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Myo-Inositol Esters of Indole-3-acetic Acid as Seed Auxin Precursors of *Zea mays* L.[1]

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ABSTRACT

Indole-3-acetyl-*myo*-inositol esters constitute 30% of the low molecular weight derivatives of indole-3-acetic acid (IAA) in seeds of *Zea mays*. [^{14}C]Indole-3-acetyl-*myo*-inositol was applied to a cut in the endosperm of the seed and found to be transported from endosperm to shoot at 400 times the rate of transport of free IAA. The rate of transport of indole-3-acetyl-*myo*-inositol from endosperm to shoot was 6.3 picomoles per shoot per hour and thus adequate to serve as the seed auxin precursor for the free IAA diffusing downward from the shoot tip. Indole-3-acetyl-*myo*-inositol is the first seed auxin precursor to be identified.

Application of either [^{14}C]IAA or ^{14}C-indole-3-acetyl-*myo*-inositol ester to the endosperm results in both free and esterified [^{14}C]IAA in the seedling shoot. Esterification of free IAA and hydrolysis of indole-3-acetyl-*myo*-inositol occurred in the shoot and not the endosperm yielding ratios of ester to free IAA which approximate the ratios of ester to free IAA normally found in corn shoot tissue. This proves, for the first time, that esterified IAA and free IAA are interconvertible in the growing shoot. Since free IAA may be limiting for plant growth, knowledge that the free hormone is in "equilibrium" with its conjugates suggests new methods for the control of plant growth.

The structure and concentrations of all of the indolylic compounds of the kernels of *Zea mays* are known (1, 13, 16, 33), and the compounds have been shown to be concentrated in the endosperm (26). The rate at which IAA is transported from endosperm to the seedling shoot has been determined (21), and it has been established that neither free IAA in the endosperm nor IAA derived from tryptophan of the endosperm moves to the seedling shoot at a rate sufficient to account for the IAA and IAA derivatives needed by the seedling (21). Esters of *myo*-inositol and IAA comprise about 15% of the total IAA of the endosperm and 30% of the low mol wt esters (33). To test the possibility that an IAA ester is the seed auxin precursor of *Zea*, we synthesized the *myo*-inositol esters of [2-^{14}C]IAA (24) and now wish to report that indole-3-acetyl-*myo*-inositol is transported from endosperm to shoot at 400 times the rate of transport of free IAA. This rate is adequate to sustain seedling needs. It was possible to convert amounts of radioactivity transported from endosperm to shoot into amounts of compound since the pool size and rate of metabolic turnover of the indolylic compounds of the endosperm was known (18).

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The rapid rate of transport (from endosperm to shoot) and the known occurrence of this ester in *Zea* endosperm establishes IAA-*myo*-inositol as a seed auxin precursor of *Zea* as studied by Cholodny (7), Skoog (31), and van Overbeek (35) in *Avena*. This study provides experimental proof for the suggestion by Went and Thimann (36) that the seed auxin precursor is an ester.

Most of the transported radioactivity remains as [^{14}C]indole-3-acetyl-*myo*-inositol during 8 h, but about 7% of the labeled ester is hydrolyzed to yield free IAA. This is the first demonstration of the hydrolysis of IAA-*myo*-inositol *in vivo* and, added to the previous demonstration (3, 21) of ester formation *in vivo*, establishes that ester and free IAA are interconvertible *in vivo*.

MATERIALS AND METHODS

All materials and methods not mentioned were as previously described (21). UV spectra were obtained with a Cary 15 spectrophotometer. Radioactivity was determined with a Packard Tri-Carb model 3003 liquid scintillation counter employing either Bray's (6) or ACS solution (Amersham) and the counting efficiencies were 90 and 82%, respectively. Total tissue radioactivity was determined by lyophilizing groups of 10 plants, and combusting them in a Packard 306 Tri-Carb sample oxidizer. The resultant $^{14}CO_2$ was trapped with a quarternary amine and counted as above. The over-all efficiency of the combustion, trapping, and counting procedure was 80%. GLC was with a Varian 2740 gas chromatograph with a flame ionization detector and using N_2 as a carrier gas.

Materials were from the following sources: [2-^{14}C]IAA (57.2 mCi/mmol): New England Nuclear; indole-3-butyric acid: Calbiochem; IAA, DEAE-cellulose Dowex 50 W-X2 200–400 mesh: Sigma; Sephadex LH-20: Pharmacia; silica gel 60 thin layer plates: Merck-Darmstadt-Brinkman; 20% sulfonated polystyrene divinylbenzene (low capacity Dowex 50): Dow Chemical Co., Midland, Mich. (15); sulfonated styrene-divinylbenzene, PA-28: Beckman; 5% SP-2401 on Supelcoport: Supelco; 2% OV-1 on Gas-chrom Z: Applied Science; *N,O*-bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane: Pierce; Stowell's Evergreen Hybrid Sweet Corn: Vaughan's Seed Co., Ovid, Mich. For synthesis of labeled (57.2 mCi/mmol), or unlabeled IAA-*myo*-inositol, the imidazolate of IAA was prepared with 1,1'-carbonyldimidazole and this reacted with sodium *myo*-inositolate as described (24). Quantitative colorimetric determination of IAA and IAA-inositol was by means of the Ehmann reagent (14, 25) using 200 μl of the reagent to, up to, 20 μl of sample, heating at 45 C for 45 min, adding 600 μl of water and determining A_{615} in a Cary 15 spectrophotometer by scanning the interval of 400 to 800 nm to verify that the spectrum is identical to that of pure IAA.

