositol) for conversion by the tip.

Early work on auxins by Cholodny (1935) and Van Overbeek (1941) suggested that the seed was the source of an auxin precursor in young seedlings. The structures and concentrations, down to a level of 10 $\mu g \cdot kg^{-1}$, of all the IAA containing compounds and the metabolic turnover rates of a number of the indolylic compounds in the kernel of *Zea mays* have been determined (cf. Cohen and Bandurski, 1982). In the *Z. mays* kernel IAA-*myo*-inositol accounts for approximately 30% of the total IAA while free IAA accounts for only about 1% of the total IAA (cf. Cohen and Bandurski, 1982). In the young dark-grown shoot of *Z. mays* ester conjugates of IAA constitute up to 80% of the total IAA present (Bandurski and Schulze, 1977). Nowacki and Bandurski (1980) demonstrated that exogenous radiolabeled IAA-myo-inositol was translocated from the endosperm to the shoot and that it was hydrolysed in the shoot releasing free IAA. They also calculated that a sufficient amount of ester IAA in the shoot was derived from IAA-myo-inositol in the endosperm to potentially supply the estimated auxin needs of the growing shoot.

At the time this study was initiated (78/79) there was evidence that the seed is the source of an auxin precursor for the young dark-grown seedling, that IAA-myo-inositol constitutes a significant amount of the the low molecular weight esters of IAA in the Z. mays kernel, and that IAA-myo-inositol can be translocated from the endosperm to the shoot and there be hydrolyzed, releasing free IAA. Further, it had been estimated that sufficient IAA-myo-inositol is translocated from the endosperm to the shoot to potentially supply the auxin needs of the growing shoot. This study then set out to further test the hypothesis that IAA-myo-inositol is a seed auxin precursor in the shoots of dark-grown Z. mays by 1) establishing the natural occurrence of IAA-myo-inositol in the shoot; by 2) determining the route of transport of IAA-myo-inositol from the endosperm to and through the shoot; and by 3) determining the pattern of distribution of IAA-myo-inositol and IAA, derived from IAA-myo-inositol, in the shoot. In order to perform these studies it was first necessary to synthesize high specific activity tritium labeled IAA-myo-inositol. IAA-myo-inositol is not available commercially, labeled or unlabeled. The tritium label was needed in order to have a sufficiently high specific activity for its use in tracer amounts. Tracer amounts were required 1) in order to be able to detect the endogenous IAA-*myo*-inositol in the vegetative tissues, which was expected to occur only at a low concentration, and 2) not to significantly perturb the normal processes of translocation of IAA-*myo*-inositol to the shoot. The experimental work is presented in the form of three individual research papers, one of which has been published while the other two are in manuscript form. The literature review gives a brief history of the work leading up to this study and more specific references can be found in the introduction sections of the research papers.

LITERATURE REVIEW

The classic concept of auxin relationships in a young seedling involves movement of a seed auxin precursor to the tip of the shoot or root, conversion of the auxin precursor to an active auxin and then transport of the active auxin to the growing region where hormonal control can be exerted (Went and Thimann, 1937, p 65). The early work leading to this concept has been thoroughly reviewed (cf. Boysen-Jensen, 1936, Went and Thimann, 1937, Went, 1951, Haagen-Smit, 1951) and more recently reexamined (Ehmann, 1973). The chemical form of the auxin precursor is however still a matter of debate.

The term auxin has been defined by a committee of the American Society of Plant Physiologists, which in part reads: "a generic term for compounds characterized by their capacity to induce elongation in shoot cells" (Tukey et al., 1954, Larsen, 1954). In this review auxin will be used to refer to preparation and compounds which have been tested for by this biological activity, regardless of whether its chemical structure is known or not.

Skoog (1937), in developing a more sensitive *Avena* curvature bioassay for auxin, first demonstrated the long held assumption that the seed is the source of precursors for auxin synthesized in the shoot tip of seedlings. By de-seeding the plant he showed that the source of auxin for the coleoptile tip is removed; the regeneration of the physiological tip is prevented in decapitated coleoptiles, and thus more sensitive test plants are obtained. He was also able to collect a substance from the decapitated coleoptile stump into agar blocks which gave no curvatures with his test method in the first 5 to 6 hours (as would be expected if the substance were and active auxin) but gave curvature 10 to 20 hours after application presumably by its conversion to an active auxin. Skoog (1937) also demonstrated that tryptophan, which Thimann (1935) had shown to be a precursor of IAA in Rhizopus suinus, and tryptamine would behave in exactly the same manner in the de-seeded Avena test as the precursor collected from the coleoptile stumps. Went and Thimann (1937, p 65) proposed that the seed auxin precursor was an auxin ester. This suggestion was, in part, based on the previous work of Kogl et al. (1934) who obtained considerable yields of auxin after the saponification of various oils prepared from seeds.

Van Overbeek (1941) compared the amounts of auxin obtained from 1 mm coleoptile tips of Zea by exhaustive diffusion and by exhaustive extraction. Assuming that auxin obtained by exhaustive extraction was active auxin and that obtained by exhaustive diffusion to be active auxin present in the tip at excision plus active auxin derived from precursor, Van Overbeek estimated that auxin precursor accounts for 92% of the potential auxin in the Zea coleoptile tip.

Haagen-Smit et al. in 1941 were the first to isolate IAA from a higher plant. Indole-3-acetic acid was isolated from alkaline hydrolyzed cornmeal and identified by its color reaction with ferric chloride and lack of melting point depression when mixed with synthetic IAA. Since the amount of IAA isolated after alkaline hydrolysis was much greater than the amount of auxin activity extracted without alkaline hydrolysis they proposed that most of the IAA must be in a bound form (presumably an ester).

Until this time IAA had been assumed to occur only in lower plants. The endogenous auxin active in higher plants was thought to be either "auxin A" or "auxin B", chemically related compounds which had been isolated from urine, malt and corngerm oil (kogl et al., 1933, 1934). The fact that no one else was ever able to repeat the isolation of these two compounds did not prevent a long debate (which lasted into the 1950's) as to which of the three compounds were the active auxins in higher plants (Haagen-Smit, 1951, Went 1951). Avery et al. (1941) demonstrated that the auxin of maize endosperm was very stable to heat and strong alkali (5N NaOH) but was completely destroyed by autoclaving in strong acid (2.5N HCl). They concluded that because of these properties the auxin could not be identical with auxins A or B. These properties are, however, consistent with the auxin being IAA. It is currently thought that IAA is the principle active auxin in plants (Bandurski and Bonner, 1952, Jacobs, 1979), and that auxin A and auxin B were artifacts.

Berger and Avery (1944a) confirmed the earlier observation of Haagen-Smit et al. (1941) of the presence of IAA in cornmeal by isolating an auxin precursor which, after alkaline hydrolysis, yielded IAA. Berger and Avery (1944b) further characterized this precursor, showing that it released IAA after mild alkaline hydrolysis (pH 9.5 for 5 min), as would be expected for and ester-linked complex. The precursor was very resistant to conversion to IAA by a variety of proteolytic enzymes and had a low nitrogen content (4.7%) suggesting that the precursor was not a protein. They also tested zein, whole corn gluten, tryptophan and three tryptophan containing proteins all of which yielded only extremely small amounts of auxin activity as compared to the isolated precursor when tested under the same conditions. They thus concluded that none of theses substances was the precursor.

This led to twenty more years of attempts at characterizing the bound auxins and auxin precursors of higher plants (cf. Bentley, 1958, 1961, Gordon, 1961, Cohen and Bandurski, 1982).

The first breakthrough was the demonstration by Andreae and Good (1955) that exogenous IAA can be converted to IAA-aspartate by pea shoot sections. This was the first chemical identification of an auxin conjugate formed *in vivo*. Its R_f value and solubility properties suggest that IAA-aspartate may account for a number af auxin active compounds previously isolated but not characterized (Bentley, 1961).

Shantz and Steward (1957, Steward and Shanz, 1959) reported the isolation of a chromatographically pure endogenous substance from immature corn kernels which, upon hydrolysis, yielded IAA and arabinose. This compound was later thought (Cohen, 1979) to be identical with and IAA-*myo*-inositol-arabinoside isolated from mature corn kernels an characterized by Labarca et al. (1965).

From 1961 to 1974 a series of papers from Bandurski's laboratory reported work which identified and determined the concentration of all of the IAA-containing compounds of mature *Zea mays* kernels present in amounts of $10 \mu g \cdot kg^{-1}$ or greater. This work has been thoroughly reviewed (Ehmann, 1973, Cohen, 1979, Cohen and Bandurski, 1982) and the results are summarized as follows: IAA accounts for approximately 1% of the total IAA-containing compounds of the kernel; IAA-*myo*-inositols, 15%; IAA-*myo*-inositol-arabinosides, 23%; IAA-*myo*-inositol-galactoside, 8%; and the Di-*O*- and Tri-*O*-IAA-*myo*-inositols and the 2-*O*-, 4-*O*-, and 6-*O*-

IAA-D-glucopyranoses all together accounting for less than 1% of the total IAA; and IAA-glucans, where the glucan is a β-1-4-cellulosic-glucan with 7 to 50 glucose units per IAA, accounting for the remaining 53% of the total IAA. The total amount of all of the IAA-containing compounds in the dry corn kernels used was approximately 76 $mg \cdot kg^{-1}$.

Epstein et al. (1980) determined the pool sizes and metabolic turnover rates of a number of the indolylic compounds in the kernel of the germinating corn seedling. The isotope dilution technique was used to measure the amounts of tryptophan, tryptamine, IAA and IAA-myo-inositol present in the kernel at zero time. Continued dilution of the applied labeled compound, as a function of incubation time, was then used to estimate turnover. Knowing the pool size and rate of turnover, it is possible to estimate the specific activity of applied radiolabeled compounds in the kernel. With this Epstein et al. (1980) were able to calculate from data of Hall and Bandurdki (1978) on the amount of radioactivity in IAA in the corn shoot derived from either radiolabeled IAA or radiolabeled tryptophan applied to the endosperm, that neither IAA or tryptophan in the endosperm supplied sufficient IAA to the shoot to supply its estimated auxin needs. Thus, other compounds in the seed must be the source of the IAA for the growing shoot.

IAA-_myo_-inositol esters constitute 30% of the low molecular weight IAA derivatives in the corn kernel and thus are attractive candidates as seed auxin precursors of the shoot. Nowacki and Bandurski (1980) studied the translocation of radiolabeled IAA-_myo_-inositol from the endosperm to the shoot of 4 day old dark-grown corn seedlings. They found that the ratio of radiolabeled free IAA to radiolabeled ester IAA in the shoot, after applying radiolabeled IAA-_myo_-inositol to the endosperm, was 7:93 which is identical to the ratio found for endogenous free to ester IAA (Bandurski and Schulze, 1974). Thus the translocated radiolabeled IAA-_myo_-inositol can be hydrolyzed in the shoot to release IAA and it appears to be equilibrating, at least to the extent of its free to ester ratio, with the endogenous IAA compounds in the shoot. Utilizing the data on pool size and turnover rates of IAA-_myo_-inositol in corn kernels (Epstein et al.,1980) they calculated that IAA-_myo_-inositol in the endosperm was traslocated to the shoot at a rate of 6.3 pmole into one shoot in one hour (Nowacki and Bandurski, 1980). This amount is sufficient to supply the estimated 5 to 9 pmole of IAA needed per shoot per hour (Nowacki and Bandurski, 1980).

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ENZYMATIC SYNTHESIS OF 5-^{3}H-INDOLE-3-ACETIC ACID AND 5-^{3}H-INDOLE-3-ACETYL-MYO-INOSITOL FROM 5-^{3}H-L-TRYPTOPHAN

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SUMMARY

Labeled l-tryptophan is converted to indole-3-acetamide and then to indole-3-acetic acid by enzymes from Pseudomonas savastanoi. Labeled indole-3-acetic acid can be converted to indole-3-acetyl-1-O-β-D-glucose and to indole-3-acetyl-myo-inositol by enzymes from kernels of Zea mays sweet corn.

Key Words: l-tryptophan, indole-3-acetamide, indole-3-acetic acid, indole-3-acetyl-1-O-β-D-glucose, indole-3-acetyl-myo-inositol.

INTRODUCTION

High specific activity ^{3}H-indole-3-acetic acid (IAA) is commercially available only by custom synthesis. However, 5-^{3}H-l-tryptophan can be converted to 5-^{3}H-indole-3-acetic acid in 70% yield, and at one-tenth the cost, by enzymes from Pseudomonas savastanoi by the following reaction sequence (1,2):

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a) l-tryptophan + O_2 $\longrightarrow$ indole-3-acetamide + CO_2 + H_2O

b) indole-3-acetamide + $2H_2O$ $\longrightarrow$ indole-3-acetic acid + NH_4OH

Labeled IAA may then be converted to indole-3-acetyl-myo-inositol by enzymes from kernels of Zea mays by the reaction sequence (3):

c) indole-3-acetic acid + UDPG $\longleftrightarrow$ indole-3-acetyl-1-O-β-D-glucose

d) indole-3-acetyl-1-O-β-D-glucose + myo-inositol $\longrightarrow$ indole-3-acetyl-myo-inositol + glucose

The enzyme catalyzing reaction a has been characterized as tryptophan 2-monooxygenase (EC 1.13.12.3) (1, 4); the enzymes catalyzing reactions b, c and d have been characterized only as to the products formed (1, 3).

A chemical synthesis of ^{14}C-indole-3-acetyl-myo-inositol employing ^{14}C-IAA-imidazole as the acylation reagent and myo-inositol as the alcohol acceptor has been published (5) as has an enzymatic synthesis of ^{14}C-IAA using a similar Pseudomonas savastanoi enzyme preparation (2). However, the enzymatic syntheses here described are adapted to a high specific activity, microscale synthesis of ^{3}H-IAA-myo-inositol and ^{3}H-IAA. ^{3}H-IAA has been used to study transport (6) and metabolism (cf. 7) of IAA in plants and in a radioimmunoassay for IAA (8). IAA-myo-inositol is a major ester of IAA in Zea (9), and the labeled compound has been used to study turnover of the ester (10) and its transport (11).

EXPERIMENTAL

Materials:

5-^{3}H-l-tryptophan, specific activity 29 Ci/mmol (±10%), was obtained from Research Products International Corp. (a product of CEA, France) and used without isotopic dilution or prior purification. It was 75% radiochemically pure as determined on thin layer chromatograms (Silica Gel 60 tlc plates, E. Merck, Darmstadt) developed with Solvent A (methylethylketone-ethylacetate-ethanol-water [3:5:1:1]). [2-ring-^{14}C]indole-3-butyric acid (^{14}C-IBA), specific activity 508 μCi/mmole, was synthesized by Dr. J.D. Cohen (12). High performance liquid chromatography (HPLC) was performed on a Whatman PXS 10/25 ODS reverse phase column (25 cm x 0.46 cm) with a Whatman CO:PELL ODS precolumn (7 cm x 0.2 cm) (Whatman Inc., Clifton, NJ). Tetrahydrofuran was redistilled over potassium metal under an N_2 atmosphere immediately prior to use. Gas-liquid chromatography was performed on a Varian 2740 gas chromatograph equipped with both a Varian TSD nitrogen specific thermionic detector and an FID with N_2 as carrier gas. Two 6 ft x 2 mm ID glass columns packed with 3% OV17 on 100/120 Gas Chrom Q (Applied Science, State College, PA) were used, one with the nitrogen specific detector for determination of the relative quantities of compound and one in conjunction with an FID for peak collection to determine radioactivity. Scintillation counting was performed with a Beckman LS7000 scintillation counter. SP Sephadex C-25 was from Pharmacia Inc., Piscataway, NJ; UDPG, indole-3-acetic acid and <u>myo</u>-inositol were products of Sigma Chemical Co., St. Louis, MO, and used without purification.

<u>Enzyme preparation</u>:

<u>Pseudomonas</u> <u>savastanoi</u> was grown as described by Kosuge et al (1), harvested by centrifugation and stored as a cell paste in liquid nitrogen. All enzyme preparative steps were performed at 0-3°. Approximately 10 g of frozen cells were thawed and suspended in 50 ml of glass distilled water, resedimented, and this procedure repeated three times. The pellet from the third centrifugation was suspended in 50 ml of 0.01 M potassium phosphate buffer, pH 7.6, containing 5 mM mercaptoethanol. The cell suspension was sonically disrupted (Branson Model 125, Branson Instruments, Inc., Danbury, Conn.) for five 30 sec pulses with 30 sec intervals between each pulse, and the resultant homogenate was centrifuged at 20,000 x g for 15 min. To the supernatant fluid (50 ml total volume), 15.6 g of solid $(NH_4)_2SO_4$ (enzyme grade, Mann Research Lab., Inc., New York, NY) was added, the resultant precipitate sedimented by centrifugation and then dissolved in 5 ml of 0.05 M Tris-HCl, pH 7.6, containing 5 mM mercaptoethanol. This solution was desalted on a 30 ml bed volume Sephadex G-50 column eluted with the buffer used to dissolve the $(NH_4)_2SO_4$

pellet. The desalted enzyme (10 ml total volume) was divided into 1 ml aliquots and stored at -70° for further use. Under these conditions both decarboxylating and hydroxylating enzymes were stable for at least 2 weeks.

An enzyme preparation from Zea, catalyzing reactions c and d, was prepared by the method of Kopcewicz et al (13). Stage I enzymes (the 10,000 x g supernatant of the crude homogenate) were made 85% saturated with respect to $(NH_4)_2SO_4$ by the addition of the solid salt. The precipitated proteins were suspended in 0.01 M Tris-HCl, pH 7.1, buffer and desalted on a Sephadex G-50 column to yield Stage II enzymes (3). This preparation, in 5.0 ml aliquots stored at -70°, was stable for at least 10 weeks.

Product characterization:

Indole-3-acetic acid: The time course for the conversion of 5-^{3}H-l-tryptophan to labeled indole-3-acetamide and IAA was followed by thin layer chromatography of aliquots of the enzyme reaction mixture. With Solvent A on Silica Gel 60, authentic l-tryptophan, indole-3-acetamide and IAA are at R_f 0.13, 0.75 and 0.87 respectively. The putative IAA was at R_f 0.87. The R_f of the putative IAA in another solvent system, chloroform-methanol-water (85:14:1), on Silica Gel 60 was also identical to that of authentic IAA (R_f 0.23). The GC retention time of the methyl ester, prepared using diazomethane (14), was identical to that of the methyl ester of authentic IAA. The identity of IAA was further established by its conversion to IAA-myo-inositol.

