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Transport and Metabolism of Indole-3-Acetyl-*myo*-Inositol-Galactoside in Seedlings of *Zea mays*[1]

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ABSTRACT

Indole-3-acetyl-*myo*-inositol galactoside labeled with ^3H in the indole and ^{14}C in the galactose moieties was applied to kernels of 5 day old germinating seedlings of *Zea mays*. Indole-3-acetyl-*myo*-inositol galactoside was not transported into either the shoot or root tissue as the intact molecule but was instead hydrolyzed to yield [^3H]indole-3-acetyl-*myo*-inositol and [^3H]indole-3-acetic acid which were then transported to the shoot with little radioactivity going to the root. With certain assumptions concerning the equilibration of applied [^3H]indole-3-acetyl-*myo*-inositol-[U-^{14}C]galactose with the endogenous pool, it may be concluded that indole-3-acetyl-*myo*-inositol galactoside in the endosperm supplies about 2 picomoles per plant per hour of indole-3-acetyl-*myo*-inositol and 1 picomole per plant per hour of indole-3-acetic acid to the shoot and thus is comparable to indole-3-acetyl-*myo*-inositol as a source of indole-acetic acid for the shoot. Quantitative estimates of the amount of galactose in the kernels suggest that [^3H]indole-3-acetyl-*myo*-inositol-[^{14}C] galactose is hydrolyzed after the compound leaves the endosperm but before it reaches the shoot. In addition, [^3H]indole-3-acetyl-*myo*-inositol-[^{14}C]galactose supplies appreciable amounts of ^{14}C to the shoot and both ^{14}C and ^3H to an uncharacterized insoluble fraction of the endosperm.

IAA, indole-3-acetyl-*myo*-inositol (IAInos),[3] and indole-3-acetyl-*myo*-inositol galactoside (IAInosgal) comprise 1, 15, and 8%, respectively, of the IAA pool of the kernels of *Zea mays* (3). The metabolism and transport of IAA and IAInos applied to the endosperm has been studied (1, 6, 7, 14).

IAA is rapidly catabolized in the germinating kernels and shoots (13) to oxindole-3-acetic acid (15, 16) and a further oxidation product, the glucoside of 7-hydroxy-oxindole-3-acetic acid (12) with only a small amount of IAA reaching the coleoptile (1). IAInos is hydrolyzed to free IAA in the kernel and is also transported into the shoot and root, there to yield free IAA (1, 14).

The present work addresses the question of whether there are differences in the metabolism and transport of IAInos and IAInosgal. Such differences, if they exist, would indicate that the conjugating moiety is important in determining the fate of the IAA conjugate. To answer the question we synthesized [^3H] IAInos-[^{14}C]gal, applied it to the endosperm of germinating corn seedlings, and then measured ^3H and ^{14}C activity in the shoot and root. In addition, we partially fractionated the seedling extracts by means of HPLC, and obtained estimates of the amount of labeled IAA, IAInos, IAInosgal, and galactose in the shoots and roots. An abstract of this work has appeared (10).

MATERIALS AND METHODS

Plant Material. Kernels of *Zea mays* L. var Stowell's Evergreen (Burpee Seed Co.) were surface sterilized with 1% NaOCl for 10 min, then soaked in running tap water (9–11°C) for 24 h, rolled in moist paper towels, and germinated in darkness at 25°C for 4 d.

Radioisotope Application. Aliquots of 5 μl of [^3H]IAInos-[^{14}C]gal in 50% propan-2-ol-water (0.085 nmol containing 4087 dpm of ^3H and 608 dpm of ^{14}C were applied to 1 by 3 mm deep holes in the kernels of 30 seedlings. The amount of IAInos gal applied was thus about 1% of the endogenous amount (3). The seedlings were rerolled in moist paper towels and incubated for 4 h at 25°C. Necessary manipulations were conducted in minimal illumination using a phototropically inactive green safe-light.

Determination of [^{14}C] and [^3H] Radioactivity in Tissues. Shoots and roots were excised 0.5 cm from the kernel and the tissues frozen with liquid N_2, weighed, and ground in acetone, using a ratio of tissue to acetone of 2:7 (w/v). Unlabeled IAA, either 0.15 or 0.3 μmol, was added and the tissue then extracted overnight at 4°C. Extracts were filtered and the residue washed three times with 80% aqueous (v/v) acetone. The washings were combined and the residue dried for estimation of dry weight and then combusted in a Packard Tri-Carb Sample Oxidizer for separate estimations of the ^3H_2O and $^{14}CO_2$ resulting from combustion.