PLANT MATERIAL AND APPLICATION OF RADIOACTIVE COMPOUNDS

Four-day-old seedlings, about 1.5–3.0 cm long, were grown aseptically at 25 C and in darkness with occasional use of photo-

tropically inactive green light, as previously described (21). About 30% (18) of the endosperm, distal to the embryo, was severed with a sterile scalpel, leaving the embryo, scutellum, and most of the aleurone intact and exposing a surface of semiliquid endosperm to which 5 μl 50% ethanol containing 15 ng [^{14}C]IAA-*myo*-inositol (equivalent to 7.8 ng, 45 pmol, of IAA or 0.5% of that present in the endosperm) and 5700 dpm was applied. The [^{14}C]IAA-*myo*-inositols were 72% radiochemically pure as determined by counting an aliquot at the origin of a TLC plate and recounting the appropriate area after development of the plate. This method underestimates radiochemical purity as [^{14}C]IAA-inositol fixed at the origin, or trailing would not be counted. Further, there are six isomeric IAA-*myo*-inositols, four of which are separable chemically (15), and it is not known whether only one or all 6 are physiologically active. With these reservations in mind, we applied the correction for radiochemical purity so that 7.8 ng × 0.72 = 5.6 ng (32 pmol) containing 5,700 × 0.72 = 4,104 dpm. The data reported for transport of [^{14}C]IAA-*myo*-inositol could thus be 28% too high (if the estimate of radiochemical purity is in error) or 600% too low (if only one of six isomers is the biologically active form). Neither correction would affect the conclusions drawn since we deal here with orders of magnitude difference in amounts transported. Seedlings were incubated for up to 8 h as previously described (21). In one experiment, employing 300 plants, the treated seedlings were pinned through the endosperm to a styrofoam sheet and incubated in a humidified glass chamber. After incubation, the seedling shoot or root tissue was cut off 1 cm from the kernel and the tissue frozen at −20 C until used for combustion or extraction.

DETERMINATION OF FREE [^{14}C]IAA OR FREE + ESTER [^{14}C]IAA IN SEEDLING SHOOT TISSUE

Free IAA. The frozen tissue was added to a mortar and pestle containing sufficient acetone to make the resultant homogenate 70% (v/v) acetone. Rapid grinding in 70% acetone was important to inactivate esterases. A known amount of unlabeled carrier IAA and 500 μg of indole-3-butyric acid was added, then the homogenate was concentrated, acidified, and free IAA extracted into $CHCl_3$ (2).

Free plus Ester IAA. For determination of free plus ester IAA the identical procedure was followed except that the concentrated homogenate was made 1 M with respect to NaOH, held for 1 h at 25 C, and then acidified and treated as above. In both cases the radioactive IAA, together with carrier, was reisolated by a procedure involving chromatography on DEAE-cellulose, LH-20, and silica gel TLC. The partially purified IAA was then silylated, fractionated by GLC, and the IAA peak collected (2). The amount of IAA recovered was determined by UV absorbancy and with the Ehmann reagent (14, 25) as described above. The total radioactivity of the sample could then be corrected for losses since the fractional recovery of the carrier IAA added could be determined (see 21 and below). This procedure has been shown to yield IAA that is pure as judged by absence of extraneous fragment ions in the mass spectrometer (2). Also, the agreement between the colorimetric Ehmann assay and assay of IAA by UV absorbancy at two wavelengths validates the assay and demonstrates that the IAA is pure.