The radiochemical purity of ^{3}H-IAA was determined by thin layer chromatography in two solvent systems (Solvent A and chloroform-methanol-water [85:14:1]) on Silica Gel 60.

The specific activity of the ^{3}H-IAA was determined by a modification of the method of Cohen and Schulze (12). Approximately 2 nmole ^{14}C-IBA was added to 1 nmole biosynthetic ^{3}H-IAA. Methylation of the mixture and gas chromatography using the nitrogen specific detector (12) showed the mole ratio of IBA to IAA to be 0.78. Chromatography of a second aliquot of the mixed methyl esters with collection of the radioactivity at the retention times of IAA and

and IBA showed the ratio of the radioactivities of IAA to IBA to be 70,800. As can be deduced from the equations of Cohen and Schulze (12), the specific activity of the IAA in the mixture can be calculated by the following equation:

$$\left(\frac{\text{dpm IAA}}{\text{dpm IBA}}\right)\left(\frac{\text{moles IBA}}{\text{moles IAA}}\right)(\text{specific activity IBA}) = \text{specific activity IAA}$$

The specific activity of the ^{3}H-IAA was determined to be 28 Ci/mmole.

Indole-3-acetyl-myo-inositol: The putative mixed isomeric IAA-myo-inositols (15) were eluted from a 0.9 x 17 cm HPLC column of sulfonated styrene-divinylbenzene copolymer (Beckman PA-28) (5,16) with retention times identical to those of authentic IAA-myo-inositols. They had the same R_f values on thin layer chromatograms (Silica Gel 60 developed in Solvent A) as did authentic IAA-myo-inositols (R_fs 0.28 and 0.32). Upon ammonolysis in 15% NH_4OH for 45 min at 45°, the methyl ester prepared from the sample had a GC retention time identical to that of the methyl ester of authentic IAA. Ammonolysis of IAA esters yields the free acid and the amide. The amide was not volatile in the GC system used and was not detected. In a previous paper (3), GC-MS analysis of the trimethylsilyl derivatives of the reaction products of Zea enzyme Stage II and unlabeled authentic IAA showed mass spectral fragmentation patterns identical to those of authentic hexakis trimethylsilyl-IAA-myo-inositol.

The radiochemical purity of ^{3}H-IAA-myo-inositol was determined by thin layer chromatography on Silica Gel 60 developed in Solvent A.

The specific activity of the ^{3}H-IAA-myo-inositol was determined after purification to 94% radiochemical purity by first chromatographing it over an SP-sephadex column (1 ml bed volume) eluted with 2-propanol-H_2O (1:1) and then chromatographing it on a reverse phase HPLC column eluted with tetrahydrofuran-H_2O (18:82). The ester was ammonolyzed to free IAA, and the procedure described above was followed exactly. Approximately 3 nmole ^{14}C-IBA was added to 0.5 nmole ^{3}H-IAA from ammonolyzed biosynthetic ^{3}H-IAA-myo-inositol. The mole ratio of IBA to IAA was 2.13 and the ratio of radioactivity in IAA to IBA was 24,500. The specific activity of ^{3}H-IAA-myo-inositol was calculated to be 27 Ci/mmole.

Enzymatic Conversions:

5-^{3}H-indole-3-acetic acid: The 5-^{3}H-l-tryptophan (1.5 mCi, 0.05 μmol) in 1.5 ml of water was incubated with 1.0 ml of the *Pseudomonas* enzyme preparation in 0.05 M TrisHCl, pH 7.6, buffer for 2 hr at 37°. The reaction proceeded to 90% completion as determined by thin layer chromatography and was terminated by the addition of an equal volume of 2-propanol. Denatured proteins were removed by centrifugation and the supernatant fluid applied to a 0.6 x 20 cm DEAE-Sephadex-acetate column. The column was developed with 300 ml of a linear gradient of 1:1 2-propanol-water to 1:1 2-propanol-0.04 M potassium phosphate (pH 3.5). Elution was at 0.04 M phosphate (140 to 154 ml). Tubes containing more than 5% of the total eluted radioactivity were combined. The IAA was extracted into ether (4 x 14 ml), the ether dried over Na_2SO_4 and evaporated to near dryness, and the IAA taken up in 5 ml of acetonitrile. The yield was 68%, the radiochemical purity was 91%, and the specific activity was 28 Ci/mmole.

5-^{3}H-indole-3-acetyl-*myo*-inositol: The 5-^{3}H-l-tryptophan (1.5 mCi, 0.05 μmol) in 1.5 ml water was incubated with 1.0 ml of the *Pseudomonas* enzyme preparation and 1.5 ml of the *Zea* enzyme preparation Stage II, together with 5 μmol of UDPG and 5 μmol of *myo*-inositol in a total volume of 4 ml. The reaction was stopped by adding an equal volume of 2-propanol; the product was freed of anionic materials and purified on the PA-28 HPLC column developed with 1:1 2-propanol-water (3). The resultant product was stored in 1:1 2-propanol-water at -20° in sealed ampules. The yield was 40% based on l-tryptophan, the radiochemical purity was 70%, and the specific activity was 27 Ci/mmole.

Incubation of the tryptophan with both enzyme preparations simultaneously provided better yields of IAA-*myo*-inositol than did converting tryptophan to IAA and later esterifying the IAA to *myo*-inositol.

DISCUSSION

Our results indicate that high specific activity ^{3}H-tryptophan can be enzymatically converted to ^{3}H-IAA and ^{3}H-IAA-myo-inositol with little or no dilution of the specific activity. The determined specific activities of the products are within 10% of that of the manufacturer's stated specific activity of 29 Ci/mmole for the ^{3}H-l-tryptophan substrate. The manufacturer's accuracy in the specific activity determination was ±10%.

The modification of the specific activity determination method of Cohen and Schulze used here is a simplification of their calculations. This simplification eliminates the requirement for knowing the amount of internal standard added to the sample (in this case ^{14}C-IBA); only the specific activity need be known. It also eliminates the requirement that the isotopically labeled internal standard be chemically related, and requires only that the standard contain nitrogen and that the relative nitrogen specific detector response to the two compounds be determined. Lastly, by reducing the number of calculations the accuracy of the method is improved.

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EXPERIMENTAL II

MYO-INOSITOL ESTERS OF INDOLE-3-ACETIC ACID ARE ENDOGENOUS COMPONENTS OF ZEA MAYS L. SHOOT TISSUE[1]

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Footnotes

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[3]Abbreviations: IAInos, Indole-3-acetyl-*myo*-inositol; FID, flame ionization detector; TSD, thermionic specific detector; PVPP, polyvinylpolypyrrolidone; [^{14}C]IBA, [2-ring-^{14}C]indole-3-butyric acid; [^{14}C]IAAsp, [2-acetyl-^{14}C]indole-3-acetyl-L-aspartate.

Abstract

Indole-3-acetyl-*myo*-inositol esters have been demonstrated to be endogenous components of etiolated *Zea mays* shoots tissue. This was accomplished by comparison of the putative compounds with authentic, synthetic esters. The properties compared were liquid and gas-liquid chromatographic retention times and the 70 eV mass spectral fragmentation pattern of the pentaacetyl derivative. The amount of indole-3-acetyl-*myo*-inositol esters in the shoots was determined to be 74 nmol kg^{-1} fresh weight as measured by isotope dilution, accounting for 19% of the ester indole-3-acetic acid of the shoot. This work is the first characterization of an ester conjugate of indole-3-acetic acid from vegetative shoot tissue using multiple chromatographic properties and mass spectral identification. It is of interest that the kernel and the seedling shoot both contain indole-3-acetyl-*myo*-inositol esters, and that these esters comprise approximately the same percentage of the total ester content of the kernel and of the shoot.

Introduction

Early work on Avena by Skoog (23) indicated that the seed was the source of an auxin precursor transported to young dark grown shoots. Went and Thimann (25) suggested that this auxin precursor might be an ester. Previous work in this laboratory has shown that the IAA ester, indole-3-acetyl-myo-inositol (IAInos)[3], is a major component of the Zea mays endosperm (12,24), and that ester conjugates of IAA constitute up to 80% of the IAA in the shoots of young dark grown Zea (3). The presence of alkali-labile auxin complexes in Zea xylem sap was demonstrated by Sheldrake (20), who suggested that the proposed seed auxin precursor was an ester transported in the xylem. Later, Nowacki and Bandurski (16) demonstrated that exogenous radiolabeled IAInos was transported from the endosperm to the young dark grown shoot and could be recovered from the shoot both as intact IAInos and as free IAA. However, until now IAInos has not been shown to be an endogenous component of the shoot tissue. In this report we demonstrate that IAInos is an endogenous component of Zea shoot tissue and provide an estimate of the amount present in the shoot tissue. This report corrects a previous abstract of these studies (4) which contained erroneously high calculations of the amounts of IAInos and free + ester IAA in the shoot tissue. This report adds to the increasing body of literature pointing to the importance of IAA conjugates in vegetative tissue (1,8,13).

Materials and Equipment

Radioactivity was measured with a Beckman LS 7000 scintillation counter. HPLC was performed with a Varian 5060 liquid chromatograph equipped with a Varian UV-5 280 nm detector. GLC was on a Varian 2740 gas chromatograph equipped with an FID and a TSD adjusted for organic nitrogen detection. GC-MS was performed with a Hewlett Packard 5985a quadrupole mass spectrometer.

Materials were from the following sources:insoluble PVPP, Dowex 50W-X2 200-400 mesh and DEAE sephadex:Sigma; sulfonated styrene-divinyl benzene PA-28 resin:Beckman; 3% OV-17 on 100/120 Gas chrom Q:Applied Science; Partisil 10, Partisil 10-ODS, Co:Pell ODS, and HC Pellosil HPLC column packing materials: Whatman; _Zea mays_ cv. Stowell's Evergreen Hybrid Sweet Corn:Burpee Seed Co.; 4-dimethylaminopyridine:Aldrich. [^{14}C]IBA (508 μCi/mmol), [^{14}C]IAAsp (54.5 μCi/μmol) and IAInos were synthesized (5,9,17) and kindly provided by J.D. Cohen (USDA, Beltsville). Indole-3-acetyl-DL-aspartate was synthesized by the method of Mollan _et al_. (15) and kindly provided by R.P. Hangarter (Michigan State University). [5-^{3}H]IAInos (two batches, 29 Ci/mmol and 27 Ci/mmol) was synthesized (14) from L-[5-^{3}H]tryptophan (29 Ci/mmol and 27 Ci/mmol, respectively) obtained from Research Products International. [1-acetyl-^{14}C]IAA (59 mCi/mmol) was obtained from Amersham and [2-acetyl-^{14}C]IAA (42.2 mCi/mmol) was obtained from New England Nuclear.

Pyridine and acetic anhydride, for acetylations, were distilled and stored in a desiccator over anhydrous $CaSO_4$. Tetrahydrofuran was freshly redistilled over potassium metal in a nitrogen atmosphere.

Methods and results

Plant material. Corn kernels were surface sterilized in 1% NaOCl for 10 min, soaked in running tap water (21°C) for 24 h and then sown in flats of vermiculite and watered with distilled water. Growth was for 5 d in darkness at 25°C. A phototropically inactive green light was used during manipulations for growth and harvest.

Isolation of IAInos. Whole corn shoots were cut approximately one to two cm above the seed and dropped into liquid nitrogen. Prior to grinding, the liquid N_2 was evaporated and the shoots weighed to provide a fresh weight estimate. The shoots were ground in a blender with sufficient acetone to give a tissue:acetone ratio of 3:7 (w/v). To the acetone was added a known amount of [^{3}H]IAInos (approximately 4 $\mu Ci \cdot kg^{-1}$) and [^{14}C]IAA (approximately 2 $\mu Ci \cdot kg^{-1}$) and sufficient 2-mercaptoethanol to make the final concentration 50 mM. The tissue was extracted for 24 h at 22°C, and the mixture filtered through Whatman No. 1 paper on a Buchner funnel. The retained acetone insoluble material was dried, weighed, and used as an estimate of the dry weight of the tissue. Aliquots of the filtrate were taken for quantitative determination of free and free + ester IAA. The acetone was distilled from the filtrate *in vacuo* and the remaining aqueous solution (pH 4) was partitioned twice with $CHCl_3$ and twice with diethyl ether. After reducing the pH of the solution to 3.5 with H_3PO_4 it was again partitioned. These partitionings removed impurities and extracted 97% of the ^{14}C. Residual solvent was removed and the remaining solution reduced in volume *in vacuo* to 130 ml. The sample was then placed on a PVPP column (4.1 cm x 22 cm) and eluted with 50 mM NaAcetate, pH 3.5, containing 10 mM 2-mercaptoethanol. ^{3}H containing fractions eluting between 250 ml and 700 ml were pooled, and the volume reduced _in vacuo_ to 90 ml. The sample was next placed on a short Dowex column (50W-X2, Na^+ form, 9.6 cm x 4 cm in a fritted disc Buchner funnel) and eluted with 50% (v/v) aqueous 2-propanol containing 10 mM 2-mercaptoethanol, using suction. Fractions of 50 to 200 ml were collected with the ^3H containing fractions eluting at 300 to 1200 ml. The first 25% of the ^3H fractions were too impure for use in the following step. Therefore, these fractions were pooled and rechromatographed on Dowex 50W-X2. The last three-fourths of the ^3H fractions from the second column were pooled with those from the first Dowex column. The volume was reduced _in vacuo_ to 3 ml and placed, in 3 batches, on a PA-28 column (0.9 x 17 cm) (7) and eluted with 50% (v/v) aqueous 2-propanol containing 10 mM 2-mercaptoethanol at a flow rate of 11 $ml \cdot hr^{-1}$. During concentration of the Dowex fractions a precipitate formed which could be removed by centrifugation without significant loss of ^3H. This was also the case during the concentration procedure preceeding all of the following liquid chromatographic steps. IAInos containing fractions (elution volume 20 to 40 ml) from the three PA-28 runs were pooled, the volume reduced _in vacuo_, loaded, in 3 batches, on a Partisil 10 ODS C_{18} reverse phase column (0.46 x 25 cm) with a Co:Pell ODS precolumn (0.21 x 7 cm) and eluted with 5% (v/v) aqueous ethanol at a flow rate of 1 $ml \cdot min^{-1}$. The mixed isomeric IAInos (10) elute in 3 peaks with maxima at 9, 12, and 15 ml. The ^3H containing fractions eluting at the IAInos elution volumes from the three Partisil 10 ODS runs were pooled and evaporated _in vacuo_ to about 30 µl of a viscous brown oily residue. This was dissolved in ethyl acetate:acetonitrile:ethanol:H_2O (5:3:3:1, v/v), volume 300 µl, and placed on a Partisil 10 normal phase column (0.46 x 25 cm) with an HC Pellosil precolumn (0.21 x 7 cm) and eluted with ethyl acetate:acetonitrile: ethanol:H_2O (10:3:3:1, v/v) at a flow rate of 0.5 ml·min^{-1}. The IAInos elutes as a single peak from 4 to 9 ml with a maximum at 6 ml. The IAInos fractions were pooled and evaporated to dryness. Then, using an N_2-flushed dry box, the residue was dissolved in 50 µl of pyridine containing 0.5% (w/v) 4-dimethylaminopyridine followed by the addition of 50 µl of acetic anhydride. The mixture was reacted for 10 min at 22°C to permit acetylation, then evaporated under a stream of N_2 to a thin brown oily residue. This was dissolved in 28% (v/v) aqueous ethanol to a final volume of 200 µl. The sample was next placed on a Partisil 10 ODS column and eluted with 28% (v/v) aqueous ethanol at a flow rate of 2 ml·min^{-1}. The acetylated IAInos elute in two peaks with maxima at 38 and 42 ml. Approximately one half of the ^3H recovered from this column eluted at volumes other than those for IAInos and was presumed to be decomposition products or underivatized material. Recovery of [^3H]IAInos at this step was approximately 20%.

Qualitative analysis

GLC. Acetylated IAInos fractions from the Partisil 10 ODS column were pooled, evaporated to dryness in vacuo, and the sample dissolved in tetrahydrofuran. GLC, using the TSD, was isothermally at 300°C on 3% OV 17 (2 mm x 1.8 m) using N_2 as the carrier gas. The acetylated derivative of authentic mixed isomeric IAInos elutes as four peaks in this system (Figure 1). In the acetylated plant sample two major peaks (peaks 1 and 3) have been detected (Figure 1) with the TSD.

It was important to determine whether the recoveries of all the isomers of IAInos by our purification procedure were equivalent. In Experiment 2 the amounts of ^3H, eluting at the retention times for the four IAInos GLC peaks, was determined for the [^{3}H]IAInos in both the pure authentic material, and after it had been reisolated from the plant sample. The [^{3}H]IAInos was diluted approximately 1:300 with IAInos, acetylated, the acetylating reagents evaporated, and the sample dissolved in tetrahydrofuran. Four hundred and sixty thousand dpm (approximately 2 nmol) were injected onto an OV 17 column, identical to the one described above, but connected to an FID with the detector flame extinguished. The radioactivity eluting between 0.25 to 18 min was collected and measured. The results are presented in the bar graph of Figure 2A, below a TSD tracing of an injection of 1 nmole from the same sample. Recovery of the injected ^{3}H was 43%. The relative amounts of ^{3}H eluting at the elution times for the four acetylated IAInos peaks was 11:2:79:9 (P_1:P_2:P_3:P_4). Forty eight thousand dpm (approximately 0.5 nmole) of the acetylated putative IAInos reisolated from the plant sample was injected as above and the radioactivity eluting between 0.25 to 32 minutes was collected and measured. The results are presented in the bar graph of Figure 2B, below an FID tracing of an injection of 7 nmoles of acetylated authentic IAInos. Recovery of injected ^{3}H was 36%. The relative amounts of ^{3} eluting at the elution times for the four acetylated IAInos peaks was 11:1:83:4 (P_1:P_2:P_3:P_4). The similarity in the ratios of the ^{3}H under the four peaks between the authentic [^{3}H]IAInos and the [^{3}H]IAInos as recovered from the plant sample suggests that the isomers eluting in peaks 1, 3, and 4 were equally recovered. The amount of ^{3}H under peak 2 in the plant sample was insufficient to rule out the possibility that the ^{3}H collected at that retention time represented tailing from peak 1.

