Enzymic synthesis of 1-*O*-indol-3-ylacetyl- β -D-glucose and indol-3-ylacetyl-*myo*-inositol

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An enzyme fraction from extracts of immature kernels of *Zea mays* catalyses the formation of 1-*O*-indol-3-ylacetyl- β -D-glucose from indol-3-ylacetic acid and UDP-glucose. A second enzyme fraction catalyses the formation of indol-3-ylacetyl-*myo*-inositol from 1-*O*-indol-3-ylacetyl- β -D-glucose and *myo*-inositol. To our knowledge, this is the first example of hydroxy-group acylation by a 1-*O*-acyl sugar. The following reaction sequence is proposed:

The enzyme catalysing reaction (1) is called UDP-glucose:indol-3-ylacetate glucosyltransferase (indol-3-ylacetylglucose synthase), and that catalysing reaction (2) is indol-3-ylacetylglucose:*myo*-inositol indol-3-ylacetyltransferase (indol-3-ylacetyl-*myo*-inositol synthase). We further show that indol-3-ylacetylglucose synthase is specific for UDP-glucose and, at the stage of purity tested, the enzyme will use either indol-3-ylacetic acid or naphthalene-1-acetic acid, but not 2,4-dichlorophenoxyacetic acid, as glucose acceptor. The indol-3-ylacetyl-*myo*-inositol synthase is specific for indol-3-ylacetylglucose and will not use naphthalene-1-acetylglucose as substrate, and it is specific for *myo*-inositol among the alcohol acceptors tested. Thus, of the auxins tested, only indol-3-ylacetic acid forms the *myo*-inositol ester.

All plants examined contained most of their indol-3-ylacetic acid (IAA-OH) as ester or amide-linked conjugates (Bandurski & Schulze, 1974, 1977). Conjugated IAA-OH serves the following functions: detoxification, since it is formed after exogenous application of excess IAA-OH (Andreae & Good, 1955; Zenk, 1961); protection, since IAA-OH conjugates are immune to peroxidative attack (Cohen & Bandurski, 1978); transport, since IAA-OH conjugates move from seed to shoot (Nowacki & Bandurski, 1980); hormonal homeostasis, since IAA-OH conjugates provide a reserve of the hormone (Bandurski *et al.*, 1977; Epstein

et al., 1980). Thus the enzymic synthesis and hydrolysis of the IAA-OH conjugates is of importance in plant hormone metabolism (Bandurski, 1979; Nowacki & Bandurski, 1980).

Kernels of *Zea mays* contain most of their IAA-OH as esters of *myo*-inositol, *myo*-inositol arabinoside and *myo*-inositol galactoside (Labarca *et al.*, 1965; Ueda & Bandurski, 1969, 1974) or as an IAA-OH ester of a cellulosic glucan (Piskornik & Bandurski, 1972). The esters are highest in concentration in the starchy endosperm of kernels of *Z. mays* (Piskornik, 1975), but are also the preponderant form of IAA-OH in vegetative tissues of *Z. mays* seedlings (Bandurski & Schulze, 1974). Acyl migration of the IAA-OH occurs readily, so that the 2-*O*-, DL-1-*O*-, DL-4-*O*- and 5-*O*-esters of *myo*-inositol with IAA-OH are all found (Ehmann & Bandurski, 1974). The arabinose or galactose residue of the IAA-*myo*-inositol glycosides is linked to the 5-*O*-position of *myo*-inositol (Ueda *et al.*,

Abbreviations used: IAA, indol-3-ylacetyl; h.p.l.c., high-pressure liquid chromatography; g.c.-m.s., gas chromatography-mass spectrometry; *m/z*, mass per unit charge.

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1970; Ueda & Bandurski, 1974). Trace amounts of the 2-*O*-, 4-*O*- and 6-*O*-esters of glucose with IAA-OH are also found (Ehmann, 1974), but the isolation conditions used would have precluded detection of the 1-*O*-ester, if present (Keglevic, 1971; the present paper).