DETERMINATION OF [^{14}C]IAA-*MYO*-INOSITOL IN SEEDLING SHOOT TISSUE

The shoots from 300 seedlings were ground in acetone, as above, the residue reextracted twice with 70% acetone, and the combined extracts filtered. Synthetic, unlabeled carrier IAA-inositol (597 μg) was added and the filtrate concentrated to 2 ml in a flash evaporator. This concentrate plus 1 ml of flask washings was applied to a column (1.2 × 5 cm) of partially sulfonated polystyrene divinylbenzene (15) and the column washed with 80 ml of 1.0 mM citrate buffer (pH 3.3) and then with 80 ml of water. Impurities were eluted with 60 ml of an acetone water gradient 0–50% and finally the IAA-inositols eluted with 40 ml 50% acetone. The acetone-water eluates were combined and dried *in vacuo*. The dried residue was dissolved in 0.5 ml of 2-propanol-water (1:1, v/v) and applied to a column (0.9 × 17 cm) of Beckman PA-28 resin. Using 50% 2-propanol in water as eluent and a flow rate of 20 ml/h at a pressure of 10 atm, the IAA-*myo*-inositols were eluted between 20 and 50 ml (9). The pooled sample was dried *in vacuo*, dissolved in 0.4 ml 50% 2-propanol, dried in a serum vial, and silylated for 30 min at 45 C using 10 μl redistilled pyridine as solvent and 20 μl of bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylchlorosilane) (15). GLC was on a column (2 mm × 1.2 m) of 2% OV-1 on 100/120 mesh Gas-chrom Z using a temperature program from 140 to 300 C at 6°/min. Under these conditions authentic synthetic IAA-*myo*-inositol isomers elute as three major and one minor peak (15) between 18 and 23 min. The radioactivity of the putative ^{14}C-labeled IAA-*myo*-inositols extracted from the shoot tissue coemerged with the added unlabeled carrier IAA-*myo*-inositols and was collected (2). The amount of IAA-*myo*-inositol recovered was determined by its *A* at 282 and 218 nm and by the Ehmann assay (14, 25). The UV spectrum was exactly that for an ester of IAA and the agreement between the UV assay and the colorimetric assay established that the IAA-*myo*-inositol esters were pure. Radioactivity was determined as above, and the radioactivity of [^{14}C]IAA-*myo*-inositol in the tissue computed as:

$$\frac{\text{Carrier IAA-}myo\text{-inositol added}}{\text{Carrier IAA-}myo\text{-inositol recovered}} \times \text{radioactivity}$$

$$= \text{Radioactivity of [}^{14}\text{C]IAA-}myo\text{-inositol in shoots}$$

RESULTS

Experimental Plan and Assumptions. We wished to determine the rate of transport of labeled IAA, tryptophan-derived IAA, and IAA-*myo*-inositol from endosperm to shoot. To do this required knowledge of: (*a*) the rate at which the radioactive compound appeared in the shoot; (*b*) the pool size of these compounds in the endosperm; and (*c*) the rate of metabolic turnover of these compounds in the endosperm. This has been accomplished in the accompanying paper (18) using the isotope dilution technique (27). Knowing (*b*) and (*c*) permitted converting radioactivity appearing in the shoot into amounts of the compound in the shoot.

It is known that most of the IAA, tryptophan, and IAA-*myo*-inositol is in the endosperm (18, 21, 26, 33). We assume that the applied radioactive compounds mix with the pools of these compounds in the endosperm. We believe this assumption to be valid since the endosperm is semiliquid at 4 days of germination; since the applied radioactive compound must diffuse through 1 cm of endosperm before reaching the scutellum; and, since an applied dye appeared to be uniformly distributed within 1 h of application. Further, if there is an error for one compound, similar errors should be applicable to all three and thus our data should serve for comparisons and as an initial approximation. A full discussion of the methodologies and assumptions used in determining pool size and rate of metabolic turnover is given in the accompanying paper (18).

Calculations.

a. There are 4,350 kernels in 1 kg of dry seed, averaging 0.23 g seed^{-1} and we remove 31% of the endosperm to permit isotope application. Thus, all data are multiplied by 1.45 to convert to an entire kernel basis.

b. Each kernel contains 308 pmol IAA and the specific radioactivity of applied IAA at 4 h, the midpoint of our experiments, is 43% of that found at zero time (18).

c. Each kernel contains 87,000 pmol of tryptophan and the specific radioactivity of applied tryptophan at 4 h is 58% of that found at zero time (18).

d. Each kernel contains 6800 pmol of IAA-*myo*-inositol and the specific radioactivity at 4 h is 80% of the specific radioactivity at zero time (18).

e. The specific radioactivity of the [^{14}C]IAA used and the [^{14}C]IAA-*myo*-inositol synthesized from it was found to be $1.28 \cdot 10^8$ dpm μmol^{-1} and had been stated by the manufacturer to be $1.27 \cdot 10^8$ dpm μmol^{-1} or 57.2 μCi μmol^{-1}.

Nonenzymic Hydrolysis. The following experiment (Table I, experiment 9) showed that our procedure for concentrating the plant extract and partitioning the extract into ether caused a negligible nonenzymic hydrolysis of [^{14}C]IAA-*myo*-inositol to free [^{14}C]IAA. Free [^{14}C]IAA in the shoot, following application of [^{14}C]IAA-*myo*-inositol to the endosperm, was thus the result of [^{14}C]IAA-*myo*-inositol hydrolysis in the shoot (see below).