GC-MS. In Experiment 2 sufficient material was recovered to permit GC-MS analysis. Analyses were at a 70 eV ionizing potential and chromatography was on 3% OV 17 (2 mm x 61 cm), temperature programmed at 200°C for 2 min followed by a temperature increase of $20°C\cdot min^{-1}$ to 300°C with He carrier gas at a flow rate of $26\ ml\cdot min^{-1}$. The retention times for peaks 1, 3, and 4 were 7.9, 9.0, and 9.4 min, respectively. Peak 2 was not observed. Table I lists the principal ions in the spectra of authentic and putative plant IAInos acetyl esters of peaks 1, 3, and 4. Figure 3 shows the spectra of the authentic and isolated pentaacetyl esters of IAInos in peak 3.

Quantitative analysis

IAInos. The amount of IAInos was determined by a modification of the double-standard isotope dilution method of Cohen and Schulze (9,14). In brief, the method utilizes coinjection into the GLC of a compound of known specific activity (the second internal standard) with a compound of unknown specific activity (the primary internal standard diluted by an unknown amount of endogenous compound). The ratio of the radioactivities collected at the elution times for the two compounds and the ratio of the peak areas of the two compounds are determined. Then if the relative molar response of the detector to the two compounds is known, the specific activity of the unknown compound can be calculated. To determine the specific activity of the [^{3}H]IAInos reisolated from the plant sample the following equation was used:

$$\frac{\text{IAAsp peak area}}{\text{IAInos peak area}} \times \begin{array}{l}\text{relative detector}\\ \text{response IAInos/IAAsp}\end{array} \times \frac{\text{dpm IAInos}}{\text{dpm IAAsp}} \times \begin{array}{l}\text{specific activity}\\ \text{of the IAAsp}\end{array}$$

$$= \text{the specific activity of the IAInos}$$

[^{3}H]IAInos was the primary internal standard, the second internal standard was [^{14}C]IAAsp methylated with ethereal diazomethane (19) forming the bis-methyl derivative. The molar response of the TSD to acetylated IAInos relative to bis-methyl-IAAsp was determined to be 0.68. The GLC system was as described for the qualitative analysis. Representative chromatograms are presented in Figure 1. Knowing the specific activity of the reisolated IAInos it was possible to calculate the amount in the plant sample using the isotope dilution equation (18):

$$Y = \left[\frac{C_o}{C} - 1\right] X$$

where C_0 is the initial specific activity of the added [^{3}H]IAInos, C is the specific activity of the reisolated [^{3}H]IAInos (determined as indicated above), X is the molar amount of [^{3}H]IAInos added to the extract, and Y is the molar amount of IAInos in the plant sample. The quantitative results for two experiments are summarized in Table II.

Free and free + ester IAA. Aliquots for free and free + ester IAA determination (approximately 5% and 3% by volume, respectively) were taken from the acetone extracts used for the IAInos quantitative analysis described above. Acetone was removed from the aliquots in vacuo. At this step the samples for free + ester IAA determination were made 1N with respect to NaOH by the addition of an equal volume of 2N NaOH and incubated for 1 hr at 22°C to hydrolyze the IAA esters (3). These hydrolysis conditions would also hydrolyze any acyl anhydride or thioester conjugated IAA, if present. The samples, both free and free + ester, were then acidified to pH 3.5 with H_3PO_4, and extracted three times with $CHCl_3$. The $CHCl_3$ layer was dried over anhydrous Na_2SO_4 and then the $CHCl_3$ was removed _in vacuo_. The residue was solubilized in 50% (v/v) aqueous 2-propanol and placed on a DEAE-Sephadex (acetate form) column (0.8 x 10 cm), washed with 20 ml of 50% (v/v) aqueous 2-propanol and eluted with a linear gradient of 50% (v/v) aqueous 2-propanol containing from 0 to 5% (v/v) glacial acetic acid. Fractions containing IAA (about 45 ml elution volume from beginning of gradient) were pooled and dried _in vacuo_. The residue was solubilized in 30% (v/v) aqueous ethanol containing 0.7% (v/v) acetic acid, loaded on a Partisil 10 ODS column and eluted with the solubilizing solution at a flow rate of 1 $ml \cdot min^{-1}$. Fractions containing IAA (about 8 ml elution volume) were pooled, dried _in vacuo_ and methylated with ethereal diazomethane. The methylated product was dried under a stream of N_2 and dissolved in tetrahydrofuran. The amount of IAA in the samples was determined by the same method as used for IAAnos. The primary internal standard for the free IAA quantitative determination was [^{14}C]IAA. When the aliquot for free + ester IAA determination was hydrolyzed, the [^{3}H]IAInos tracer in the sample was hydrolyzed, along with the plant ester IAA, yielding [^{3}H]IAA. Therefore, both [^{14}C]IAA and [^{3}H]IAInos (hydrolyzed to [^{3}H]IAA) served as parallel primary internal standards (requiring differential counting for the two isotopes) in the determination of free + ester IAA. [^{14}C]IBA, methylated with ethereal diazomethane, was the second internal standard. The GLC system was as described for the acetylated IAInos qualitative analysis except that chromatography was isothermal at 200°C. The results for two experiments are presented in Table II. The average and relative amounts of IAInos, free IAA, and free + ester IAA are summarized in Table III.

Discussion

Endogenous IAInos was isolated from 5 day old _Z. mays_ shoot tissue. It was identified by comparison of its physico-chemical properties with authentic IAInos, and measured quantitatively by an isotope dilution method. The mass spectra of the isolated compound were identical to those of the authentic compound, both having a molecular ion at m/z 547. Other ions, characteristic of acetylated _myo_-inositol, were at m/z 373 (M^+-IAA), m/z 157, and m/z 103 (Ac_2OH^+) (21). Ions characteristic of the indole moiety were at m/z 130 (the quinolinium ion) and m/z 103 (the styryl ion) (26). The putative IAInos cochromatographed with authentic [^{3}H]IAInos in 5 liquid chromatographic systems. As the acetyl derivative it cochromatographed with the authentic tritiated derivative in the liquid chromatographic and GLC systems tested. Two of the 4 acetylated authentic IAInos peaks eluting in the GLC system were detected with a TSD in the acetylated putative IAInos sample.

Since the isomers of IAInos elute as more than one peak in at least one of the chromatographic purification steps, the possibility of preferential loss of one or more of the isomers exists. Data suggesting that preferential isomer loss was not a problem is presented in figure 2 and discussed in the Methods and Results section. However, acyl migration, observed in inositol esters (2,22), has previously been noted in IAInos esters (12). This might account for similar ratios of [^{3}H]IAInos in the 4 GLC peaks as long as acyl migration occurred after the step in which a preferential loss might have occurred. If preferential loss did occur the most likely isomers to be lost would have been those eluting in peaks 2 or 4 in the GLC system. Loss of these isomers was more apt to be unnoticed since the labeled tracer contained relatively little of them and it was the label which was followed during purification. If the shoot tissue contained relativly more of these isomers than that found in the labeled tracer and preferential loss of these isomers did occur, then the estimate for the amount in the tissue may be low. If acyl migration occurred before preferential isomer loss the isomers of the endogenous IAInos and the isomers of the added [^{3}H]IAInos would have come to the same equilibrium mixture and all of the isomers would be equal in specific activity. Thus, the specific activity of any one isomer would reflect the amount of isotope dilution encountered by the entire sample, and preferential loss of an isomer would have had no effect on the accurate determination of the amount of IAInos in the tissue.

IAInos accounts for about 15% of the free + ester IAA or about 19% of the ester conjugated IAA of the shoot. Thus, about 80% of the ester IAA of the shoot is some ester(s) other than IAInos. This ratio of IAInos to ester IAA is similar to that found in the kernel after 4 days of germination (25%) (11). Other IAA esters in the shoot may be the same as those found in the kernel, for example, IAInos-arabinosides, IAInos-galactosides, di- and tri-O-IAInos, and IAA-glucosides (8). Due to the difficulty of isolating these types of neutral compounds, which would only be present in very low amounts in the tissue, identification and quantification of the other IAA ester conjugates in the shoot awaits the synthesis of suitable radiolabeled internal standards.

The close agreement in the amount of free + ester IAA in the shoot whether [^{14}C]IAA or [^{3}H]IAInos was used as the internal standard validates the customary use (cf. 3) of free IAA as the internal standard in the determination of free + ester IAA. This confirms an earlier validation noted by Cohen (6) when he compared values for total (free + ester + amide) IAA in seeds of <u>Glycine max</u> L. determined using [^{14}C]IAA and [^{14}C]IAAsp as internal standards.

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Table I. Characteristic ions in the 70 eV mass spectra of the pentaacetyl derivatives of authentic IAInos and of IAInos isolated from *Z. mays* shoot tissue.

	Relative Abundance (%)					
	Peak 1		Peak 3		Peak 4	
m/z	Authentic	Isolated	Authentic	Isolated	Authentic	Isolated
103	4.5	3.3	5.0	3.4	9.6	3.9
130	100.0	100.0	100.0	100.0	100.0	100.0
157	45.8	41.2	74.1	69.6	76.1	66.4
373	-	1.5	0.5	0.8	-	-
547	10.6	9.9	5.8	5.7	7.5	7.3

Table II. Quantitative analyses of IAInos, free IAA and free + ester IAA in *Z. mays* shoot tissue

	Tissue Extracted g dry wt	Tissue Extracted g fresh wt	Compund Assayed and Standard Used	Radioactivity Added dpm	Amount Added nmol	C_o dpm·nmol^{-1}	C dpm·nmo^{-1}	Amount in Tissue nmol·kg fresh wt^{-1}
Experiment 1			IAInos					
			[^{3}H]IAInos	5.9×10^{6}	0.092	6.4×10^{7}	1.7×10^{5}	61
			Free IAA					
	23	568	[1-acetyl-^{14}C]IAA	2.2×10^{6}	17	1.3×10^{5}	2.7×10^{4}	110
			Free + ester IAA					
			[1-acetyl-^{14}C]IAA	2.2×10^{6}	17	1.3×10^{5}	6.5×10^{3}	570
			[^{3}H]IAInos	5.9×10^{6}	0.092	6.4×10^{7}	2.2×10^{4}	470
Experiment 2			IAInos					
			[^{3}H]IAInos	1.6×10^{7}	0.27	6.0×10^{7}	1.0×10^{5}	87
			Free IAA					
	57	1855	[2-acetyl-^{14}C]IAA	7.8×10^{6}	83	9.4×10^{4}	2.5×10^{4}	120
			Free + ester IAA					
			[2-acetyl-^{14}C]IAA	7.8×10^{6}	83	9.4×10^{4}	7.5×10^{3}	520
			[^{3}H]IAInos	1.6×10^{7}	0.27	6.0×10^{7}	1.8×10^{4}	490

Table III. The average and relative amounts of IAInos, free IAA and free + ester IAA in _Z. mays_ shoot tissue.

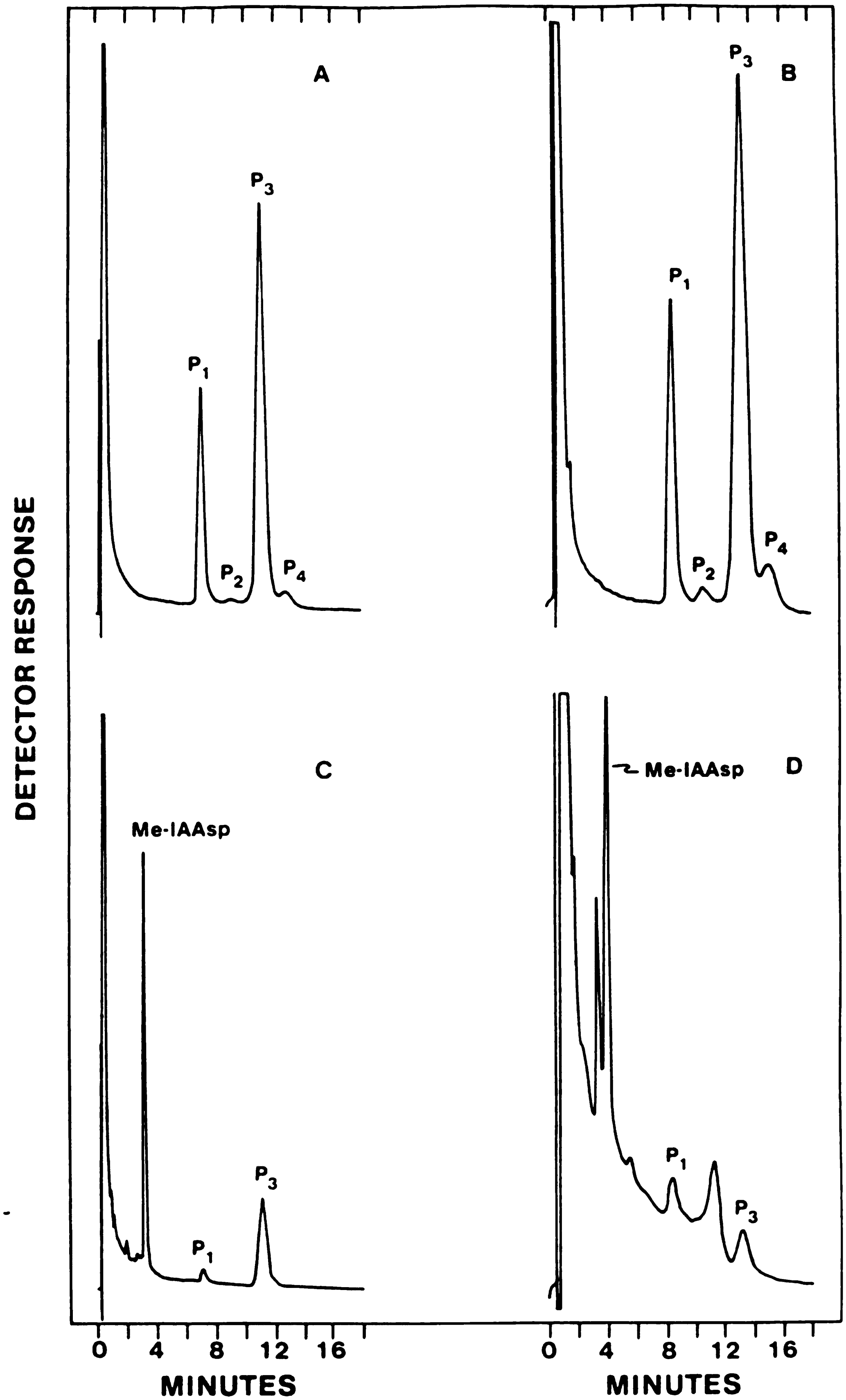
Compound	Amount in Tissue nmol/kg fr. wt.	% of Total
IAInos	74	15
Free IAA	120	24
Free + ester IAA	510	100