Determination of IAA, IAInos, and IAInosgal. Kernel extracts required a prepurification step on a 16 cm^3 bed volume column of Dowex X 2-50W (5) using 1:1, propan-2-ol:water as the eluting agent. Materials eluting in the first 20 ml, were pooled as the galactose fractions, and between 20 and 320 ml were pooled, as the indolylic fraction.

The prepurified kernel extract and the extracts from roots and shoots were separately chromatographed on a 0.9 × 17 cm PA-28 column (4) using 1:1, 2-propanol:water as eluent. Galactose eluted in the first 22 ml and the fractions containing galactose from the PA-28 column and the galactose-kernel fraction from the Dowex-50 column were dried and radioactivity determined by liquid scintillation counting. Material eluting from the PA-28 column between 23 to 70 ml was collected, dried, dissolved in ethylacetate:acetonitrile:ethanol:water (65:21:7:7, v/v) and eluted from a 0.46 × 25 cm Partisil-10 HPLC column with this solvent (4). Absorbancy at 280 nm was monitored and ^3H and ^{14}C activities determined by counting using differential channel-counting where necessary.

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[3] Abbreviations: IAInos, indole-3-acetyl-*myo*-inositol; IAInosgal, indole-3-acetyl-*myo*-inositol galactoside.

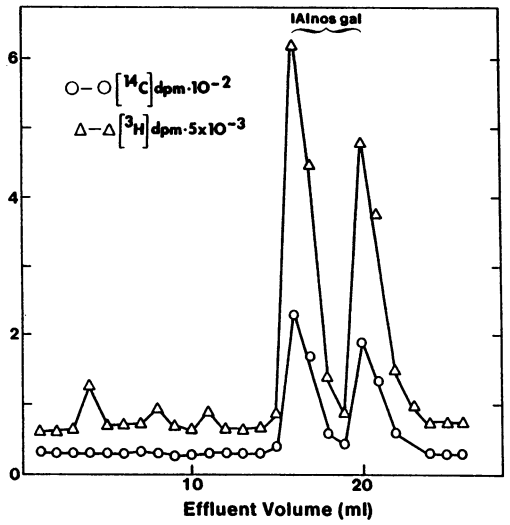

FIG. 1. HPLC profile of enzymically synthesized 5-[^{3}H]indole-3-acetyl-*myo*-inositol-[U-^{14}C]galactose chromatographed on a straight phase Partisil-10 column using a flow rate of 1 ml/min. Injection was with a 0.3 ml loop using ethylacetate:acetonitrile:ethanol:water (65:21:7:7). The two peaks of isomeric IAInosgal eluting between 15 to 22 ml were pooled for use in the feeding experiments. The residual contaminant peaks of IAA at 3 min and the isomeric IAInos peaks at 7 and 11 min were discarded.

Determination of Galactose Pool Size. For determination of the endogenous pool size of galactose in germinating kernels of *Zea mays*, 1.00 μCi (0.24 μmol) of [^{14}C]galactose was added (as above) to 12 germinating kernels, (5 g dry weight), and the kernels homogenized in 80% aqueous ethanol. Insoluble material was rinsed with 10 ml of 80% ethanol and the extracts filtered and reduced in volume to 4 ml. This solution was acidified to pH 2 and passed through a 1.2 cc bed volume column of Dowex 50 (H^+ form). The filtrate and column washings were combined and adjusted to pH 8 with NH_4OH and passed through a similar column of Dowex 1-OH form. The eluent was concentrated to 500 μl, and 5 μl was chromatographed on a 4 × 250 mm HPLC column of Aminex HPX-87 C (Ca^{2+} form, 8% cross-linked, cation exchange resin, Bio-Rad, Richmond, CA). Radioactive fractions at or near the solvent front were pooled, dried, and sugars converted to the trimethylsilyl ethers by treatment with 100 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide-1% trimethylchlorosilane plus 15 μl of 1% 4-dimethylaminopyridine in pyridine (17) at 45°C for 2 h and then 16 h at 25°C. GC was on a 2 mm by 1.8 m OV-1 column programmed from 130 to 230°C at 4°C per min. The α-peak of galactose emerges at 8.35 min in front of the much larger glucose peak which would otherwise obscure the small amount of galactose present. The specific activity of the extracted galactose peak was 0.74 $\mu Ci \cdot \mu mol^{-1}$, whereas the specific activity of the galactose added to the plant extract was 4.17 $\mu Ci \cdot \mu mol^{-1}$. Thus, using the isotope dilution equation (6), the amount of galactose in the sample was Y = ([4.17/0.74]-1.0) 0.24 = 1.11 μmol per 12 kernels, or 0.0925 μmol per kernel.