[^{14}C]IAA-*myo*-inositol (3,592 dpm) was mixed with 3 μg unlabeled IAA-*myo*-inositol (an approximation of the concentrations during an actual isolation of IAA-*myo*-inositol from shoot tissue) and the mixture chromatographed on a thin layer plate of MN-Polygram Cel 300 (Brinkmann) using 1-butanol saturated with water as the developing solvent. No free IAA was present in the IAA-*myo*-inositol eluted from the plate between R_F 0.28 and 0.46 since IAA is at R_F = 1.0. The purified IAA-*myo*-inositol was added to a 70% acetone homogenate of 60 shoots and the isolation procedure used for free IAA, as described above, was used. As shown in Table I, experiment 9, our procedure for concentrating the extract and partitioning into ether caused a 1.2% (44 dpm/3,592 dpm) hydrolysis of [^{14}C]IAA-*myo*-inositol to [^{14}C]IAA. This nonenzymic hydrolysis of [^{14}C]IAA-*myo*-inositol to free [^{14}C]IAA amounts to only 3.7% of the endosperm-catalyzed rate of hydrolysis of IAA-inositol to IAA (17, 18) and would account for a negligible amount of the observed free IAA of the shoot derived from IAA-inositol in the endosperm (Table II).

Movement of Radioactivity from Endosperm to Shoot following Application of [^{14}C]IAA-*myo*-Inositol to Endosperm. The experiment of Figure 1 shows that radioactivity from [^{14}C]IAA-*myo*-inositol moves from the endosperm into the shoot and root at a rate of 6.8 pmol shoot^{-1} h^{-1} and 2.6 pmol root^{-1} h^{-1}, respectively (see below).

At zero time, 4,104 dpm (5.6 ng) of labeled IAA-*myo*-inositol was applied to each cut kernel and at 1, 2, and 8 h the shoots and roots were harvested, dried, and combusted as described above. The applied label would be diluted by the 823 ng of IAA-inositol in each cut kernel so the initial specific radioactivity would be 4,104 dpm/823 ng or 4.98 dpm/ng or 0.87 dpm/pmol. Since new IAA-inositol is being formed in the endo-

Table II. *Distribution of Radioactivity in Shoot after Application of [^{14}C]IAA-inositol to the Endosperm of a Z. mays Seedling*

Radioactivity in shoot 8 h after application of 5.8 ng (33 pmol) [^{14}C]-IAA-*myo*-inositol to endosperm. Each endosperm initially contained 6800 pmol of IAA-*myo*-inositol so the perturbation owing to applied [^{14}C]IAA-*myo*-inositol was only 33/6800 = 0.5%.

Parameter	Amount	Total
	pmol·shoot^{-1}·h^{-1}	*%*
Radioactivity in shoot	6.8	100
IAA plus ester IAA	6.3	93
IAA	0.5	7
Ester IAA	5.8	85
IAA-*myo*-inositol	3.8	56
Esters other than IAA-*myo*-inositol	2.0	29
Radioactivity not accounted for and presumably used in growth	0.5	8

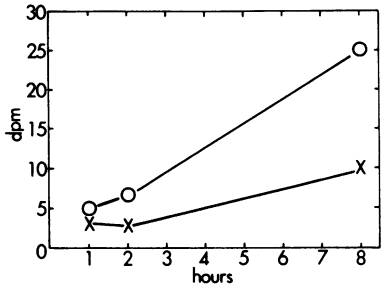

FIG. 1. Movement of radioactivity from endosperm to shoot (O——O) or root (×——×), following application of [2-^{14}C]indole-3-acetyl-*myo*-inositol to the endosperm of a 4-day-germinated seedling of *Z. mays*. Results are shown as dpm in one shoot or one root.

Table I. *Reisolation of [^{14}C]IAA and [^{14}C]IAA-inositol from the Shoot after Application of [^{14}C]IAA-inositol to the Endosperm of Z. mays Seedlings*

Experiment Isolation of [^{14}C]IAA from Shoot after Application of [^{14}C]IAA-Inositol to Endosperm	Recovery of Carrier IAA added: by A_{282}		Recovery of Carrier IAA added: by Ehmann color		Mean	Radioactivity in Sample	Radioactivity in One Shoot in an Hour		IAA Transported into One Shoot in an Hour
	μg	*%*	*μg*	*%*	*μg*	*dpm*	*dpm*	*mean*	*pmol*
1. Hydrolyzed 60 shoots	71	14	71	14	71	1,085	2.3		
2. Hydrolyzed 60 shoots	36	7.1	34	6.8	35	2,622	5.5	3.0	6.3
3. Hydrolyzed 60 shoots	29	5.9	27	5.4	28	574	1.2		
4. Unhydrolyzed 60 shoots	18	3.7	17	3.4	18	114	0.24	0.24	0.5
5. unhydrolyzed 60 shoots	29	5.8	30	6.0	30	117	0.24		
Experiment: Isolation of [^{14}C]IAA-Inositol from Shoot after Application of [^{14}C]IAA-Inositol to Endosperm	Recovery of Carrier IAA-Inositol added: by A_{282}		Recovery of Carrier IAA-Inositol added: by Ehmann color			Radioactivity in one Shoot in an Hour			5.8 ester
	μg	*%*	*μg*	*%*	*μg*	*dpm*	*dpm*	*mean*	
6. Unhydrolyzed IAA-inositol reisolated from 300 shoots	7.8	2.5	7.6	2.5	7.7	4365	1.82		
7. Unhydrolyzed IAA-inositol reisolated from 300 shoots	11	3.5	10	3.3	10	5197	2.16	1.8	3.8
8. Unhydrolyzed IAA-inositol reisolated from 300 shoots	4.5	1.4	3.8	1.2	4.3	3600	1.50		
9. Non-enzymic hydrolysis of [^{14}C]IAA-inositol to [^{14}C]IAA	44	8.8	43	8.5	44	44	0.73	0.73	