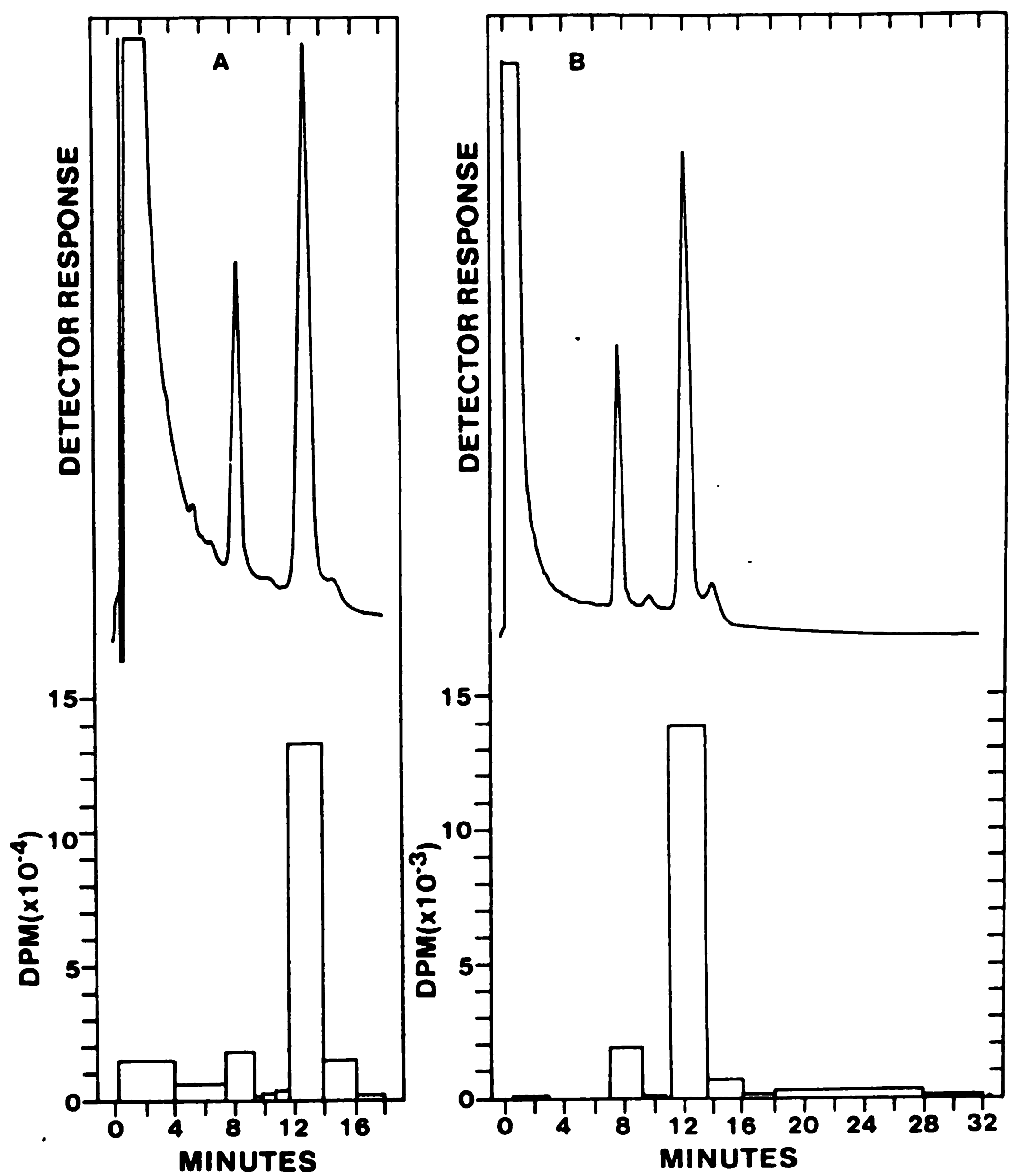
Figure Legends

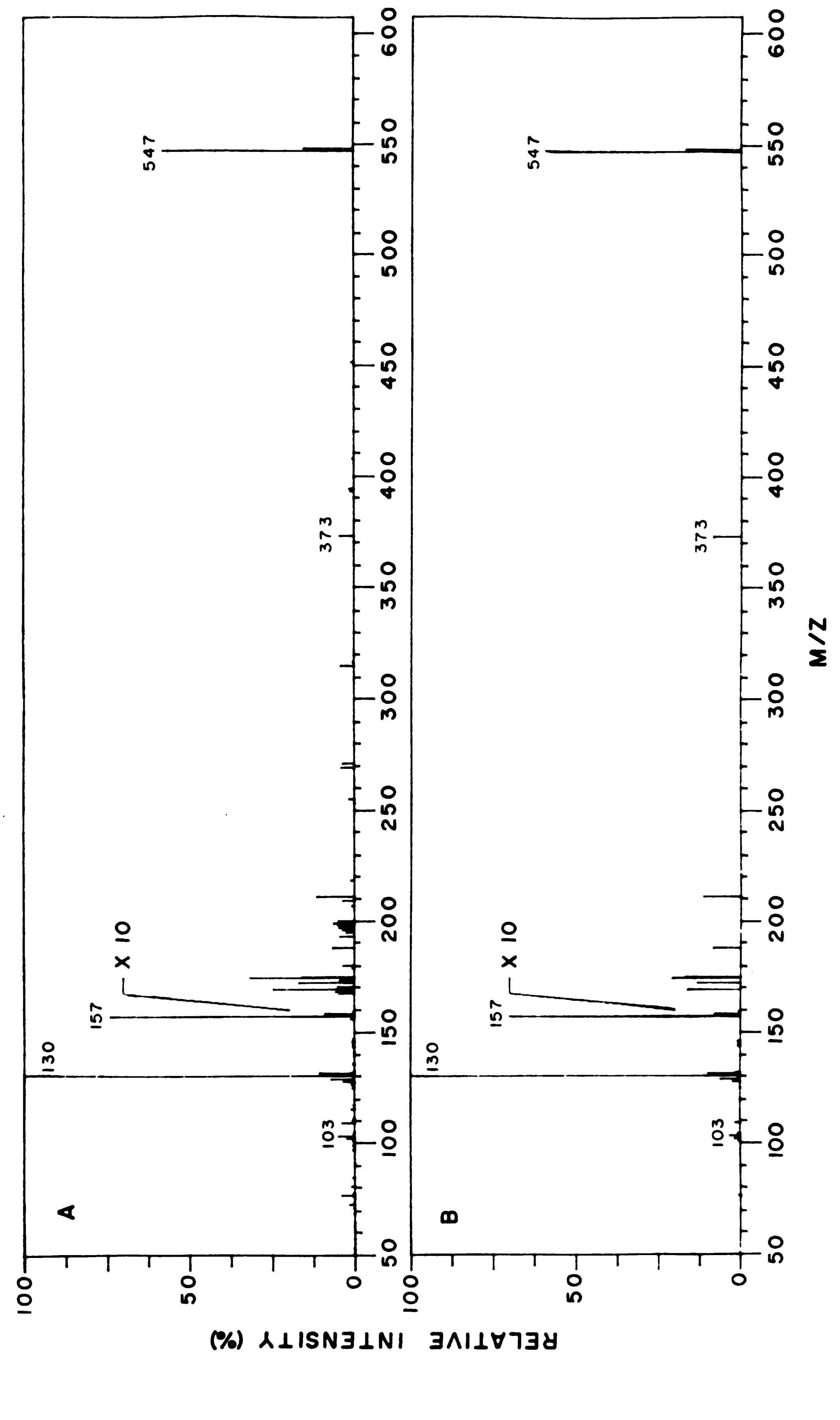
Fig. 1. TSD chromatograms of acetylated authentic IAInos and of acetylated putative IAInos isolated from _Z. mays_ shoot tissue. Chromatograms A and C are the authentic and putative IAInos, respectively, from Experiment 1. Chromatograms B and D are the authentic and putative IAInos, respectively, from Experiment 2. The four resolvable IAInos peaks (P_1-P_4) are seen in chromatograms A and B. Only P_1 and P_3 are seen in the plant samples, chromatograms C and D. Peaks between P_1 and P_3 in chromatogram D are thought to be contaminants (see Discussion). Also seen in chromatograms C and D is the second internal standard bis-methyl-[^{14}C]IAAsp (Me-IAAsp).

Fig. 2. The GLC elution profiles of ^{3}H from injections of [^{3}H]IAInos (A) and of putative [^{3}H]IAInos reisolated from _Z. mays_ shoot tissue (B). The ^{3}H elution profile resulting from the injection of 460,000 dpm (approximately 2 nmol) of acetylated [^{3}H]IAInos diluted 1:300 with acetylated IAInos is presented below a TSD tracing of an injection of 1 nmol of the same sample (A). The ^{3}H elution profile resulting from the injection of 48,000 dpm of acetylated putative [^{3}H]IAInos reisolated from the plant sample is presented below an FID tracing of a 7 nmol injection of acetylated authentic IAInos (B).

Fig. 3. The 70 eV spectrum of pentaacetyl IAInos. Spectrum A: GLC peak 3 of authentic pentaacetyl IAInos. Spectrum B: GLC peak 3 of putative pentaacetyl IAInos isolated from _Z. mays_ shoot tissue.


A
P3
P1
P2
P4
B
P3
P1
P2
P4
C
Me-IAAsp
P3
P1
D
Me-IAAsp
P1
P3
DETECTOR RESPONSE
0 4 8 12 16
MINUTES
0 4 8 12 16
MINUTES


A
B
DETECTOR RESPONSE
DETECTOR RESPONSE
DPM(x10-4)
DPM(x10-3)
0
5
10
15
0 4 8 12 16
0 4 8 12 16 20 24 28 32
MINUTES
MINUTES


A
B
547
373
157
X 10
130
103
50
100
150
200
250
300
350
400
450
500
550
600
M/Z
0
50
100
RELATIVE INTENSITY (%)

EXPERIMENTAL III

TRANSLOCATION OF RADIOLABELED INDOLE-3-ACETIC ACID AND INDOLE-3-ACETYL-*MYO*-INOSITOL FROM ENDOSPERM TO SHOOT OF *ZEA MAYS* L.[1]

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Footnotes

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[4]Abbreviations: IAInos, Indole-3-acetyl-_myo_-inositol; MeIAA, the methyl ester of IAA.

Introduction

It is well documented that the tip of a growing shoot plays a special role in regulating the growth rate of the lower portions of the shoot (2,21). The studies of Went, Cholodny, Snow and others indicated that the shoot tip supplied the growth hormone auxin (presumably indole-3-acetic acid) to the lower portion of the shoot. However, the exact chemistry of IAA production by the tip is unclear. It has not been established whether the tip is synthesizing IAA *de novo* (W.L. Pengelly, personal communication), or from tryptophan (9), or by hydrolysis of an IAA conjugate (14). This work is addressed to that question by studying one of the possible precursors of IAA for conversion by the tip.

Early work on auxins by Cholodny (4) and van Overbeek (25) in *Avena* suggested that the seed was the source of an auxin precursor for young seedlings. Skoog (19) developed a bioassay for a "seed auxin precursor" in which the substance was collected into agar as a diffusate from decapitated coleoptile stumps. Went and Thimann (27) suggested that this seed auxin precursor was an ester. The endosperm of kernels of *Zea mays* contains small amounts of IAA and large amounts of esterified IAA (1,11,16,22,23), and Sheldrake, (18) demonstrated that an alkalai-labile IAA compound is present in the xylem sap of *Z. mays*, thus, potentially being a seed auxin precursor. This laboratory has determined the structure and concentration of all the IAA containing compounds in the *Z. mays* kernel, down to a level of 10 $\mu g \cdot kg^{-1}$ (see 5 for references). IAA-*myo*-inositol (IAInos)[4] comprises approximately 15% of the total IAA of the kernel and 30% of the low molecular weight IAA esters (22). More recently this laboratory has determined the rate of metabolic turnover of the indolylic compounds in the kernel (8). With this knowledge of the structure, amount and turnover of IAA compounds in the *Zea* kernel, it was determined from the data of Hall and Bandurski (12) that the amount of IAA translocated to the shoot from the kernel is insufficient to supply the auxin needs of the growing shoot (14). Nowacki and Bandurski (14) using radiolabeled IAInos applied to the endosperm, demonstrated that IAInos is translocated to the shoot and that it can be hydrolyzed in the shoot releasing free IAA, thus qualifying it as a seed auxin precursor. They also calculated that a sufficient amount of ester IAA in the shoot had arisen from IAInos in the endosperm to supply the estimated auxin needs of the growing shoot.

In this paper we present data on the distribution in the shoot of total radioactivity, radiolabeled IAA and radiolabeled ester IAA arising from radiolabeled IAInos and radiolabeled IAA applied to the endosperm. We find differences in the distribution and chemical form of the radiolabeled IAA in the shoot depending upon whether radiolabeled IAA or radiolabeled IAInos was applied to the endosperm. We can confirm that IAInos applied to the endosperm provides free IAA to both the mesocotyl and coleoptile. However, further data on amounts and sites of hydrolysis will be required before it can be determined whether the IAInos translocated from the endosperm is hydrolyzed in the shoot tip, thus explaining the special role of the tip as a source of growth substance.

Materials and Methods

I. Radioactivity in the shoot from [^{14}C]IAA applied to the endosperm.

Plant Material. *Zea mays* L. var. Stowells Evergreen Hybrid, a white sweet-corn, was obtained from Ferry-Morse Seed Company, Mountain View, California. Seeds were surface sterilized in 1% NaOCl for 10 min and then soaked in running tap water (21°) for 22 to 32 h. The seeds were rolled in paper towels, and placed in buckets, containing a small amount of distilled water. Germination was in darkness at 25°C for 83 to 93 h at which time the shoot mesocotyls were 4 to 8 cm and the coleoptiles 1.5 to 2.5 cm long.

Application of [^{14}C]IAA. All manipulations were performed at 25°C with the use of a phototropically inactive green safelight. The top end of the kernel (approximately 20% by weight) was cut off, leaving the scutellum intact, so that an endosperm surface was exposed for isotope application (see Figure 1). Five µl of [2-actyl-^{14}C]IAA (51.7 mCi·mmol^{-1}, New England Nuclear) in 50% (v/v) aqueous 2-propanol was applied to the cut endosperm surface. Each 5 µl aliquot contained approximately 4000 dpm equal to 35 pmol of ^{14}C. Radiochemical purity was not determined, but, based upon previous experience with commercially supplied [2-acetyl-^{14}C]IAA, was estimated to be 80%. Thus 28 pmol of IAA was added to the kernel, which is equal to approximately 11% of the endogenous IAA remaining in the kernel (8). The seedlings were pinned through the endosperm to a styrofoam sheet with their roots dipping into a tray of distilled water. The entire plant holder was draped with moistened paper toweling. The seedlings (60 per time point) were incubated for 0.5, 1, 2, and 4 h with duplicate samples used for each point.

Determination of ^{14}C in the shoot. The shoots were cut off 0.5 cm above the seed and dissected into 3 pieces: 1) the coleoptile, containing the primary leaves and the apical 0.5 cm of the mesocotyl, 2) the vascular stele of the mesocotyl (probably separated from the cortex at the endodermis (10)), and 3) the mesocotyl cortex plus epidermis. The pieces were placed immediately into beakers sitting on dry ice, and the tissue was lyophilized and weighed. The samples were combusted in a Packard model 306 Sample Oxidizer, and the $^{14}CO_2$ collected and counted in a Packard Tri-Carb Scintillation Counter. Corrections were made for both combustion recovery and scintillation counting efficiency.

II. [^{3}H]IAA, free and free + ester, translocated to the shoot from [^{3}H]IAA applied to the endosperm.

Plant Material. *Z. mays* Stowells Evergreen Hybrid (Lot 80 for 1980) was obtained from the Burpee Seed Co., Clinton, Iowa. Plant material was grown and prepared as described in the preceeding section.

Application of [^{3}H]IAA. [5-^{3}H]IAA (29 Ci$\cdot$mmol^{-1}) was enzymatically synthesized from [5-^{3}]L-tryptophan (29 Ci$\cdot$mmol^{-1}) (13). Radiochemical purity was determined by the percentage of the radioactivity recoverable from a TLC plate at the R_f of the compound. The validity of this radiochemical purity determination was confirmed by reverse phase HPLC, where the radiochemical purity equaled the percentage of the total eluting radioactivity emerging at the elution volume for the compound. Application of the [^{3}H]IAA and manipulation of the seedlings was as described in the previous section. To one batch of seedlings (39 in number) 3.3 X 10^6 dpm (51 pmol) of [^{3}H]IAA (at 82% radiochemical purity, equaling 4.0 X 10^6 dpm ^{3}H) in 5 μl of 50% (v/v) aqueous 2-propanol was applied to the cut endosperm surface and the seedlings were incubated for

2 h. For another batch of seedlings (45 in number) 2.1×10^6 dpm (33 pmol) [^{3}H]IAA (at 93% radiochemical purity, equaling 2.3×10^6 dpm ^{3}H) in 2 µl of 50% (v/v) aqueous 2-propanol was applied to the cut endosperm surface and the seedlings were incubated for 4 h. Fifty one pmol of IAA is equal to approximately 21% of the endogenous IAA in the kernel and 33 pmol is equal to approximately 13%.

Determination of free and free + ester [^{3}H]IAA in the shoot tissue. The shoots were harvested and dissected as described above. The frozen tissue was weighed to determine the "fresh" weight and the tissue was ground in sufficient acetone to result in a tissue to acetone ratio of 30:70 (w/v). The acetone contained a known amount (about 3 µmol) of IAA as cold carrier. The tissue was extracted for 24 h at 23°C, then filtered through tared Whatman No. 1 filter paper, and the acetone insoluble material dried, and used as an estimate of the dry weight of the tissue. Acetone was removed from the filtrate _in vacuo_ and the aqueous solution divided, one half for determination of free IAA and one half for determination of free + ester IAA. The free + ester samples were made 1N with respect to NaOH and incubated for 1 h at 23°C to hydrolyze the IAA esters. The pH of both free and free + ester samples was adjusted to 3.5 using H_3PO_4, and the samples were partitioned three times with $CHCl_3$. The combined $CHCl_3$ fractions were dried over Na_2SO_4 and evaporated to dryness _in vacuo_. The residue was dissolved in 50% (v/v) aqueous 2-propanol and adsorbed on a DEAE Sephadex (Sigma, St. Louis, MO) (acetate form) column (5 ml bed vol), and eluted with a linear gradient of 50% (v/v) aqueous 2-propanol containing from 0 to 10% (v/v) glacial acetic acid. Fractions containing IAA were identified by spotting 10 µl aliquots on TLC plates and staining for indoles using

Ehmann's Van Urk-Salkowski Reagent (7). The IAA containing fractions were pooled, evaporated to dryness in vacuo, and then dissolved in 2-propanol. The sample was streaked on a 20 X 20 cm Silica Gel 60 TLC plate (Merk) and the plate developed with $CHCl_3$:CH_3OH:H_2O (85:14:1, v/v). A guide strip from the TLC plate was stained with Ehmann's reagent, as above, to locate the IAA. The band containing IAA was scraped off the TLC plate and the IAA eluted with 50% (v/v) aqueous 2-propanol. The solution was filtered through Whatman No. 1 filter paper to remove the silica gel, and the sample was loaded on a Sephadex LH-20 (Sigma) column (2 ml bed volume) and eluted with 50% (v/v) aqueous 2-propanol. Fractions containing IAA, identified as described for the DEAE fractions, were pooled and evaporated to dryness in vacuo. The residue was dissolved in CH_3OH and methylated using ethereal diazomethane (17) and dried under a stream of N_2. The residue was dissolved in tetrahydrofuran which had been freshly redistilled over potassium and under an N_2 atmosphere. The sample was then chromatographed on 3% OV 17 on' 60/50 Gas Chrom Q (Anspec, Ann Arbor, MI), 6 mm x 122 cm, isothermally at 188°C, using N_2 as carrier gas. Substances eluting at the retention time for MeIAA (approximately 3 to 4 min) were collected in an open ended glass tube plugged with silanized glass wool (Anspec). The MeIAA was eluted from the collection tube with methanol and its UV absorbance spectrum determined using a Cary 15 spectrophotometer. Aliquots were taken from the spectrophotometer cuvette and counted for ^3H in a Beckmann LS 7000 scintillation counter. The shape of the UV absorbance spectrum was used to confirm the purity of the MeIAA collected, and its molar extinction coefficient at 280 mm (E = 6060) was used to determine the MeIAA concentration of the solution. The concentration of the MeIAA in the solution along with the concentration of the ^3H in the solution were used to calculate the specific activity of the reisolated IAA. The specific activity of the reisolated IAA multiplied by the amount of cold carrier IAA added equalled the amount of [^{3}H]IAA, free or free + ester, in the shoot at the time of harvest.

III. [^{3}H]IAA, free and free + ester, translocated to the shoot from [^{3}H]IAInos applied to the endosperm.

Plant Material. Growth and preparation of the plant material was as described previously.