The esters of IAA-OH are formed as the kernels ripen (Corcuera, 1967), and then hydrolysed (Ueda & Bandurski, 1969; Epstein *et al.*, 1980) or transported (Nowacki & Bandurski, 1980) as the kernels germinate. An enzyme-catalysed CoA-requiring synthesis of IAA-*myo*-inositols has previously been reported (Kopcewicz *et al.*, 1974), and the CoA-requiring reaction is distinct from the reactions studied in the present investigation. There has been a prior published report, without details, of the enzymic synthesis of 1-*O*-IAA- β -D-glucose (Zenk, 1964), and a brief report of the occurrence of these reactions has been published (Michalczuk & Bandurski, 1980).

Experimental

Materials

Zea mays (sweet corn), at the stage used for cooking and eating, was obtained from local green-grocers. [*Ac*-2-¹⁴C]IAA-OH (50 mCi/mmol) and [2-³H]*myo*-inositol (2.8 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). UDP-[6-³H]glucose (3.1 Ci/mmol), [4-¹⁴C]UTP (49.5 mCi/mmol), 2,4-dichlorophenoxy[1-¹⁴C]acetic acid (54 mCi/mmol), UDP-[U-¹⁴C]glucose (200 mCi/mmol) and naphthalene-1-[2-¹⁴C]acetic acid (15.7 mCi/mmol) were from Amersham International (Arlington Heights, IL, U.S.A.). [*ring*-2-¹⁴C]Indol-3-ylbutyric acid was supplied by Dr. J. D. Cohen (U.S. Department of Agriculture, Beltsville, MD, U.S.A.). Unlabelled ATP, GTP, CTP, UTP, UDP-galactose, UDP-glucose, ADP-glucose, CDP-glucose, GDP-glucose, reduced glutathione and *myo*-inositol were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-*O*-IAA- β -D-glucose was a gift from Dr. Dina Keglevic (Keglevic, 1971). (NH₄)₂SO₄ (enzyme grade) was from Mann Research Laboratories (New York, NY, U.S.A.). DEAE-Sephadex A-25, Sephadex G-50 and Sephadex G-150 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Chromatography and mass spectrometry

T.l.c., except as otherwise indicated, was on Silica Gel 60 (Merck-Darmstadt, from EM Laboratories, Elmsford, NY, U.S.A.), with the following solvents: *A*, ethyl acetate/butan-2-one/ethanol/water (5:3:1:1, by vol.); *B*, chloroform/methanol/water (85:14:1, by vol.); *C*, butan-1-ol saturated with water.

G.l.c. was with a Hewlett-Packard 402 apparatus and a 1.6 m $\times$ 2 mm column of 3% OV-17 on Gas-

Chrom Q (Applied Science Laboratories, State College, PA, U.S.A.), with an N₂ carrier gas flow of 60 ml/min and a flame ionization detector with the temperature programmed from 180 to 270°C. A Hewlett-Packard 5985 A quadrupole mass spectrometer was used for g.c.-m.s. to obtain 70 eV spectra and was coupled to a 1.1 m $\times$ 2 mm column of 3% OV-17 on Gas-Chrom Q.

Trimethylsilyl derivatives were prepared by treating the compound with Regisil [*NO*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane] (Regis Chemical Co., Morton Grove, IL, U.S.A.) for 15 min at 25°C.

H.p.l.c. was performed as described by Cohen (1979) and was on a PA-28 resin (sulphonated polystyrene-divinylbenzene; Beckman Instruments, Palo Alto, CA, U.S.A.) column (bed volume 0.9 cm $\times$ 17 cm) with propan-2-ol/water (1:1, v/v) as eluent, a pressure of 1 MPa (about 10 atm), a flow rate of 12 ml/h and an injection-loop volume of 0.5 ml.