sperm, the specific radioactivity at the midpoint of the experiment would be 80% of that at zero time (18) or 0.69 dpm/pmol.

Figure 1 shows the amount of radioactivity in the shoots and roots as a function of incubation time. Each point represents the average radioactivity found in three groups of 10 shoots or three groups of 10 roots, and thus is the average for 30 plants. After 8 h, about 26 dpm were found in each shoot (3.25 dpm shoot^{-1} h^{-1}) and 10 dpm (1.25 dpm shoot^{-1} h^{-1}) in each root. Thus, for shoots, 3.25 dpm transported/0.69 dpm pmol^{-1} = 4.71 pmol per cut kernel or 6.8 pmol radioactivity from IAA inositol into one intact shoot in 1 h. For roots, 1.25 dpm transported/0.69 dpm pmol^{-1} = 1.8 pmol/cut kernel or 2.6 pmol radioactivity from IAA-inositol into one intact root in 1 h.

Inasmuch as (see below) 6.30 pmol shoot^{-1} h^{-1} can be accounted for as IAA or IAA-*myo*-inositol in the shoots, 93% of the radioactivity transported can be accounted for as these two compounds. For roots, 0.95 pmol are accounted for as IAA and IAA-inositol so that only 0.95/2.60, or 36% of the transported radioactivity remains as IAA or ester IAA.

[^{14}C]IAA plus ^{14}C-Ester IAA in the Shoot following Application of [^{14}C]IAA-*myo*-Inositol to Endosperm. The data of Table I, experiments 1 through 3, show that the amount of IAA plus ester IAA accumulated in the shoot after application of labeled IAA-*myo*-inositol to the endosperm was 6.3 pmol shoot^{-1} h^{-1} thus accounting for 93% of the radioactivity (Table II). Experiments 4 and 5 show that the amount of free IAA accumulated in the shoot after application of IAA-*myo*-inositol to the endosperm was 0.5 pmol shoot^{-1} h^{-1}. Thus, labeled IAA-*myo*-inositol applied to the endosperm appears as 93% ester and 7% free IAA in the shoot—exactly the normal ratio of ester to free IAA in *Zea* shoots (2).

In experiments 1, 2, and 3 the acetone extract of the tissue was hydrolyzed with 1 N NaOH and, following addition of carrier unlabeled IAA, the [^{14}C]IAA reisolated as pure IAA. An average of 3.0 dpm shoot^{-1} h^{-1} could be recovered as IAA following the hydrolysis so that 3.0 dpm/0.69 dpm pmol^{-1} × 1.45 = 6.3 pmol shoot^{-1} h^{-1} of ester plus free IAA were present in the shoot. In experiments 4 and 5, [^{14}C]IAA was isolated from the shoot without prior alkaline hydrolysis. In this case 0.24 dpm/0.69 dpm pmol^{-1} × 1.45 = 0.5 pmol shoot^{-1} h^{-1} of free IAA, derived from IAA-*myo*-inositol in the endosperm, was present in the shoot.

[^{14}C]IAA-*myo*-Inositol in the Shoot following Application of [^{14}C]IAA-*myo*-Inositol to Endosperm. The data of Figure 1 and Tables I and II, experiments 1 through 3, show that 93% of the radioactivity of [^{14}C]IAA-*myo*-inositol applied to the endosperm, that appeared in the shoot, was either ester or free IAA. The following experiments were undertaken to see if the ester was still IAA-*myo*-inositol. The results of experiments in which IAA-*myo*-inositol applied to the endosperm was reisolated as the single chemical entity, IAA-*myo*-inositol, from the shoots are summarized in Table I, experiments 6–8. It was found that 66% of the radioactive esters of the shoot could be accounted for as the isomeric IAA-*myo*-inositols. Thus, 34% of the radioactive esters of the shoot are esters other than the IAA-*myo*-inositols.