Application of [^{3}H]IAInos. [5-^{3}H]IAInos (27 Ci/mmol) was enzymatically synthesized from [5-^{3}H]L-tryptophan (27 Ci/mmol, Research Products International) by the method of Michalczuk and Chisnell (13). The application of 4 μl aliquots of the [^{3}H]IAInos and incubation conditions were as described in Section I. Each 4 μl aliquot contained approximately 2.2 X 10^6 dpm (37 pmol) of [^{3}H]IAInos (at 83% radiochemical purity, equaling 2.7 X 10^6 dpm ^3H) in 50% (v/v) aqueous 2-propanol. Thirty seven pmol equals about 1% of the endogenous IAInos in the kernel (8). The seedlings were incubated for either 2 or 4 h. The shoots were dissected as previously described and immediately placed in beakers containing liquid-N_2.

Determination of free and free + ester [^{3}H]IAA in the shoot tissue.

Prior to grinding, the liquid-N_2 was evaporated and the tissue weighed to determine "fresh" weight. The tissue was ground in a glass homogenizer with sufficient acetone to give a tissue to acetone ratio of 3:7 (w/v). The acetone contained a known amount of unlabeled IAA (approximately 1.2 μmol) added as carrier. The tissue was extracted, divided into free and free + ester samples, the free + ester sample hydrolyzed, and the samples partitioned as described in Section II. The residue remaining after evaporation of the $CHCl_3$ layer was dissolved in 20% (v/v) aqueous ethanol containing 1% (v/v) glacial acetic acid and placed on a Partisil 10 ODS C_{18} reverse phase HPLC column (0.46 X 25 cm, Whatman) with a Co:Pell ODS precolumn (0.21 X 7 cm, Whatman). The sample was eluted with 20% (v/v) aqueous ethanol containing 1% (v/v) glacial acetic acid at a flow rate of 1 ml$\cdot$min^{-1} in a Varian 5060 liquid chromatograph equipped with a Varian UV-5 280 nm detector. The large amount of impurities prevented monitoring IAA elution by 280 nm absorption, therefore, a tlc spot assay, as described in Section II, was used to locate the IAA containing fractions. The fractions eluting at the edges of the IAA peak (less than 5% of the peak) were discarded and the remaining peak fractions pooled. The pooled sample was evaporated to dryness _in vacuo_ and methylated using ethereal diazomethane. The methylation reagents were evaporated under a stream of N_2 and the residue dissolved in 30% (v/v) aqeous ethanol. The sample was placed on the reverse phase HPLC column, as previously described, and eluted with 30% (v/v) aqueous ethanol at a flow rate of 1 ml$\cdot$min^{-1}. The IAA elutes as a single fully resolved peak, as detected by 280 nm absorption, at approximately 14 to 16 ml. The relative amounts of IAA in the fractions collected at the IAA retention volumes were determined by the tlc plate spot assay previously described. The main fractions (approxiamtely 90% of the IAA in the peak) were pooled, the UV absorption spectrum was determined, and aliquots were taken from the spectrophotometer cuvette for determination of ^3H by liquid scintillation counting. The amounts of [^3H]IAA, free and free + ester, in the shoot sections were calculated as described in Section II.

IV. Longitudinal distribution of ^3H and [^3H]IAA, free and free + ester, in the shoot translocated from [^3H]IAInos applied to the endosperm.

Plant Material. Growth and preparation of the plant material was as described in Section II.

Application of [^3H]IAInos. To each kernel 1.2 X 10^6 dpm (19 pmol) of [5-^3H]IAInos (29 Ci/mmol; at 90% radiochemical purity, equaling 1.3 X 10^6 dpm ^3H) in 2 µl 50% (v/v) aqueous 2-propanol was applied.

Distribution of the ^3H in the shoot. Initially, a time course was run measuring the distribution of ^3H in the shoot in lots of 10 seedlings incubated for 1, 2, 4, 6, and 8 h. The shoots were cut from the seedlings approximately 0.5 cm above the kernel and then dissected into 6 pieces with 5 lateral cuts (see Figure 2A). The shoot pieces (10 pieces per vial) were placed in 20 ml scintillation vials sitting on dry ice, and the tissue was weighed to determine the "fresh" weight. NCS Tissue Solublilizer (Amersham) was added to the tissue, and the vials were capped and agitated for 16 h at 45°C to digest the tissue. Scintillation cocktail (6 g/l 2,5-diphenyloxazole, 81 mg/l 1,4 bis(2-(4-methyl-5-phenyloxazolyl))-benzene in toluene) was added to the mixture and the sample was counted for ^3H. The counting efficiency ranged from 14 to 35% as determined by both external and internal standards. The 4 h time point was repeated once with a slightly different dissection as was used when the distribution of the [^3H]IAA, free and free + ester, was determined (see Figure B). In the 4 h time point repeat experiment each shoot piece was placed in a separate vial in order to determine the variation in translocated ^3H from shoot to shoot.

Distribution of [^{3}H]IAA, free and free + ester. Fifty seedlings were incubated for 4 h after which the shoots were excised and dissected into 6 pieces as shown in figure 2B. The shoot pieces were placed in vials sitting on dry ice and then weighed to determine the "fresh" weight.

Determination of free and free + ester [^{3}H]IAA in the shoot pieces. The procedure was as described in section III.

V. Distribution of ^{3}H in the kernel after incubation with applied [^{3}H]IAA or [^{3}H]IAInos.

Ten kernels were excised from the seedlings, used for the [^{3}H]IAA and [^{3}H]IAInos translocation experiments described above. The kernels were dissected into 3 pieces of about equal size (see Figure 1). The uppermost section contained the cut endosperm surface to which the labeled compound had been applied. The pieces were collected in vials sitting on dry ice and weighed to determine the "fresh" weight. The tissues were then digested with NCS Tissue Solubilizer and counted for ^{3}H as described previously.

RESULTS

The overall intent of this work is to examine 1) the special function of the tip of etiolated seedlings in controlling the growth of the subapical elongating regions, and 2) the role seed auxin precursors play in this regulation. The purpose of the experiments presented here was to identify the paths of translocation in the shoot of free IAA and a putative auxin precursor, both originating in the endosperm. To this end, we present data on the distribution of free and ester radiolabeled IAA and total radiolabel in the shoot as a function of time after applying radiolabeled IAA and IAInos to the endosperm.

Experimental Design. In initial experiments a tissue oxidizer was used in determining the total radioactivity in shoot pieces. In later work, however, a tissue solubilizer was used because of the ease in processing multiple samples simultaneously.

The procedure used to determine the amount of free and free + ester [^{3}H]IAA required 1) that radiolabel not in IAA be removed, and 2) that any remaining non-radiolabeled impurities not absorb significantly at 280 or 220 nm. The initial procedure used (Section 2), which consisted of 5 purification steps (partitioning, DEAE, TLC, LH-20, GLC) and required 10 days to perform, had previously been shown to meet these criteria (14). This procedure was simplified (Sections III and IV) to one involving only 3 purification steps (partitioning, and 2 reverse phase HPLC steps), requiring 4 days to perform. The specific activity of reisolated [^{3}H]IAA, as determined by the simplified procedure, did not change significantly (less than 10%) when the IAA was further purified by GLC. The sensitivity of the procedure was approximately doubled by reducing, by one-half, the amount of cold carrier IAA added. This reduction was made possible due to the improved recovery of IAA resulting from fewer purification steps, and especially to the elimination of the TLC and GLC steps.

Raw data from experimental sections I through IV are presented in Tables I, II, III, and IV.

Results I. Results from experimental section I are presented graphically in figure 3 on a dpm per shoot section basis normalized to the amount of ^{14}C applied to the endosperm. ^{14}C was detectable in all parts of the shoot within one half hour, with the largest amount found in the mesocotyl stele. When the relative dry weight ratios of stele to cortex or coleoptile section (1:4) or the relative fresh weight ratios of stele to cortex or stele to coleoptile (1:6 and 1:4, respectively) were considered, the concentration of ^{14}C located in the mesocotyl stele was much higher.

At 1 h there was a 4 fold increase in the total amount of ^{14}C in the shoot compared to that found at 0.5 h. The stele still contained more ^{14}C per piece than the other shoot sections, and there was approximately a 3 fold increase in the amount of ^{14}C in this section over that found at 0.5 h. The coleoptile section contained about 80% as much ^{14}C and the cortex contained about 40% as much ^{14}C as that found in the stele. At 2 h, there was again an approximately 4 fold increase in the total amount of ^{14}C in the shoot as compared to 1 h. The coleoptile section now contained the largest amount of ^{14}C of the 3 sections, followed by the stele and then the cortex sections. At this time approximately 50% of the total ^{14}C in the shoot was located in the coleoptile section with 30% in the stele and 20% in the cortex. In one of the 2 h incubation experiments (I 27) the mesocotyl was not dissected. At 4 h there was a further 1.5 fold increase in the total amount of ^{14}C in the shoot as compared to 2 h. The coleoptile section still contained the largest amount of ^{14}C of the 3 sections (about 55% of the total), but now the relative amount in the cortex section had increased to about 30% of the total shoot ^{14}C while the relative amount in the stele section had decreased to about 15% of the total shoot ^{14}C.

Results II. Results from experimental section II on the amount of intact [^{3}H]IAA, free and free + ester per shoot section, are presented in figure 4, again, normalized to the amount of [^{3}H]IAA applied to each endosperm. The low radioactivities in cortex and especially in the coleoptile sections (see Table II) leaves open the possibility of inaccuracies in the amounts of [^{3}H]IAA determined for these sections. At both the 2 h and the 4 h time points most (85 and 80%, respectively) of the recoverable [^{3}H]IAA remained as the free acid, the same chemical form as applied to the endosperm. At 2 h 85% of the [^{3}H]IAA in the shoot was located in the stele and 85% of this was as free [^{3}H]IAA. This ratio of free to ester [^{3}H]IAA was also obtained for the cortex and coleoptile sections, which contained 13% and 2%, respectively, of the free + ester [^{3}H]IAA of the shoot. At 4 h there was a 1.5 fold decrease in the amount of [^{3}H]IAA, free + ester, found in the shoot contrasting sharply with a 1.5 fold increase in total radioactivity in the shoot over this time period when [^{14}C]IAA was applied to the endosperm. Further, there was a redistribution of both the location and the form (free or ester) of the [^{3}H]IAA from that found at 2 h. The stele section contained only about 56% of the total free + ester [^{3}H]IAA in the shoot, while the amount in the cortex increased to 40%. There still was only a trace amount of [^{3}H]IAA found in the coleoptile section (about 4%). Only the cortex section contained any ester [^{3}H]IAA, present in an amount approximately equal to the free [^{3}H]IAA. The relative distribution in the shoot of free + ester [^{3}H]IAA, at both 2 h and 4 h time points, when [^{3}H]IAA was applied to the endosperm contrasts sharply with the relative distribution in the shoot of total ^{14}C when [^{14}C]IAA was applied to the endosperm. The most striking contrast was in the coleoptile section which contained a large amount of the radiolabel, from radiolabeled IAA applied to the endosperm, but only trace amounts of intact radiolabeled IAA. Based upon the ratio of label recovered in the entire shoot at 2 h to that applied to the endosperm approximately 25% of the radiolabel was located in in intact IAA, free or ester, while at 4 h only about 10% of the radiolabel was located in intact IAA, free or ester. Thus, at 2 h [^{3}H]IAA, free + ester, accounts for 70% of the ^{3}H present in stele, 16% in the cortex and 1% in the coleoptile. At 4 h [^{3}H]IAA accounts for 37% of the ^{3}H present in the stele, 13% in the cortex and 1% in the coleoptile.

When this data is presented on an apparent concentration basis the relatively high amount of [^{3}H]IAA in the stele is highlighted (Figure 5).

Results III. The results from section III on the amount of intact [^{3}H]IAA, free and free + ester, per shoot piece are presented graphically in figure 4 normalized to the amount of [^{3}H]IAInos applied to each endosperm. Low radioactivity in the free [^{3}H]IAA samples, especially in the cortex at 2 h (see Table III), make the amounts of [^{3}H]IAA calculated for these sections subject to large error. When [^{3}H]IAInos was applied to the endosperm more than 90% of the recoverable [^{3}H]IAA in the shoot remained as ester IAA. In general the pattern of distribution of free + ester and ester [^{3}H]IAA in the shoot was similar to that found for total ^{14}C in the shoot when [^{14}C]IAA was applied to the endosperm (figure 4B). The ratios of the free + ester [^{3}H]IAA in the shoot sections were 33:22:45 (stele:cortex:coleoptile) at 2 h and 24:32:44 at 4 h when [^{3}H]IAInos was applied to the endosperm compared to the ratios of ^{14}C found in the shoot sections of 29:21:50 at 2 h and 13:32:55 at 4 h when [^{14}C]IAA was applied to the endosperm. The pattern of free [^{3}H]IAA distribution in the shoot was markedly different from that found when [^{3}H]IAA was applied to the endosperm. At 2 h the ratios of free IAA in the 3 shoot sections were approximately 10:1:4 (stele:cortex:coleoptile) and at 4 h these ratios were approximately 9:4:11 when [^{3}H]IAInos was applied to the endosperm. This contrasts with ratios of 39:6:1 at 2 h and 25:9:2 at 4 h when [^{3}H]IAA was applied to the endosperm. The total amounts of both free and free + ester [^{3}H]IAA in the shoots increased almost 4 fold from 2 to 4 h.

When this data is presented on an apparent concentration basis (Figure 5) both free and ester [^{3}H]IAA are seen to have the highest concentration in the stele, followed by that in the coleoptile section.

Results IV. The results of the experiment on the longitudinal distribution of ^{3}H and [^{3}H]IAA, free and free + ester, when [^{3}H]IAInos was applied to the endosperm are presented graphically in figures 6, 7 and 8. The changes in the distribution and amounts of ^{3}H per shoot section over time are presented in Figure 6A. The total amount of ^{3}H doubled from 1 to 2 h, and then increased 5 fold to peak at 4 h after which it decreased slightly. The ratios of the total ^{3}H in the shoot from 1 to 2 to 4 to 6 to 8 h was 1:2:10:9:8. The general pattern of ^{3}H distribution changed little after 2 h. The lower quarter of the mesocotyl (section A) contained a large amount of ^{3}H. The middle two quarters of the mesocotyl (sections B and C) contained relatively little ^{3}H. The top quarter of the mesocotyl (section D), including the coleoptilar node, contained about the same amount of ^{3}H as the lower quarter (section A). The lower half of the coleoptile and primary leaves (Section E) changed the most over the time course. Starting at 2 h this section contained approximately one half as much ^{3}H as the upper quarter of the mesocotyl (Section D) and progressively gained ^{3}H until at 8 h it was approximately equal to that in the upper quarter of the mesocotyl.

The amount of ^3H in the upper one half of the coleoptile and primary leaves (Section F) increased 11 fold from 2 to 4 h then remained at approximately the same level through 8 h.

When this data is presented on an apparent concentration basis (figure 6B), relatively higher concentrations of ^3H are seen in the coleoptile pieces reflecting their lower "fresh" weights.

The longitudinal distribution of ^3H and [^3H]IAA, free and free + ester, in the shoot at 4 h is presented in figure 7. A slightly different dissection was used in this experiment to separate the coleoptilar node from the mesocotyl. Considering the different dissection, the distribution of the average amounts of ^3H in the shoot in this experiment was very similar to that found prevously at the 6 h time point. The main difference is the relatively lower amount of ^3H in the lowest mesocotyl section found in this experiment. In general the ^3H accumulated in the upper third of the mesocotyl to the lower half of the coleoptile (sections C, D and E). The coleoptilar node, even though relatively small, contains more than half as much ^3H as the upper one third of the mesocotyl and almost an equal amount of ^3H to that found in the lower half of the coleoptile and primary leaves.

This experiment was designed to determine variation from shoot to shoot and within each shoot of the amount of ^3H translocated. There were large variations in the amount of ^3H translocated to the entire shoot (450 to 5580 dpm) and in the distribution in the shoot. Thus, an analysis of variance for a randomized complete block design experiment with 10 replications was carried out on the data to determine the significance of the differences in amount of ^3H in the shoot sections. The $LSD_{0.1}$ value indicates that section C contained significantly more

^3H than the other sections, and that sections D and E contain significantly more ^3H than sections A, B, and F.

In general the distribution of free + ester [^3H]IAA reflects that found for ^3H (Figure 7). In the whole shoot [^3H]IAA, free + ester, accounts for approximately 35% of the ^3H. Within the shoot pieces the notable exceptions are the coleoptilar node (section D) where [^3H]IAA, free + ester, accounts for only 25% of the ^3H present, and in the upper half of the coleoptile and primary leaves (section F) where [^3H]IAA, free + ester, accounts for almost 50% of the ^3H present. As was found in the stele, cortex, coleoptile dissection, free [^3H]IAA in the whole shoot accounts for approximately 9% of the free + ester [^3H]IAA. Within the shoot sections variations from this average were found in the lower third of the mesocotyl (section A, 21%) and the upper third of the mesocotyl and the upper half of the coleoptile and primary leaves (sections C and F, respectively, both 5%).

When the results for the [^3H]IAA, free and free + ester, and the ^3H are presented on an apparent concentration basis (Figure 8) the only relative differences from the dpm per section results appear in the coleoptilar node section and the coleoptile and primary leaves sections (sections D, E and F). The apparent free + ester [^3H]IAA concentrations in sections C, D and F were approximately the same and approximately 3 fold higher than that found in sections A and B and 20% less than that found in section E. The relative apparent concentrations of ^3H in the shoot pieces were similar to those found previously at the 6 h time point where the ^3H was most concentrated in the upper half of the shoot. The coleoptilar node (section D) is unique in having the highest apparent concentration of ^3H.

Results V. The results of the distribution of ^3H in the corn kernel after incubation for 2 to 8 h with [^3H]IAA or [^3H]IAInos are presented in Table V. There was essentially no difference in the distribution of ^3H in the seed whether the seed was incubated for 4 or 8 h, or whether [^3H]IAA or [^3H]IAInos was applied to the seed. The average ratio of the ^3H in the seed pieces at 4 and 8 h was 85:13:2, top to middle to bottom pieces. In the one experiment, where the seeds were incubated for only 2 h with [^3H]IAA, relatively more radioactivity was located in the top piece. The ratios of ^3H in the top to middle to bottom pieces were 97:7:1.