Preparation of enzymes

Corn kernels (150 g) were homogenized for 1 min in 150 ml of 0.1 M-Tris/HCl buffer, pH 7.6, in a Sorvall Omni-Mixer. The homogenate was centrifuged at 10000 *g* for 10 min, and the supernatant fluid was separated into small portions and stored at -80°C for use as a crude enzyme, designated stage I preparation, or proteins were precipitated by making the solution 85% saturated with respect to (NH₄)₂SO₄. The precipitate was dissolved in 50 mM-Tris/HCl buffer, pH 7.6, dialysed for 20 h against three changes of buffer, adjusted to a total volume of 200 ml and stored in small portions at -80°C as enzyme stage II preparation.

Enzyme stage III preparation was prepared from the 40–85% satn.-(NH₄)₂SO₄-precipitable fraction. Solid (NH₄)₂SO₄ (60 g) was added to 250 ml of crude enzyme preparation and the precipitate was removed by centrifugation at 10000 *g* for 10 min. Then 92 g of solid (NH₄)₂SO₄ was added to the supernatant fluid, and the precipitated proteins were collected by centrifugation at 10000 *g* for 10 min and dissolved in 75 ml of 50 mM-Tris/HCl buffer, pH 7.6, to yield enzyme (stage III preparation). About 75 ml of enzyme stage III preparation was applied to a 5 cm $\times$ 50 cm column of Sephadex G-150, and the column was eluted with 1000 ml of 50 mM-Tris/HCl buffer, pH 7.6. The fractions eluted between 400 and 825 ml were pooled, and proteins were precipitated by making the solution 85% saturated with (NH₄)₂SO₄. The resultant precipitate was dissolved in 5 ml of 50 mM-Tris/HCl buffer, pH 7.6, and applied to a 2.5 cm $\times$ 100 cm column of Sephadex G-100. With 50 mM-Tris/HCl buffer, pH 7.6, as eluent the IAA-glucose synthase (UDP-glucose:indol-3-ylacetate glucosyltransferase) was eluted at 250–300 ml (stage

III-A preparation), whereas the IAA-*myo*-inositol synthase (indol-3-ylacetylglucose:*myo*-inositol indol-3-ylacetyltransferase) was eluted at 180–240 ml (stage III-B preparation). These fractions were stored at -80°C for as long as a month, until used.

Protein was determined by the method of Bradford (1976). Enzyme stage I preparation contained 20 mg of protein/ml, stage II preparation 13 mg/ml, and stage III preparation 47 mg/ml.

Maximum enzyme activity was obtained from corn kernels at, or slightly beyond, the maturity stage desirable for eating. Unfilled immature kernels and overly mature starchy kernels yielded little or no enzyme activity. Thus the amount of ester formed may vary by 2-fold between different enzyme preparations.

Assay of enzyme activities

Enzyme preparation (0.3 ml at the stage indicated) was incubated with substrate and cofactors in a total volume of 0.5 ml for 1 h at 37°C . The reaction was terminated by the addition of 0.5 ml of propan-2-ol; the mixture was centrifuged and the combined supernatant fluid and washings were passed through a column (1 ml bed volume) of DEAE-Sephadex (acetate form). This column served to remove unesterified anionic IAA- O^{-} . The column was washed with 2 ml of 50% (v/v) propan-2-ol, the eluate was adjusted to 5 ml, and 1 ml was used for determination of non-anionic radioactivity. To the remaining 4 ml, 100 μg of synthetic IAA-*inositol* esters was added, the mixture was concentrated to 0.2–0.3 ml and it was applied, with washings in a total volume of 0.5 ml, to a PA-28 h.p.l.c. column and eluted as described below for determination of ^{14}C -labelled IAA-glucose and IAA-*myo*-inositol.