We were interested in the possibility of transacylation of the IAA moiety from *myo*-inositol to a mono or disaccharide acceptor since *Zea* endosperm contains traces of the 2-0, 4-0 and 6-0 IAA esters of glucose (13) and also contains IAA esters of *myo*-inositol glycosides (34). The isolation procedure used to reisolate [^{14}C]-IAA-*myo*-inositol from the tissue would not have totally separated such esters from the *myo*-inositol ester until the last GLC step. Thus, we collected all of the radioactivity emerging from the GLC column. About 76% of the radioactivity emerged in the region of the isomeric IAA-*myo*-inositols which is in reasonable agreement with the 66% observed by column chromatography. Interestingly, 11% of the emerging radioactivity came in the region where an IAA-*myo*-inositol glycoside would occur (15). As in the accompanying paper (18), this again indicates the 5-0 glycosylation may be reversible although the IAA-*myo*-inositol glycosides were not specifically studied.

The mean amount of [^{14}C]IAA-*myo*-inositol recovered from the shoots was 1.8 dpm shoot^{-1} h^{-1}. This amounts to 1.8 dpm/0.69 dpm pmol^{-1} × 1.45 = 3.8 pmol shoot^{-1} h^{-1} of IAA-*myo*-inositol in the shoot. Now the total ester in the shoot is (Table I) 6.3 pmols (total IAA plus ester) minus 0.5 pmol free IAA = 5.8 pmol of ester. Then 3.8/5.8 = 66% of the ester is IAA-*myo*-inositol.

Metabolic Fate of [^{14}C]IAA-*myo*-Inositol Transported from Endosperm to Shoot. Our abiding interest is in what happens to the IAA-*myo*-inositol transported from endosperm to shoot. Table II presents a summary of that data derived from Figure 1 and Table I. It shows the following: if the total radioactivity appearing in the shoot, following application of [^{14}C]IAA-*myo*-inositol to the endosperm, is normalized to 100%, then 7% of that is free IAA in the shoot; 56% is IAA-*myo*-inositol in the shoot; 29% is in esters other than IAA-*myo*-inositol; and 8% is unaccounted for—without loss of the methylenic carbon.

Evidence That the Hydrolysis of [^{14}C]IAA-*myo*-Inositol Applied to Endosperm Occurs in Shoot. We wished to determine whether hydrolysis of [^{14}C]IAA-*myo*-inositol to free [^{14}C]IAA occurred in the endosperm or in the seedling shoot, and to determine whether the ratio of [^{14}C]IAA to [^{14}C]IAA-*myo*-inositol approximated the normal ratio of free to ester IAA we previously found in shoot tissue (2). We know the amount of [^{14}C]IAA-*myo*-inositol applied, and we determined the pool size of IAA-*myo*-inositol and IAA in the endosperm (18) and the amount of labeled IAA and IAA-*myo*-inositol appearing in the shoot (21 and this paper). Since only 5 × 10^{-4}% of the IAA and IAA-*myo*-inositol esters occur in the shoot (2, 26, 33) with the rest in the endosperm, we can determine whether hydrolysis and/or esterification occurred in the endosperm or shoot because hydrolysis in the endosperm would yield enormously more dilution of the applied isotope than would transport followed by hydrolysis in the shoot.

From these considerations we see that the free IAA in the shoot coming from IAA-*myo*-inositol applied to the endosperm had to arise by ester hydrolysis in the shoot or isotope dilution would have precluded detection. Similarly the [^{14}C]IAA ester in the shoot following application of [^{14}C]IAA to the endosperm had to have been produced by esterification in the shoot, or dilution in the endosperm would have precluded its detection (21).

The application of [^{14}C]IAA-inositol to the endosperm resulted in 0.50 pmol free IAA appearing in the shoot in 1 h (Table I). Had hydrolysis to free IAA occurred in the endosperm, then 1.52 × 10^{-2} pmol h^{-1} seedling^{-1} (ref. 21 and Table III)—from a pool of 308 pmol free IAA endosperm^{-1}—would have been transported to the shoot. Thus:

$$\frac{1.52 \times 10^{-2} \text{ pmol of IAA transported shoot}^{-1}\text{ h}^{-1}}{308 \text{ pmol of free IAA endosperm}^{-1}} 0.24 \text{ dpm}$$

$$= 1.2 \times 10^{-5} \text{ dpm shoot}^{-1}\text{ h}^{-1}$$

Table III. *Transport of IAA, Tryptophan-derived IAA, or IAA-myo-Inositol from Endosperm to Shoot of Z. mays*

Basis for Calculation	pmol shoot^{-1} h^{-1}
[^{3}H]IAA applied to endosperm and appearing as [^{3}H]-IAA in shoot after dilution with free IAA pool of endosperm and with new IAA being produced by hydrolysis of esters in the endosperm (18, 21, and this paper).	0.015
[^{3}H]Tryptophan applied to endosperm and appearing as [^{3}H]IAA in shoot after dilution with tryptophan pool of endosperm and with new tryptophan being produced by hydrolysis of protein in the endosperm (18, 21, and this paper).	0.15
[^{14}C]IAA-*myo*-inositol applied to endosperm and appearing as [^{14}C]IAA or [^{14}C]IAA esters in the shoot after allowing for dilution with the IAA-inositol pool in the endosperm and with new IAA-inositol being produced by hydrolysis of IAA-inositol-glycosides in the endosperm (18 and this paper).	6.3
Amount of IAA needed by shoot (19, 21, and this paper).	5 to 9

a quantity we would not have detected. Thus, the IAA-inositol was transported and hydrolysis of the IAA-inositol occurred in the seedling and not in the endosperm.