In three of the ^3H distribution in the seed experiments the % recoveries of ^3H applied to the seeds were significantly greater than 100% (III 45, III 59 and III 101). However, the distribution of ^3H in these experiments was the same as that found in the other experiments where the % recoveries were either less than 100% or, within experimental error, essentially equal to 100%. The high recoveries may have been due to chemiluminescence.

Discussion

It is well established that the tip of a plant serves as a source of IAA for the subapical portions. For example, the tip contains more IAA than lower coleoptile sections, as determined by bioassay (20,24) and cessation of growth of the coleoptile and mesocotyl due to decapitation can be reversed by exogenous IAA (6,23). It is not known, however, where the IAA in the tip comes from. In an etiolated seedling, all of the compounds of the shoot must have originated in the seed since the seedling is not photosynthetic. But, the chemical form of the substance(s) moving from the seed to the shoot have not been extensively studied. Thus we do not know whether tryptophan is translocated from the seed to the shoot tip and there converted to IAA; whether simple non-aromatic precursors are translocated to the shoot tip for *de novo* IAA synthesis, or whether IAA conjugates move from the seed to the shoot tip and are hydrolyzed to yield free IAA.

Our initial hypothesis in these studies was based upon the classic concept of auxin relationship in young seedlings in which the seed auxin precursor moves to the tip of the shoot where is is converted to an active auxin, and then transported to the growing region where hormonal control is expressed (27). Went and Thimann (27) suggested that this seed auxin precursor is an ester. That the seed auxin precursor in *Z. mays* is an IAA ester is supported by Sheldrake's observation of an alkalai-labile IAA compound in the xylem sap (18).

Previous work in this laboratory has identified the structure and concentrations of all the IAA containing compounds of the *Z. mays* kernel down to a level of 10 $\mu g \cdot kg^{-1}$ (see 5 for references). IAInos comprises about 15% of the total IAA and 30% of the low molecular weight IAA esters of the endosperm (22) and has been calculated to be translocated from the endosperm to the shoot in sufficient amounts to serve as a seed auxin precursor (14). It has also been demonstrated that insufficient amounts of free IAA were translocated from the endosperm in *Z. mays* to supply the auxin needs of the growing shoot (12,14).

The data of this paper, using the assumptions of Nowacki and Bandurski (14) (that there is complete equilibration of applied [^{3}H]IAInos with the endogenous IAInos and that the half-life of the IAInos pool in the *Zea* kernel is 12 h, as determined by Epstein et al.

(8)), indicates that IAInos in the endosperm was supplying approximately 1 to 2 pmol of IAA, free + ester, per h to each shoot. That IAInos supplies 1 to 2 pmol IAA, free + ester, per h is in fairly close agreement with Nowacki and Bandurski's calculation of 6.3 pmol$\cdot$h^{-1} and would supply 10 to 40% of the estimated 5 to 9 pmol$\cdot$h^{-1} needed per shoot (14). Data from other experiments (to be published elsewhere) indicate that IAInos accounts for approximately 17% of the ester IAA of the shoot. Thus, it is possible that other IAA esters are also serving as seed auxin precursors and account for our, less than sufficient transport rate of IAA, free + ester, from IAInos in the kernel. Most of the [^{3}H]IAA, free + ester, in the shoot was located in the coleoptile plus primary leaves and the upper one third of the mesocotyl (regions of higher growth rate (15)) when [^{3}H]IAInos was applied to the endosperm. This contrasts sharply with the only trace amounts of [^{3}H]IAA, free + ester, found in the coleoptile when [^{3}H]IAA was applied to the endosperm, thus supporting the hypothesis that IAA in the shoot tip arises from ester IAA in the endosperm and not from free IAA in the endosperm. However, the two incubation times chosen for these experiments in which [^{3}H]IAA was reisolated from the shoots do not allow us to determine if the [^{3}H]IAInos translocated to the coleoptile was hydrolyzed to free [^{3}H]IAA and then translocated basipetally to the growing region. Because of the high concentrations of ester [^{3}H]IAA in the vascular stele it is likely that it was being translocated in this tissue. Large amounts of ester [^{3}H]IAA were translocated to the coleoptile plus primary leaves section of the shoot but the percent free [^{3}H]IAA in this section was only intermediate between that found for the mesocotyl stele in the mesocotyl cortex. This intermediate level may reflect the mix of the mostly non-vascular tissue of the coleoptile and the large amount of vascular tissue in the primary leaves of this shoot section. In addition, our data on the uniformity of the longitudinal distribution of free [^{3}H]IAA in the shoot 4 h after [^{3}H]IAInos was applied to the endosperm is not in agreement with the hypothesis that the ester IAA should be hydrolyzed to free IAA in the shoot tip. However, if experiments with longer incubation times are performed, a wave of free [^{3}H]IAA moving down from the shoot tip might be observed. Also, if the coleoptile were separated from the primary leaves it might allow the observation of the hydrolysis of translocated ester IAA in the coleoptile tip that otherwise might be obscured by potentially high concentrations of translocated radiolabeled IAA in the vascular tissue of the primary leaves.

Radiolabeled IAA, free and ester, assumed strikingly different proportions and distributions in the 4 day old etiolated _Z. mays_ shoot depending upon whether they were derived from radiolabeled IAA or radiolabeled IAInos applied to the endosperm. However, total radioactivity in the shoot derived from either radiolabeled IAA or radiolabeled IAInos applied to the endosperm was distributed in essentially the same manner. Free plus ester radiolabeled IAA in the shoot derived from radiolabeled IAInos applied to the endosperm was distributed in approximately the same pattern as the total radioactivity in the shoot. There was little conversion from free to ester (15 to 20%) and even less from ester to free (5 to 10%) of the applied radiolabeled compounds. The radial distribution of free [^{3}H]IAA, at both 2 and 4 h, between the mesocotyl vascular stele and the mesocotyl cortex-epidermis, whether it came from [^{3}H]IAA or [^{3}H]IAInos applied to the endosperm, approximately reflects that found for endogenous free IAA (15). That most of the free IAA is localized in the vascular stele, whereas the surrounding cortex-epidermis contains very low levels, has also been demonstrated in Z. mays roots (3,10). The distributions of ester [^{3}H]IAA did not reflect that found for endogenous ester IAA. In all of the translocation experiments, except one, the relative concentrations of ester [^{3}H]IAA were much higher in the stele than the cortex as compared to the approximately 1:1 ratio of the concentrations of endogenous ester IAA (15). However, it is possible that insufficient time had elapsed to allow the translocated ester [^{3}H]IAA to equilibrate with the endogenous ester IAA in the shoot. In the [^{3}H]IAInos experiments the ratio of the amount of ester [^{3}H]IAA in the stele to that in the cortex-epidermis increased from approximately 1.5:1 to 1:1.5, and with longer incubation times might continue to increase to the 1:15 ratio found for the endogenous ester IAA.

The longitudinal distribution of free [^{3}H]IAA and ester [^{3}H]IAA in the shoot, 4 h after the application of [^{3}H]IAInos to the endosperm, reflected an opposite trend to that found for endogenous free IAA and ester IAA (15). Free [^{3}H]IAA was fairly evenly distributed up the shoot contrasting with the 2 to 3 fold higher concentration of endogenous free IAA in the apical one fourth of the mesocotyl. Ester [^{3}H]IAA was almost 3 fold more concentrated in the apical one third of the mesocotyl contrasting with a uniform concentration along the mesocotyl of endogenous ester IAA.

Further experiments are also needed to investigate the apparent cessation of the translocation of applied radiolabeled compounds from the endosperm to the shoot after approximately 4 h. This was observed in the translocation of total radioactivity when [^{3}H]IAInos was applied to the endosperm. Since only approximately 0.2% of the applied radioactivity had translocated to the shoot the remaining radiolabeled compounds may have been metabolized to compounds which are not translocated or in some other way immobilized in the kernel.

It is obvious from the above discussion that a very rapid method of measuring, and determining the specific activity of IAA and its conjugates is needed to continue and complete this investigation. Only in that way can sufficient time points be established to accurately reflect the identity, source, routes of translocation and turnover rates of the possible IAA precursors. Despite the paucity of data, we wish to propose two working hypotheses that are consistent with our present knowledge. In the first hypothesis, at least some of the IAA of the shoot originates in the seed as IAInos. This ester moves in the vascular system - probably the xylem - to the apex of the mesocotyl and the tip of the coleoptile. At these points, a portion of the easter is hydrolyzed to free IAA, and the free IAA begins its downward movement still in the vascular bundle but now in or near the phloem (26). Most of the free IAA now leaks from the vascular bundles in the coleoptile and the stele in the mesocotyl into the surrounding cortex. In the cortex the IAA is either quickly used up in the growth promoting reaction or it is reconjugated. The special function of the coleoptile tip and the mesocotyl apex may lie in serving as crossover points from xylem to phloem and as points of conjugate hydrolysis.

In our second working hypothesis, again, some of the IAA of the shoot originates in the seed as IAInos and moves to and through the shoot in the vascular system - probably the xylem. However, as the IAInos moves up the shoot it leaks out, from the mesocotyl stele into the mesocotyl cortex and from the vascular bundles into the coleoptile cortex, in proportion to other metabolites as needed for growth in that region. The IAInos in the cortical tissue could then serve as an IAA precursor being converted to IAA as needed.

The data in this paper indicates that free IAA in the seed is the source of some of the IAA of the mesocotyl but that IAA from the endosperm supplies essentially none of the IAA in the coleoptile or primary leaves. Further, the coleoptilar node appears to be a gate closed to the acropetal movement of free IAA. These conclusions are incorporated into the above two hypotheses.

The above are simply working hypotheses and they are complex. However, the hypotheses are subject to experimental tests, and we believe that ultimately a better understanding of the regulation of hormone concentrations will result.

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Table I. Raw data on total radioactivity in the shoot from [^{14}C]IAA applied to the endosperm.

Experiment No.	Tissue Piece	Incubation Time (h)	DPM ^{14}C Applied per Seed	Number of Shoots per sample	Dry Wt.[a] (mg)	CPM-bkg.[a]	DPM[a] ^{14}C
I 38		0.5	3950	60			
	Stele				123.6	28	49
	Cortex				495.5	22	39
	Coleoptile				530.4	15	26
I 47		0.5	3920	60			
	Stele				147.2	21	39
	Cortex				586.3	3	6
	Coleoptile				551.6	12	22
I 35		1	3960	60			
	Stele				141.7	84	152
	Cortex				549.1	39	71
	Coleoptile				523.2	72	130
I 44		1	4020	60			
	Stele				137.1	93	172
	Cortex				531.5	25	46
	Coleoptile				551.3	75	138
I 27		2	3800	60			
	Mesocotyl				641.4	331	637
	Coleoptile				473.6	369	710
I 41		2	4100	60			
	Stele				127.5	199	351
	Cortex				501.1	142	250
	Coleoptile				-	338	596
I 16		4	3910	56			
	Stele				74.4	75	190
	Cortex				367.4	174	441
	Coleoptile				417.7	291	737
I 20		4	3930	60			
	Stele				121.2	113	286
	Cortex				530.0	287	727
	Coleoptile				490.5	507	1284

[a]Data is expressed as units per sample.

Table II. Raw data on free $[^3H]$IAA and free + ester $[^3H]$IAA in the shoot from $[^3H]$IAA applied to the endosperm.

Experiment No.	Tissue Piece and IAA Fraction	Incubation Time (h)	DPM $[^3H]$IAA Applied per Seed	Number of Shoots per Sample	Wet Wt.[a] (g)	Dry Wt.[b] (mg)	CPM-bkg.[c]	Sample Volume (μl)	DPM $[^3H]$IAA[d] per ml	Sample IAA Concentration (mM)	Amount[e] Carrier IAA Added (μmol)
II 84		2	3.3×10^6	39							
	Stele Free				1.2	60	71	100	1710	3.60×10^{-2}	2.6
	Stele Free + Ester						104	100	2480	4.55×10^{-2}	
	Cortex Free				7.7	120	8	100	180	2.41×10^{-2}	2.6
	Cortex Free + Ester						11	100	240	2.82×10^{-2}	
	Coleoptile Free				3.5	180	1	100	13	1.10×10^{-2}	2.6
	Coleoptile Free + Ester						2	100	40	2.81×10^{-2}	
III 101		4	2.01×10^6	45							
	Stele Free				1.2	69.7	27	100	777	5.26×10^{-2}	2.7
	Stele Free + Ester						22	100	630	4.29×10^{-2}	
	Cortex Free				8.5	156.8	11	100	323	6.32×10^{-2}	2.7
	Cortex Free + Exter						21	100	613	5.78×10^{-2}	
	Coleoptile Free				4.5	219.5	3	100	90	7.51×10^{-2}	2.7
	Coleoptile Free + Ester						3	100	77	7.24×10^{-2}	

[a]Wet weight of the sample before grinding and separating in one-half for free and free + ester $[^3H]$IAA determination.

[b]Dry weight of the acetone insoluble material of the entire sample.

[c]The cpm above background per volume indicated.

[d]Calculated from the cpm-bkg corrected for counting efficiency and the sample volume counted.

[e]This was added to the entire sample before grinding and splitting the sample for free and free + ester $[^3H]$IAA determination.

Table III. Raw data on free [^{3}H]IAA and free + ester [^{3}H]IAA in the shoot from [^{3}H]IAInos applied to the endosperm.

Experiment No.	Tissue Piece and IAA Fraction	Incubation Time (h)	DPM [^{3}H]IAAnos Applied per Seed	Number of Shoots per sample	Wet Wt.[a] (g)	Dry Wt.[b] (mg)	CPM-bkg.[c]	Sample Volume (μl)	DPM (^{3}H)IAA[d] per ml	Sample IAA Concentration (mM)	Amount[e] Carrier IAA added (μmol)
VI 135		2	2.10×10^6	60							
	Stele Free				1.3	65.5	38	500	260	1.37×10^{-1}	1.2
	Stele Free + Ester						214	500	1510	1.56×10^{-1}	
	Cortex Free				7.7	141.2	4	500	30	1.59×10^{-1}	1.2
	Cortex Free + Ester						90	500	640	1.29×10^{-1}	
	Coleoptile Free				4.6	246.7	11	500	80	1.19×10^{-1}	1.2
	Coleoptile Free + Ester						225	500	1590	1.32×10^{-1}	
VI 143		2	2.08×10^6	60							
	Stele Free				1.2	60.2	42	500	280	1.17×10^{-1}	1.2
	Stele Free + Ester						203	500	2080	1.32×10^{-1}	
	Cortex Free				7.3	145.1	4	500	30	1.21×10^{-1}	1.2
	Cortex Free + Ester						217	500	1460	1.25×10^{-1}	
	Coleoptile Free				4.1	231.5	20	500	140	1.41×10^{-1}	1.2
	Coleoptile Free + Ester						418	500	2810	1.18×10^{-1}	
VI 107		4	2.23×10^6	60							
	Stele Free				1.5	137	23	400	210	4.16×10^{-2}	1.2
	Stele Free + Ester						49	400	430	1.32×10^{-2}	
	Cortex Free				8.1	155	17	400	140	4.46×10^{-2}	1.2
	Cortex Free + Ester						505	400	4500	8.38×10^{-2}	
	Coleoptile Free				5.0	238	40	400	350	4.92×10^{-2}	1.2
	Coleoptile Free + Ester						414	500	3080	4.26×10^{-2}	
VI 123		4	2.38×10^6	60							
	Stele Free				1.6	74.7	16	600	90	2.15×10^{-2}	1.2
	Stele Free + Ester						775	500	5420	1.59×10^{-1}	
	Cortex Free				8.3	143.3	15	500	100	1.23×10^{-1}	1.2
	Cortex Free + Exter						644	500	4370	1.11×10^{-1}	
	Coleoptile Free				5.3	259.4	50	500	330	9.39×10^{-2}	1.2
	Coleoptile Free + Exter						1308	500	8560	1.62×10^{-1}	

[a]Wet weight of the sample before grinding and separating in one-half for free and free + ester [^{3}H]IAA determination.

[b]Dry weight of the acetone insoluble material of the entire sample.

[c]The cpm above background per volume indicated.

[d]Calculated from the cpm-bkg corrected for counting efficiency and the sample volume counted.

[e]This was added to the entire sample before grinding and splitting the sample for free and free + ester [^{3}H]IAA determination.

Table IV. Raw data on the longitudinal distribution of free [^3H]IAA and free + ester [^3H]IAA in the shoot from [^3H]IAInos applied to the endosperm. Tissue pieces A to F are from the base to the apex of the shoot as indicated in figure 2B.

Experiment No.	Tissue Piece and IAA Fraction	Wet Wt.[a] (g)	Dry Wt.[b] (mg)	CPM-bkg.[c]	Sample Volume (μl)	DPM (^3H)IAA[d] per ml	Sample IAA Concentration (mM)	Amount[e] Carrier IAA added (μmol)
IV 73								
	A Free	2.67	66.5	16	1000	57	4.92×10^{-2}	1.1
	A Free + Ester			184	700	897	1.28×10^{-1}	
	B Free	2.57	54.4	19	800	89	1.11×10^{-1}	1.1
	B Free + Ester			92	600	547	8.25×10^{-2}	
	C Free	2.48	60.2	19	600	107	1.18×10^{-1}	1.1
	C Free + Ester			430	700	2190	1.23×10^{-1}	
	D Free	1.07	61.0	11	600	62	1.27×10^{-1}	1.1
	D Free + Ester			155	700	796	1.06×10^{-1}	
	E Free	1.35	80.7	17	700	87	9.19×10^{-2}	1.1
	E Free + Ester			37	600	222	1.94×10^{-2}	
	F Free	0.81	43.0	6	700	29	1.05×10^{-1}	1.1
	F Free + Ester			30	600	193	3.40×10^{-2}	

[a]Wet weight of the sample before grinding and separating in one-half for free and free + ester [^3H]IAA determination.

[b]Dry weight of the acetone insoluble material of the entire sample.

[c]The cpm above background per volume indicated.

[d]Calculated from the cpm-bkg corrected for counting efficiency and the sample volume counted.

[e]This was added to the entire sample before grinding and splitting the sample for free and free + ester [^3H]IAA determination.