RESULTS

Enzymic Synthesis of 5-[^{3}H]IAA-*myo*-Inositol-[U-^{14}C]galactose. Approximately 5 mg of enzyme protein in 0.5 ml of 25 mM Hepes buffer (ph 7.6) containing 0.05% Triton X-100, 5 mM mercaptoethanol, and 5 mM $CaCl_2$ was incubated with 24 nmol of 5-[^{3}H]IAA-*myo*-inositol (11) and 1.6 μmol of UDP-[U-^{14}C] galactose for 1 h at 37°C. This incubation mixture is modified and improved over that used by Corcuera *et al.* (4). The reaction was terminated by the addition of 0.5 ml of propan-2-ol and protein removed by centrifugation (10 min × 1300*g*). The supernatant fluid and washings of the precipitate were combined, dried *in vacuo* using a bath temperature of 50°C, and the residue dissolved in 0.5 ml of 50% propan-2-ol:water. The sample was applied to a 0.9 × 17 cm PA-28 column and eluted with the same solvent. Radioactive material eluting between 25 to 70 ml was collected, dried *in vacuo* and applied to a Partisil-10 HPLC column and eluted with ethyl acetate:acetonitrile:ethanol:water (65:21:7:7). Fractions between 15 to 22 ml were pooled as is shown in Figure 1. They contained the two isomeric peaks of IAInosgal at 17 and 20 min, clearly separated from [^{3}H]IAA, at 3 min, and residual [^{3}H]IAInos peaks at 7 and 11 min. The product contained 4.25 nmol (204,345 dpm) of [^{3}H]IAA and 6.1 nmol (30,406 dpm) of [^{14}C]galactose so that the molar ratio of IAInos to galactose was 0.7.

Rechromatography of the product on Partisil-10 removed extraneous ^{14}C containing material and yielded a product separated by baseline resolution from the traces of IAA and IAInos. Chromatography on Silica Gel G (TLC) using the same solvent as for HPLC showed a product identical to authentic IAInosgal isolated from corn kernels.

Radioactivity in Soluble and Insoluble Fractions of Roots, Stems, and Kernels following Feeding with 5-[^{3}H]IAInos-[^{14}C]-gal. A summary of the overall distribution of ^{3}H and ^{14}C in the soluble and insoluble fractions of roots, stems, and kernels is shown in Table I. Recovery of ^{3}H and ^{14}C based upon the radioactivity placed in the kernels was 77 and 90%, respectively.

Distribution of Labeled Galactose, IAA, IAInos, and IAInosgal in Roots, Shoots, and Kernels following Application of [^{3}H] IAInos[^{14}C]gal to Kernels. Table II shows the distribution of the various components following application of IAInosgal to the kernels. Less ^{3}H and ^{14}C activity appears in the roots than in the shoots. Between 35 to 53% of the ^{14}C activity applied to the kernels appeared in the shoots at the HPLC retention time of galactose. Thus, IAInosgal appears to be an excellent donor of galactose to the shoots, with the remainder of the galactose remaining in the kernel. Between 0.9 to 1.8% of the ^{3}H applied to the kernel as IAInosgal appears in the shoots as IAInos, with 0.4% (single experiment) appearing in the shoot as free IAA. However, 24 to 52% of the ^{3}H applied as IAInosgal to the kernel appears as IAInos in the kernel. Since no intact IAInosgal appears in the shoot, these data suggest that IAInosgal is hydrolyzed to IAInos before entering the vegetative shoot.

Figure 2 illustrates the separation attained for the putative IAA, IAInos, and IAInosgal on the Partisil-10 ODS column.