A similar calculation may be applied to the data of Hall and Bandurski (21) to determine whether the esterification of [^{3}H]IAA applied to the endosperm occurred in the endosperm or the shoot. Using their data of Table I, and subtracting the value "not hydrolyzed," an average of 15,106 dpm of esterified IAA 60 shoots^{-1} 8 h^{-1} is found or 3.15×10^1 dpm shoot^{-1} h^{-1}. They applied 7.6 ng [^{3}H]IAA containing 2.26×10^6 dpm. Had this esterified in the endosperm it would have equilibrated with a pool of 823 ng 2/3 kernel,$^{-1}$ plus the 7.6 ng applied, so the specific radioactivity at zero time would have been:

$$\frac{2.26 \times 10^6 \text{ dpm}}{823} = 2.75 \times 10^3 \text{ dpm ng}^{-1}$$

Now

$$\frac{3.15 \times 10^1 \text{ dpm shoot}^{-1} \text{ h}^{-1}}{2.75 \times 10^3 \text{ dpm ng}^{-1}} = 1.14 \times 10^{-2} \text{ ng}$$

$$= 0.07 \text{ pmol 2/3 shoot}^{-1} \text{ h}^{-1}$$

$$= 0.10 \text{ pmol shoot}^{-1} \text{ h}^{-1}$$

$$\text{and } (0.10/6.30)100 = 1.6\%$$

Thus, hydrolysis followed by esterification of IAA in the endosperm and subsequent transport to the shoot could account for only 1.6% of the observed transport of radioactive IAA from endosperm to shoot following application of [^{14}C]IAA-*myo*-inositol to the endosperm.

DISCUSSION

Our studies provide a chemical identification of the "seed auxin precursor" and establish, for *Zea*, that the precursor is IAA-*myo*-inositol and not tryptophan or tryptamine. It is now important to determine whether hydrolysis of IAA-*myo*-inositol occurs in the tip of the seedling shoot. One possible interpretation is that IAA-*myo*-inositol moves from the endosperm to the shoot tip, there it is hydrolyzed to free IAA and the amount of hydrolysis is determined by environmental impacts upon the shoot (33). The resultant free IAA then diffuses to the elongating zone—there to control growth and then be destroyed (5, 10). The large amounts of IAA derived by exhaustive diffusion of coleoptile tips (*cf.* 2, 35) would result from continuous hydrolysis of the 10-fold larger amounts of IAA ester in the shoots (2). We have previously established that our values for the content of ester plus free IAA in *Zea* seedlings accord with the content of IAA available by exhaustive diffusion (2, 35).

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 (36). The concept that a stimulating substance moved down from the tip was developed by Ciesielski (8) in studies of root geotropism, and Darwin, stimulated by Ciesielski's experiments, concluded that a downward diffusing stimulus was involved in shoot phototropism (11). Downward diffusion of IAA has been extensively studied (20) but studies of the upward diffusing seed auxin precursor are few. The precursor was studied by Skoog who showed that a substance, collected from coleoptile stumps, was slowly converted into a downward diffusing active auxin (31). The precursor was variously regarded to be an auxin ester (35, 36) or a tryptophan or tryptamine-like substance (31, 32). It was known that seeds were rich sources of substances convertible to IAA by alkaline hydrolysis (4, 35, 36). The discovery that tryptophan could be converted to IAA (32) overrode the possibility that IAA-esters were the seed auxin precursor and knowledge that tryptophan in proteins could be converted to IAA by strong alkaline hydrolysis further complicated the matter (29). Now, with knowledge of the chemical identity of all these substances (1, 16) it is possible to determine which of these substances is a seed auxin precursor.

The data of this paper establish that IAA-*myo*-inositol is transported from endosperm to shoot at a rate of 6.3 pmol into one shoot in 1 h. This is 400 times the rate of transport of free IAA from endosperm to shoot and more than 40 times the rate at which tryptophan-derived IAA appears in the shoot (Table III). It would supply precursor for the 5 pmol shoot^{-1} h^{-1} of free IAA moving down from the tip (19). It is also adequate to maintain a constant concentration of ester IAA in the growing shoot since our previous estimate of 9 pmol shoot^{-1} h^{-1} is almost certainly high (21). These data, together with the demonstration of the occurrence of IAA-*myo*-inositols in *Zea* endosperm (26, 34), establish IAA-*myo*-inositol as a seed auxin precursor.