Table V. Raw data on the distribution of radioactivity in the kernel after incubation with applied [^{3}H]IAA and [^{3}H]IAInos. The top seed piece is that piece which contains the cut endosperm surface to which the labeled compound was applied. See figure 1 for description of the kernel dissection.

Experiment No.	Seed Piece	Compound Applied	DPM ^{3}H Applied per Seed	Incubation Time (h)	Wet Wt. (g)	DPM Recovered
II 85		[^{3}H]IAA	4.02×10^7	2		
	Top				0.8	2.57×10^7
	Middle				0.7	1.89×10^6
	Bottom				0.5	3.55×10^5
III 45		[^{3}H]IAA	1.90×10^7	4		
	Top				1.9	2.59×10^7
	Middle				2.0	4.34×10^6
	Bottom				1.6	1.21×10^5
III 59		[^{3}H]IAA	2.07×10^7	4		
	Top				1.9	3.17×10^7
	Middle				2.4	5.54×10^6
	Bottom				1.8	6.33×10^5
III 101		[^{3}H]IAA	2.16×10^7	4		
	Top				1.9	3.74×10^7
	Middle				2.0	4.61×10^6
	Bottom				1.8	9.69×10^5
III 139		[^{3}H]IAInos	1.35×10^7	4		
	Top				0.9	1.20×10^7
	Middle				1.1	1.56×10^6
	Bottom				0.8	1.69×10^5
III 139		[^{3}H]IAInos	1.35×10^7	8		
	Top				0.8	1.21×10^7
	Middle				1.0	2.09×10^6
	Bottom				0.8	1.60 x 105

Figure Legends

Fig. 1. Diagram of a 4-d-old dark grown _Zea mays_ seedling. The location of the endosperm surface exposed for isotope application, and the dissection of the kernel for experimental section V are shown.

Fig. 2. Location of the tissue sections used in the longitudinal distribution experiments. The dissection used in determining the distribution of total radioactivity as a function of time (Fig. 6) is indicated in figure 2A. The dissection used in determining the distribution of [^{3}H]IAA, free and ester, and total radioactivity at 4 h (Fig. 7) is indicated in figure 2B.

Fig. 3. The distribution of radioactivity in the shoot as a function of time after application of [^{14}C]IAA to the endosperm. The values at each time point, except 2 h which was not repeated, are the average of two determinations. The data are normalized to the amount of ^{14}C applied per endosperm. St = mesocotyl stele, Cr = mesocotyl cortex + epidermis, and Cl = apical 0.5 cm of the mesocotyl and the coleoptile containing the primary leaves.

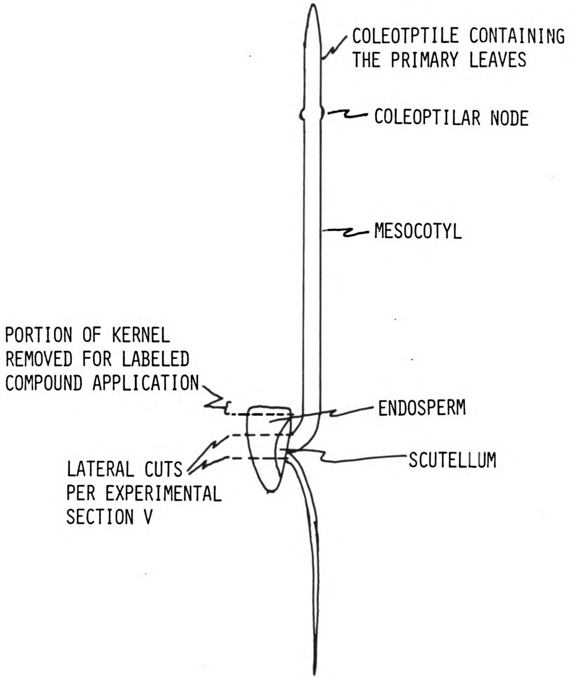
Fig. 4. The distribution of [^{3}H]IAA, free and ester, in the shoot 2 h and 4 h after the application of [^{3}H]IAA and [^{3}H]IAInos to the endosperm. The values, when [^{3}H]IAInos was applied, are the average of two determinations. The data are normalized to the amount of [^{3}H]IAA or [^{3}H]IAInos applied per endosperm. The amount of ester [^{3}H]IAA was calculated by subtracting the amount of free [^{3}H]IAA determined from the amount of free + ester [^{3}H]IAA determined. St = mesocotyl stele, Cr = mesocotyl cortex + epidermis, and Cl = apical 0.5 cm of the mesocotyl and the coleoptile containing the primary leaves.

Fig. 5. The concentration of [^{3}H]IAA, free and ester, in the shoot 2 h and 4 h after the application of [^{3}H]IAA and [^{3}H]IAInos to the endosperm. The values, when [^{3}H]IAInos was applied, are the average of two determinations. The data are normalized to the amount of [^{3}H]IAA or [^{3}H]IAInos applied per endosperm. The amount of ester [^{3}H]IAA was calculated by subtracting the amount of free [^{3}H]IAA determined from the amount of free + ester [^{3}H]IAA determined. St = mesocotyl stele, Cr = mesocotyl cortex + epidermis, and Cl = apical 0.5 cm of the mesocotyl and the coleoptile containing the primary leaves.

Fig. 6. The longitudinal distribution of radioactivity (A) and concentrations of radioactivity (B) in the shoot as a function of time after application of [^{3}H]IAInos to the endosperm. Shoot dissection (tissue pieces A-F) was as indicated in figure 2A.

Fig. 7. The longitudinal distribution of [^{3}H]IAA, free and ester, and total radioactivity in the shoot 4 h after application of [^{3}H]IAInos to the endosperm. Shoot dissection (tissue pieces A-F) was as indicated in figure 2B. L.S.D. value at the 0.1 level for total radioactivity = 192.

Fig. 8. The longitudinal distribution of the concentration of [^{3}H]IAA, free and ester, and total radioactivity in the shoot 4 h after application of [^{3}H]IAInos to the endosperm. Shoot dissection (tissue pieces A-F) was as indicated in figure 2B.


COLEOTPTILE CONTAINING
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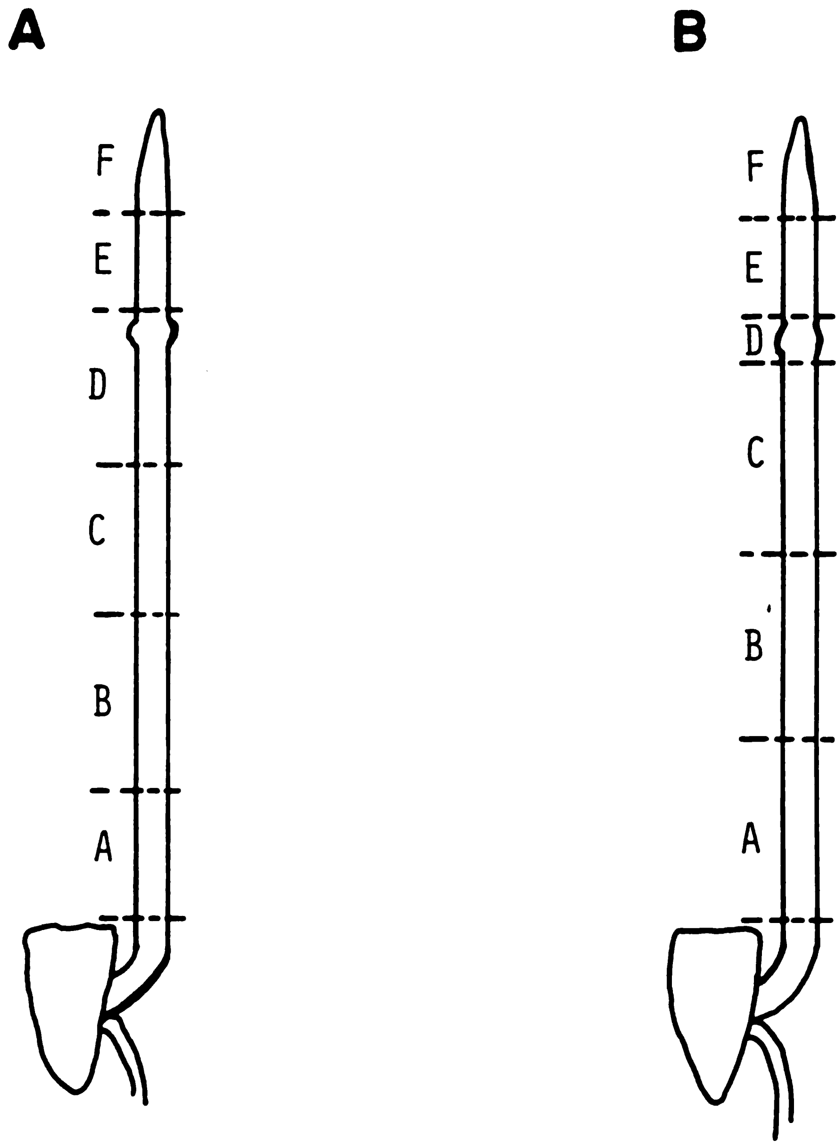
ENDOSPERM
SCUTELLUM
LATERAL CUTS
PER EXPERIMENTAL
SECTION V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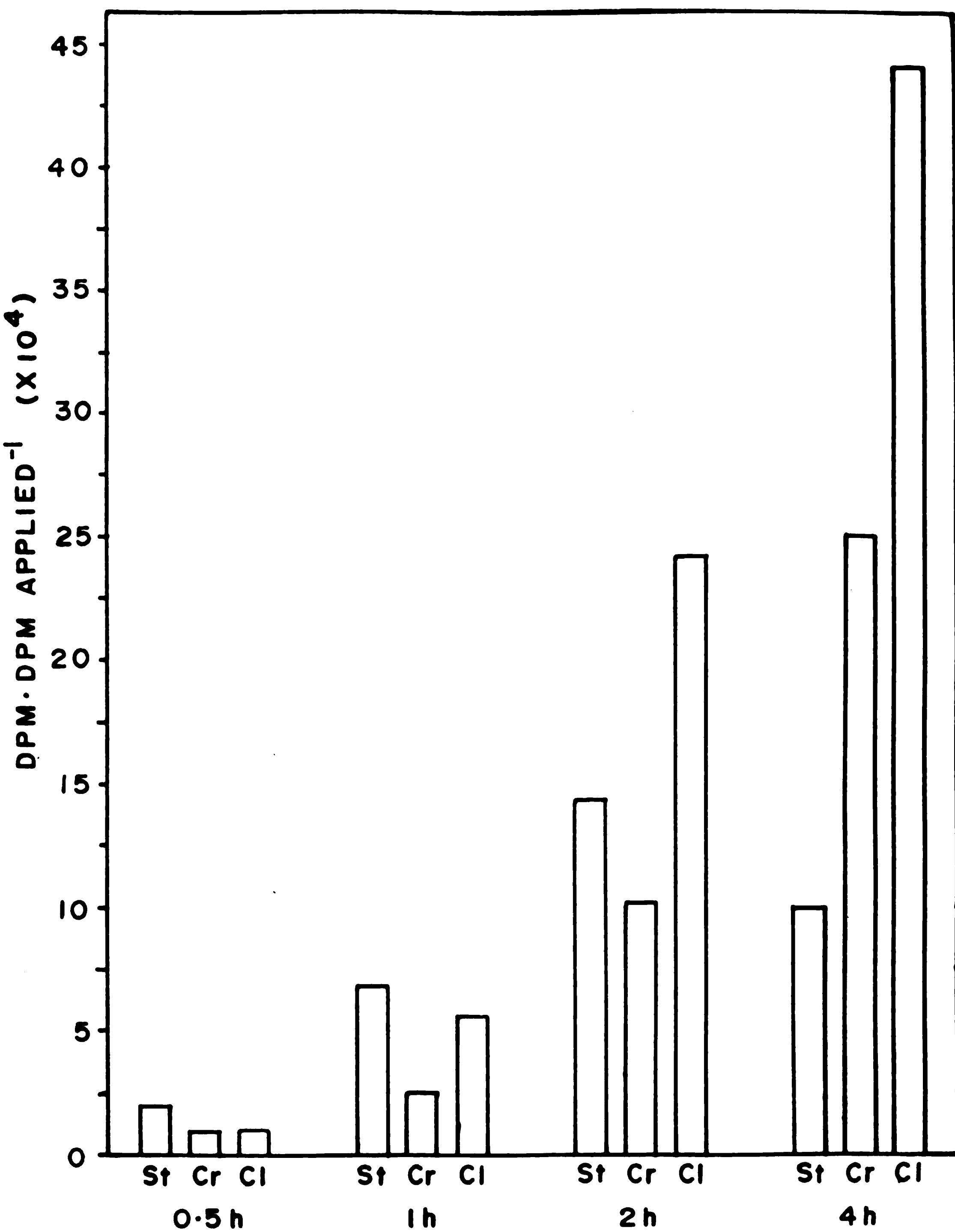
DPM·DPM APPLIED^{-1} (X10^4)
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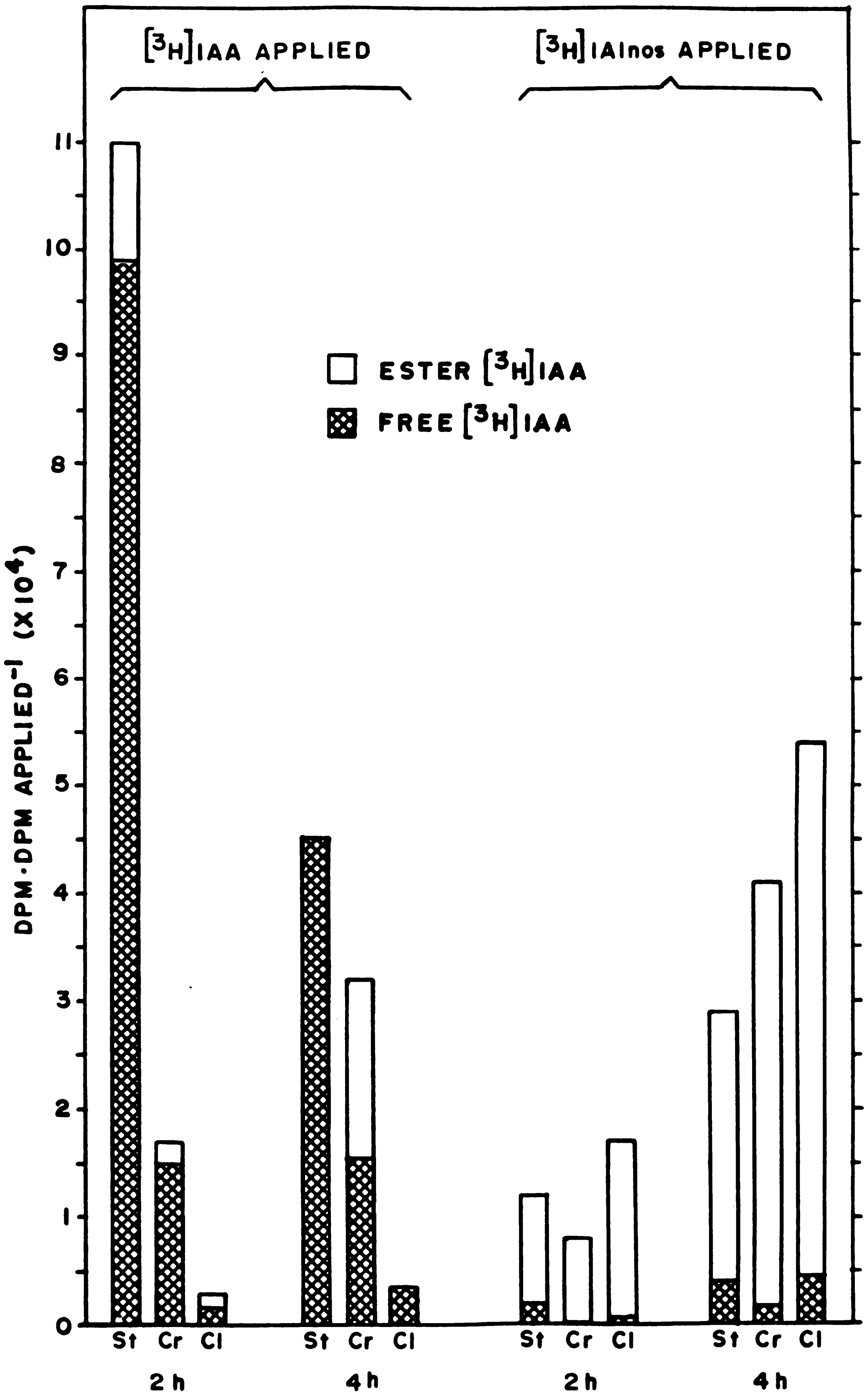
[3H]IAA APPLIED
[3H]IAInos APPLIED
ESTER [3H]IAA
FREE [3H]IAA
DPM·DPM APPLIED-1 (X10-4)
0
1
2
3
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11
St Cr Cl St Cr Cl St Cr Cl St Cr Cl
2 h
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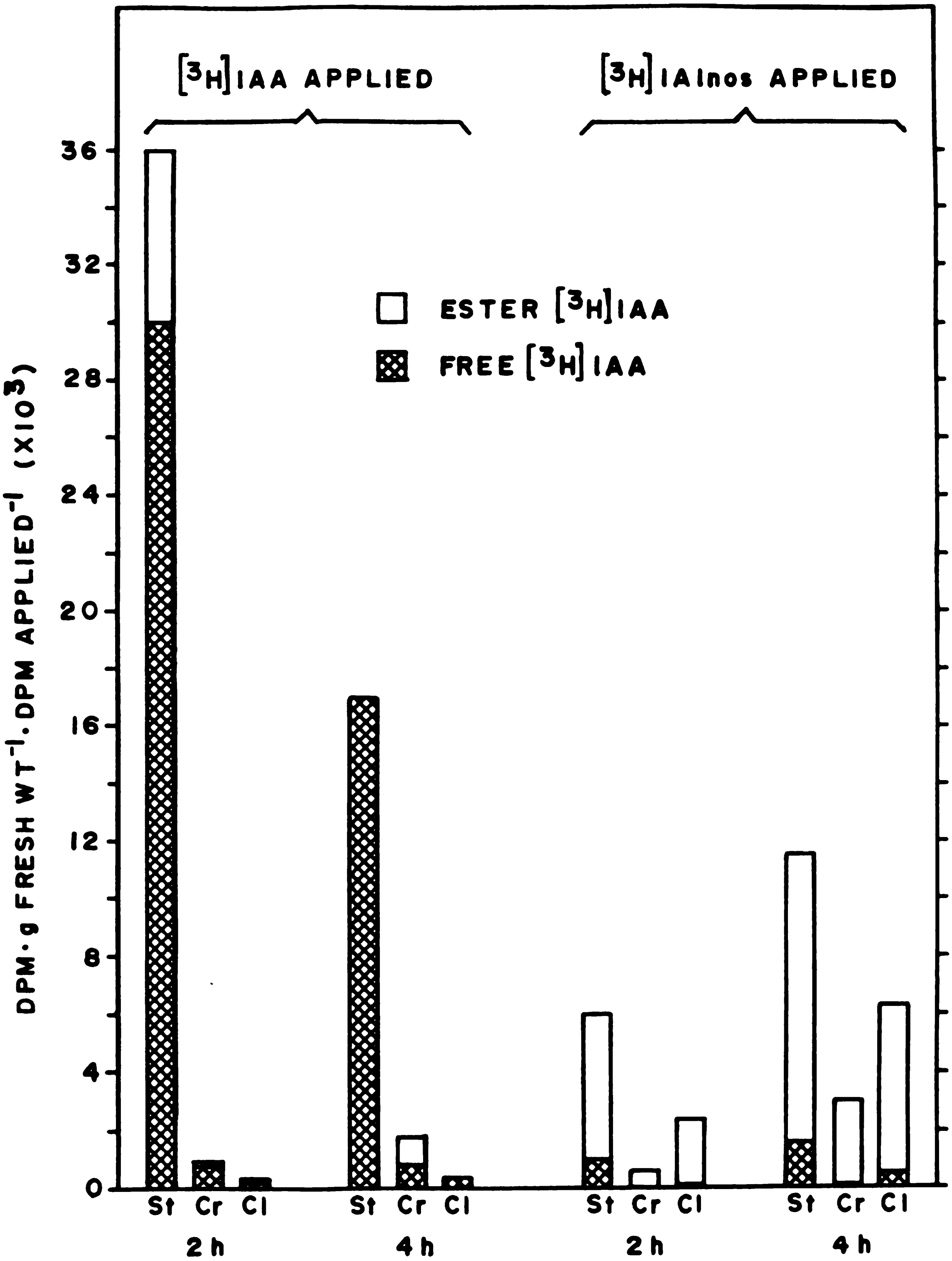
4 h