Measurement of ^{14}C -labelled IAA-glucose and IAA-*myo*-inositol

Deproteinized samples were applied, in a 0.5 ml injection loop, to a PA-28 h.p.l.c. column and eluted with 50% (v/v) propan-2-ol. The column had been calibrated with authentic synthetic samples of 1-*O*-IAA- β -*D*-glucose (Keglevic, 1971) and IAA-*myo*-inositol (Nowacki *et al.*, 1978), and the respective elution volumes of 8–16 ml and 21–37 ml were found to be reproducible to better than ± 1 ml. Fractions of volume 1 ml were collected into scintillation vials, and, after measurement of u.v. absorption at 282 nm, 5 ml of scintillation fluid (ACS; Amersham International) was added to each vial and the radioactivity measured. The amount of ester formed was calculated by using the following equation:

$$a = b \times \frac{c}{d}$$

where a is the amount of ester formed, b is the non-anionic radioactivity passing through the DEAE-

Sephadex column, c is the radioactivity in the IAA-*myo*-inositol peak or that in the IAA-glucose peak, and d is the total radioactivity eluted from the PA-28 column. This procedure corrects for the approx. 15% loss caused by h.p.l.c. on the PA-28 column.

Formation of IAA-glucose demonstrated with ^{14}C -IAA-OH and UDP-[6- ^3H]glucose

^{14}C IAA-OH (10 $\mu\text{Ci}/\mu\text{mol}$; 0.05 μmol), UDP-[6- ^3H]glucose (20 $\mu\text{Ci}/\mu\text{mol}$; 1 μmol), reduced glutathione (5 μmol) and enzyme stage I preparation (0.3 ml) were incubated for 1 h at 37°C . The reaction was terminated with propan-2-ol, and the supernatant fluid was subjected to h.p.l.c. on a PA-28 column. The fractions obtained at 10–18 ml contained both ^{14}C and ^3H radioactivity and were combined, dried and subjected to t.l.c. on Avicel (Analtech, Wilmington, DE, U.S.A.) with solvent *C*. The cellulose layer was removed and eluted with water, and the radioactivity of the eluate was counted. One doubly labelled spot was found at R_F 0.69; this material was eluted, and the fraction was dried and dissolved in 50 μl of conc. aq. NH_3 (sp.gr. 0.880). After the mixture had been heated at 45°C in a Teflon-capped vial for 30 min, the hydrolysis products were spotted on a t.l.c. plate and developed with solvent *B* to confirm that the doubly labelled spot yielded ^{14}C -labelled IAA-OH and IAA- NH_2 and ^3H]glucose.

Formation of IAA-glucose from IAA-OH and UDP-glucose

A 1 μmol portion of IAA-OH and 20 μmol of UDP-glucose were incubated with 4 ml of enzyme stage III-A preparation or 4 ml of enzyme stage III-B preparation, as indicated, for 4 h at 37°C . The reactions were terminated with propan-2-ol, and small samples of the supernatant fluid were spotted on silica-gel t.l.c. plates and developed in solvent *A*. Incubation with enzyme stage III-A preparation yielded a product that migrated on t.l.c. identically with authentic IAA-glucose. Enzyme stage III-B preparation did not form an ester under these conditions. The putative IAA-glucose was further purified on a column (80 ml bed volume) of Sephadex LH-20, eluted with 50% propan-2-ol, and its identity was established by g.c.-m.s., as described in the Results and discussion section.

Formation of IAA-*myo*-inositol from ^{14}C IAA-OH, UDP-glucose and *myo*-inositol

The reaction mixture contained ^{14}C IAA-OH (10 $\mu\text{Ci}/\mu\text{mol}$; 0.002 μmol), UDP-glucose (1 μmol), *myo*-inositol (5 μmol) and enzyme stage III-A or III-B preparation (0.3 ml) or a mixture of the two enzyme fractions (0.15 ml each) in a total volume of 0.5 ml. The amount of esters formed was assayed by DEAE-Sephadex gel filtration and h.p.l.c.