Labeled IAA and tryptophan (21) or, in this work, labeled IAA-*myo*-inositol (24), were introduced into the shoot by application of the labeled compound to a cut surface of the endosperm. This technique was utilized because it has been established that the compounds that yield IAA upon alkaline hydrolysis are localized in the endosperm (26). Skoog used the Anne Boleyn technique to study the upward diffusion of the seed auxin precursor and the formation and subsequent downward diffusion of the active auxin. This decapitation technique has yielded most of our knowledge of plant auxin metabolism. It is not perfect in that it requires that the diffusing substances leave and enter membrane barriers not normally encountered by the auxin and requires that the precursor and auxin diffuse through a layer of cell and cell debris containing, of course, esterases and oxidative enzymes.

Our technique, like the Anne Boleyn technique, presents difficulties in that endosperm pool sizes, pool turnover, and pool homogeneity must be established. These are surmountable difficulties and no new membrane barriers are interposed between the seed auxin precursor and the shoot. In addition, at 4 days of germination, the endosperm is fairly liquid, and mixing occurs as the labeled compound moves from the cut endosperm surface to the scutellum where absorption into the shoot occurs. Since the endosperm is rich in indolylic compounds it becomes necessary to allow for dilution of the labeled compound by unlabeled indolylic compounds. Studies of the dilution are described in references 17 and 18. It is this knowledge of the identity of the seed indoles (1, 16), their amounts (3, 33), and their metabolic turnover (17, 18) which permits conversion of radioactivity into amounts of compound transported.

We previously postulated that the growth rate of plants is controlled by varying the ratio of free to conjugated IAA (1, 3, 21, 23) and have shown, in the case of photoinhibition of corn seedling growth, that such control occurs (3). It was further shown that enzymes to make and hydrolyze the conjugates could be demonstrated (22, 23). To this we now add the demonstration that IAA and IAA-*myo*-inositol are in equilibrium with each other in the shoot tissue and that equilibrium can be attained starting either with free [^{14}C]IAA or [^{14}C]IAA-*myo*-inositol. This conclusion was established as follows:

Labeled IAA-*myo*-inositol was applied to the endosperm and appeared as labeled IAA and labeled IAA-*myo*-inositol in the shoot. Hydrolysis of [^{14}C]IAA-*myo*-inositol to free [^{14}C]IAA in the endosperm and subsequent transport of the [^{14}C]IAA could be excluded since, had hydrolysis occurred in the endosperm, the resultant dilution by the large free IAA pool and the subsequent slow rate of IAA transport would yield only an undetectable 10^{-5} dpm shoot^{-1} h^{-1}. Further the [^{14}C]IAA-*myo*-inositol has been isolated as the ester from shoot tissue. Thus, we conclude that transport of [^{14}C]IAA-*myo*-inositol from endosperm to shoot occurs as transport of ester and not free IAA, followed by subsequent reesterification. Prior studies show 93% of the IAA of *Zea* shoots to be esterified and 7% free (2) which is identical to the 93% ester, 7% free, observed in the shoot in these studies following application of labeled IAA-*myo*-inositol to the endosperm.

Similarly labeled IAA applied to the endosperm appears as

labeled ester in the shoot (21). Had esterification occurred in the endosperm equilibration would occur with the large endosperm ester pool and only 1.5% of the observed ester in the shoot could be accounted for. We conclude that the slight transport of free IAA from endosperm to shoot occurs as transport of free IAA, and not as ester with subsequent hydrolysis. The proportion of ester to free IAA in the shoot following application of labeled IAA to the endosperm is 72% ester and 28% free (21). This value is low and variable and we attribute this to lack of knowledge of the rapidity of ester hydrolysis at the time of these earlier studies. However, it establishes that free IAA can be converted to ester IAA in the shoot.

Collectively then, the *in vitro* (22, 23) and *in vivo* studies (3, 21, and this paper) establish that free IAA and IAA-*myo*-inositol are in equilibrium with each other in shoot tissue of *Zea*. This, to our knowledge, is the first demonstration in biology of reversible synthesis and hydrolysis of a co-valently bonded hormone conjugate.

Whether transport of IAA as an ester is a general phenomenon or one restricted to *Zea* is not known. Sheldrake has observed an alkali-labile IAA compound in the xylem sap of *Zea* (30). Much earlier studies showed that a growth regulating chemical, 2,4-D, was not transported out of the leaf of a bean plant in the dark but was transported in the light or if sugar was applied to the darkened plant (28). Esterification of 2,4-D is now well studied (12). Unpublished studies by Schulze and Bandurski show an IAA ester and an IAA amide in vascular exudates of pumpkins but amounts adequate for characterization were not obtained. Thus, the generality of upward transport of IAA as an ester remains to be demonstrated.

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