[3H]IAA APPLIED
[3H]IAInos APPLIED
ESTER [3H]IAA
FREE [3H]IAA
DPM·g FRESH WT-1·DPM APPLIED-1 (X10^3)
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16
20
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28
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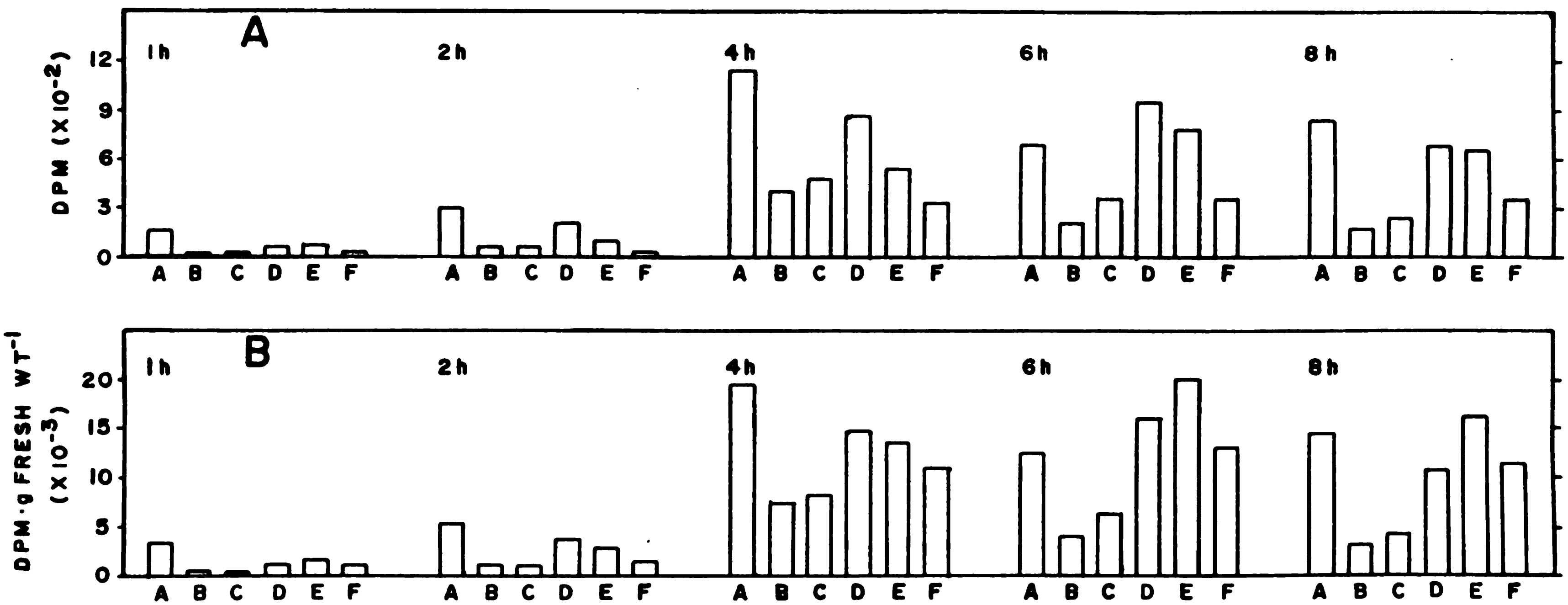
36
St Cr Cl
2 h
4 h


A
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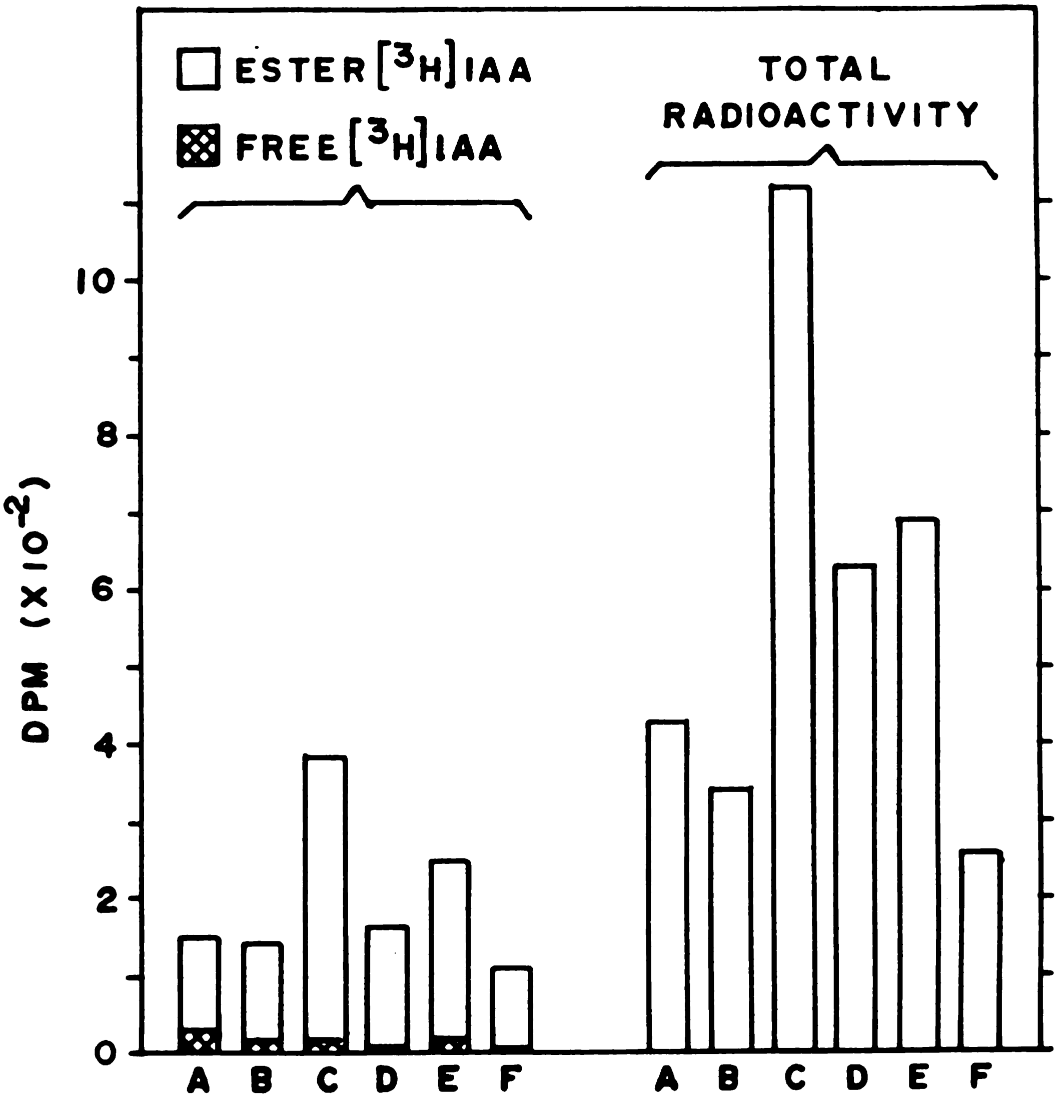
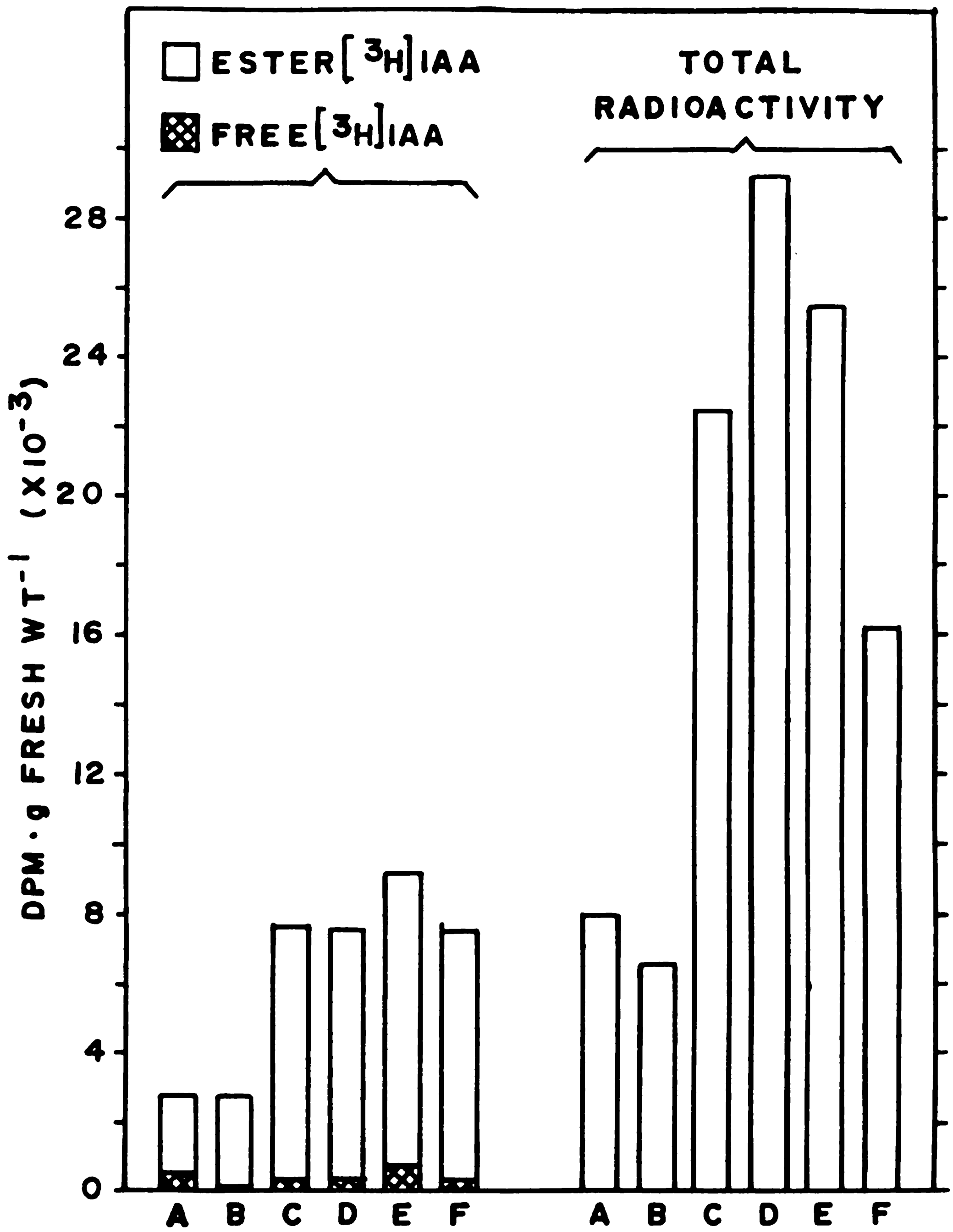
8h
A B C D E F



ESTER [3H] IAA
FREE [3H] IAA
TOTAL RADIOACTIVITY
DPM (X10-2)
0
2
4
6
8
10
A B C D E F
A B C D E F



ESTER [^{3}H] IAA
FREE [^{3}H] IAA
TOTAL RADIOACTIVITY
DPM · g FRESH WT^{-1} (X10^{-3})
0
4
8
12
16
20
24
28
A B C D E F
A B C D E F

CONCLUSIONS

At the outset of this study the working hypothesis in use was based upon the classic concept of auxin relationships in young seedlings in which the seed auxin precursor moves to the tip of the shoot where it is converted to an active auxin, and then transported to the growing region where hormonal control is expressed. It had been proposed that IAA-*myo*-inositol was a seed auxin precursor in the shoots of young dark-grown *Z. mays* seedlings. This was based upon the observations that 1) high concentrations of IAA-*myo*-inositol are found in the kernel (comprising about 15% of the total IAA), 2) that radiolabeled IAA-*myo*-inositol can be translocated from the endosperm to the shoot and there be hydrolyzed releasing IAA, and 3) the calculation that IAA-*myo*-inositol in the kernel supplied a sufficient amount of ester IAA to the shoot to serve as a seed auxin precursor.

In further investigating the proposition that IAA-*myo*-inositol is a seed auxin precursor in the shoots of young dark-grown *Z. mays* this study has established that IAA-*myo*-inositol is an endogenous component of the shoot tissue. It is present in the five-day-old shoot at a concentration of approximately 74 nmole per kg fresh weight and accounts for about 15% of the free + ester IAA or 19% of the ester conjugated IAA found there. This ratio of IAA-*myo*-inositol to ester conjugated IAA is similar to that found in the kernel after four days of germination (25%) (Epstein et al., 1980). This study also provides data on the translocation of radiolabeled IAA-_myo_-inositol and radiolabeled IAA from the endosperm to the shoot and the subsequent distribution of free and ester radiolabeled IAA in the shoot. The estimate in this study that IAA-_myo_-inositol in the endosperm supplies 1 to 2 pmole free + ester IAA to each shoot per hour is in fairly close agreement with Nowacki and Bandurski's (1980) calculation of 6.3 pmole per hour. The calculated transport rate of this study would be sufficient to supply 10 to 40% of the estimated 5 to 9 pmole needed per hour per shoot. As indicated above, IAA-_myo_-inositol accounts for only 19% of the endogenous ester conjugated IAA in the shoot. Thus, it is possible that other IAA esters are also serving as seed auxin precursors and account for the less than sufficient transport rate of free and ester IAA from IAA-_myo_-inositol in the kernel.

Radiolabeled IAA and radiolabeled ester IAA assumed strikingly different proportions and distributions in the shoot depending upon whether they were derived from radiolabeled IAA or radiolabeled IAA-_myo_-inositol applied to the endosperm. Both compounds appeared to be transported, at least initially, in the vascular stele of the mesocotyl. Radiolabeled free and ester IAA in the shoot, derived from radiolabeled IAA applied to the endosperm was almost entirely restricted to the mesocotyl. The coleoptilar node appeared to be a gate closed to the acropetal movement of IAA. This was in sharp contrast to when radiolabeled IAA-_myo_-inositol was applied to the endosperm, more than one-third of the free and ester IAA in the shoot was found at or above the coleoptilar node in the coleoptile and primary leaves. There was little conversion from free to ester (15 to 20%) and even less from ester to free (5 to 10%) of the applied radiolabeled compounds.

In order to accomplish the above work a synthesis for high specific activity tritiated IAA and IAA-myo-inositol was developed. This was an enzymatic synthesis on a nanomolar scale with little or no dilution of the initial specific activity of the L-[5-^{3}H]tryptophan substrate.

Two working hypotheses are presented which incorporate the data of this study and hopefully point the direction for further research on the source of IAA in etiolated seedlings. There are a number of questions which need to be investigated in the future. First, a much more rapid and possibly more sensitive method of measuring and determining the specific activity of IAA and its conjugates needs to be developed. This would enable the analysis of an increased number of time points and dissections of the shoot, which are required to accurately determine the identities, sources, routes of translocation, locations and turnover rates of the possible IAA precursors. Second, the identities and concentrations of the remaining 80% of the endogenous IAA esters in the shoot need to be determined. Also, it would be interesting to investigate the apparent cessation of the translocation of applied radiolabeled compounds approximately four hours after application. Finally, the question of which of the IAA-myo-inositol isomers are involved in the regulation of IAA levels and the sites of their hydrolysis needs to be determined. This work is currently being pursued by Hall and Bandurski (1981, 1983) who have demonstrated IAA-myo-inositol hydrolysing activity in a partially purified enzyme preparation from dark-grown *Z. mays* shoots.

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