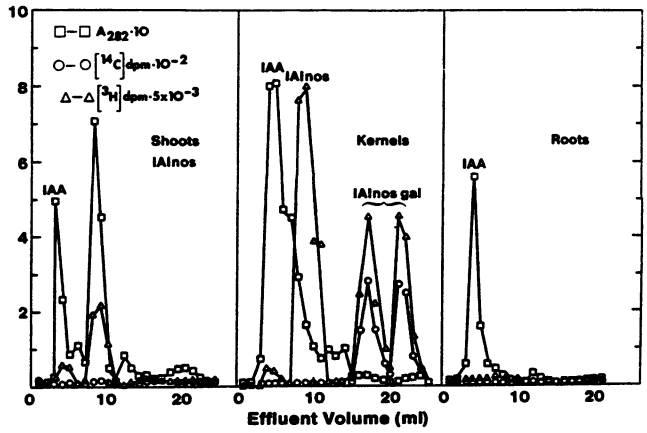

FIG. 2. HPLC separation of the products formed upon feeding 5-[^{3}H] indole-3-acetyl-*myo*-inositol-[U-^{14}C]galactose to kernels of *Zea mays* and permitting metabolism and transport to occur for 4 h. Chromatographic conditions were as described for Figure 1.

Table I. *Distribution of Radioactivity in Shoots, Kernels and Roots, 4 Hours after Application of 5-[^{3}H]indole-3-acetyl-myo-inositol-[U-^{14}C] galactose to Germinating Zea mays Kernels*

Following incubation the seedlings were dissected and combusted and the resultant ^3H_2O and $^{14}CO_2$ collected for scintillation counting. Thirty seedlings were used for each experiment and each sample of 30 seedlings received 2.55 nmol of [^{3}H]-IAInos-[^{14}C]gal containing 123,000 dpm of ^{3}H and 18,250 dpm of ^{14}C. Data represent the average of two or three experiments except for the soluble root fractions which represent a single experiment.

Isotope	Unit	Soluble Fraction			Insoluble Fraction			Yield	
		Shoots	Kernels	Roots	Shoots	Kernels	Roots	nmol	%
^{3}H	dpm	2,350	71,000	900	288	20,050	190		
	nmol	0.05	1.5	0.02	0.006	0.4	0.004	1.98	77
	%	1.9	58	0.8	0.23	15	0.15		
^{14}C	dpm	7,193	6,940	320	39	1,895	151		
	nmol	1.0	0.9	0.04	0.005	0.26	0.02	2.2	90
	%	39	38	1.7	0.2	10	0.8		

Table II. *Radioactivity in the Putative IAA, IAInos, Galactose, and IAInosgal Fractions, 4 Hours after Application of 5-[^{3}H]-indole-3-acetyl-myo-inositol-[U-^{14}C]galactose to the Kernels*

Fractionation procedures were as described in the text and for Figure 1. A zero indicates no detectable radioactivity. The results are the average of two or three experiments except for IAA and IAInos in the shoots which represents a single experiment.

Compound	Tissue								
	Shoots			Kernels			Roots		
	dpm	nmol	%	dpm	nmol	%	dpm	nmol	%
Galactose	7193	1.0	39	Not measured	Not measured	Not measured	300	0.04	1.6
IAA	523	0.01	0.4	1,650	0.03	1.3			
IAInos	1070	0.02	1.3	35,300	0.73	29.0	900	0.02	0.7
IAInosgal									
^{3}H	0	0	0	37,650	0.8	30.0			
^{14}C	0	0	0	6,010	0.83	33.0	0	0	0

DISCUSSION

IAInos constitutes about 15% of the IAA ester compounds of the germinating kernel and about 19% of the IAA esters of the shoot (2). It was this close agreement between the percent composition in the shoot and kernel that led to the present experiments. They were designed to determine whether the ester conjugates would diffuse up into the shoot in proportion to their concentration in the kernel or whether specificity of transport and metabolism would be shown. The present data demonstrate that both the transport and metabolism of IAInosgal and IAInos are different and thus specificity in metabolism and transport is implied. Labeled IAInos is transported from the kernel into both the shoot and the roots, although with greater efficiency into the shoots (14). By contrast, labeled IAInosgal is not transported into either the shoot or root. Instead, it is hydrolyzed to IAInos. The IAInos and up to one-half of the galactose is then transported into the shoot. There is less transport of radioactivity into the root. We thus conclude that IAInos and IAInosgal are differently metabolized and transported.

In experiments I and II between 0.9 to 1.8% of the ^{3}H radioactivity of [^{3}H]IAInos-[^{14}C]gal applied to the kernel appeared as [^{3}H]IAInos in the shoot. Provisionally, as shown in Table III, these data may be translated into the amounts of IAInos transported into the shoot as the result of application of [^{3}H]IAInos-[^{14}C]gal to the kernel. To calculate the amount of compound present in the shoot from the amount of radioactivity we assume that the applied activity is fully diluted by the IAInosgal pool in the kernel. With this assumption the calculated amounts of IAInos in the shoot as the result of IAInosgal applied to the endosperm is 2 pmol·plant^{-1}·h^{-1}. Similarly, using the 523 dpm of free IAA found in the shoot in experiment II one may calculate how much IAA appears in the shoot as the result of application of [^{3}H]IAInos-[^{14}C]gal to the kernel. A value of about 1 pmol·plant^{-1}·h^{-1} of IAA appears in the shoot as the result of [^{3}H]IAInos-[^{14}C]gal applied to the endosperm. Jackson and McWha (9) have recently pointed out that the endosperm liquifies primarily in a zone proximal to the aleurone layers and thus applied isotope will not fully equilibrate with endosperm components. We observed that the extent of endosperm liquefaction during 4 d of germination varies depending upon the variety and vigor of the seeds. However, whether the applied radioactivity moves as on a planar surface from the point of application on the endosperm to the scutellum is not a major factor since it is the specific activity of the material reaching the scutellar surface which is used in the calculation. If only a hollow cylinder of radioactivity, representing the liquid layer under the aleurone, moves to the scutellum, then less isotope will enter the shoot but the specific activity will be as calculated above. However, it must be emphasized that these and past quantitative estimates of material moving from endosperm to shoot (1, 6, 14) are first approximations. Ultimately, it will be necessary to determine the specific activity of materials in a thin layer of endosperm proximal to the scutellum.

A further point meriting discussion is the appearance of ^{3}H and ^{14}C in the insoluble fraction of the kernel. The ^{3}H radioactivity could represent IAA or an IAA derivtive that has become covalently attached to insoluble material of the endosperm and the ^{14}C activity might represent galactose or a derivative which is covalently bound to insoluble material. Both fractions will require further study.

A striking point in the data of Table II is the 35 to 53% of ^{14}C activity appearing in the shoot, probably as the free sugar. This

Table III. *Calculation of the Amount of Compound Transported from Kernel to Shoot Based upon Radioactivity Appearing in the Shoot*

Amounts of ^3H and ^{14}C radioactivity and putative [^{3}H]IAA, and [^{3}H]IAInos in the shoots of *Zea mays* plants after application of 5-[^{3}H]indole-3-acetyl-*myo*-inositol-[U-^{14}C]galactose to the kernel. [^{3}H]IAA and [^{3}H] IAInos fractions were obtained by HPLC chromatography. Data for radioactivity were obtained by liquid scintillation counting and the amount of compound transported was calculated by assuming full dilution of the applied isotope by endogenous compound in the kernel.

Compound Applied to Kernel	Amount in Shoot			
	Determined by radioactivity transported to shoot		Determined by HPLC purified compound extracted from shoot	
	pmol·plant^{-1}·h^{-1}			
	^{3}H activity	^{14}C activity	[^{3}H]IAA	[^{3}H]IAInos
[^{3}H]-IAA-*myo*-inositol-[^{14}C]galactose				
Assuming dilution with endogenous IAInosgal	4	80	0.9	1.8
Assuming dilution with galactose pool in endosperm		3,070		
[^{14}C]galactose applied to kernel		360		

high percentage of ^{14}C transported represents only about 10 pmol·plant^{-1}·h^{-1} and thus would be small compared to the 92 nmol of endogenous galactose in the endosperm. However, this finding of the high proportion of ^{14}C transported to the shoot indicates that hydrolysis of [^{3}H]IAInos-[^{14}C]gal to yield free ^{14}C, must occur at some point after the [^{3}H]IAInos-[^{14}C]gal has left the endosperm but before it has entered the shoot. Hydrolysis probably does not occur in the endosperm since if it did, then IAInosgal and galactose should be equivalent sources of galactose for the shoot. Similarly, hydrolysis must occur before the shoot since no intact IAInosgal is found in the shoot. A likely site for hydrolysis is the scutellum. If this conclusion is correct than IAInos is a transport form for IAA, as has been previously concluded (1, 2, 14), and the IAInos glycosides must first be hydrolyzed before providing IAA and IAInos to the shoot.

A last precautionary statement must be made concerning the identification of the metabolic products of IAInosgal. In prior studies we have utilized multiple criteria of purity as, for example, the absence of unaccountable mass spectral ions (11, 15), or we have purified to constant specific activity (1). In the present case, owing to the number of fractions involved, we have relied upon HPLC retention times. Since the retention times of the free acid, IAA, IAInos, and, the IAInos glycosides, are in the ratio of 1:2.3 to 5.6 we believe identifications are sufficiently clear to establish the differences in transport and metabolism between IAInos and IAInosgal.

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