Formation of IAA-myo-inositol from 1-O-IAA- β -D-glucose and myo-inositol

A 1 μ mol portion of synthetic authentic 1-O-IAA- β -D-glucose and 20 μ mol of *myo*-inositol were incubated with enzyme stage III-A preparation or with enzyme stage III-B preparation for 4 h at 37°C. The reactions were terminated with propan-2-ol, and samples of the supernatant fluids were subjected to t.l.c. with solvent A to check for the formation of IAA-*myo*-inositol. Only incubation mixtures containing enzyme stage III-B preparation yielded products that migrated on t.l.c. together with authentic IAA-*myo*-inositol. The products of the reaction were further purified on a Sephadex LH-20 column as described above for IAA-glucose, and their identity was confirmed by g.c.-m.s., as described in the Results and discussion section.

Determination of the pH optima for formation of IAA-glucose and of IAA-myo-inositol

Enzyme stage III-A and III-B preparations were mixed (5 ml of each) and equilibrated with 1 mM-Tris by passage of the proteins through a column (30 ml bed volume) of Sephadex G-50 suspended in 1 mM-Tris/HCl buffer, pH 7.6. Batches of 0.25 M-Tris/HCl buffer at pH 6.3, 6.7, 7.1, 7.5, 7.9, 8.3 and 8.9 were prepared and used as below in a reaction mixture consisting of (a) 0.3 ml of enzyme, 50000 d.p.m. of [14 C]IAA-OH (1 μ Ci/ μ mol), 1 μ mol of UDP-glucose and 150 μ l of one of above buffers, or (b) the same as in (a) but with the addition of 5 μ mol of *myo*-inositol. Incubation was for 15 min and was terminated with propan-2-ol, and esters were assayed by DEAE-Sephadex chromatography and PA-28 h.p.l.c. Since IAA-glucose formation was not rate-limiting, it was possible to determine pH optima for both IAA-glucose and IAA-*myo*-inositol biosynthesis.

Determination of kinetic constants

Michaelis constants were determined for IAA-OH and UDP-glucose for the IAA-glucose synthase. Determination of the K_m for the IAA-*myo*-inositol synthase was not possible owing to limited availability of 1-O-IAA- β -D-glucose.

K_m for IAA-OH. The reaction mixture consisted of UDP-glucose (5 or 10 μ mol as indicated), enzyme stage III-A preparation (0.3 ml) and [14 C]IAA-OH (1 μ Ci/ μ mol; in amounts of 0.050, 0.060, 0.083, 0.125 and 0.250 μ mol) in a total volume of 0.5 ml. The pH of the reaction mixture was 7.1 and after 5.0 min at 37°C the reaction was terminated by the addition of propan-2-ol. The amount of IAA-glucose formed was determined from the total amount of non-anionic radioactivity passing through the DEAE-Sephadex column. This simpler assay method, omitting the PA-28 h.p.l.c., was possible

since in the absence of added *myo*-inositol the only radioactive uncharged compound present was labelled IAA-glucose. Results were plotted as a double-reciprocal plot (Lineweaver & Burk, 1934; Michaelis & Menten, 1913).

K_m for UDP-glucose. The reaction mixture consisted of [14 C]IAA-OH (0.1 μ Ci/mol; 7 μ mol), enzyme stage III preparation (0.3 ml) and UDP-glucose (in increasing amounts of 0.20, 0.25, 0.33, 0.50 and 1.0 μ mol) in a total volume of 0.5 ml. The time of incubation and assay conditions were as described above.

Time course of the reaction

The reaction mixture contained [14 C]IAA-OH (0.05 μ Ci/ μ mol; 8.25 μ mol), UDP-glucose (15 μ mol), *myo*-inositol (45 μ mol), enzyme stage III-A preparation (0.3 ml) and enzyme stage III-B preparation (0.3 ml) in a total volume 1.5 ml. At 5, 10, 20, 30, 50, 70 and 120 min, 200 μ l samples were taken, each was mixed with 200 μ l of propan-2-ol and the amounts of IAA-glucose and IAA-*myo*-inositol formed in the reaction were determined as described above.

Determination of substrate specificity

Acyl specificity. A 2 μ mol portion of the desired acid, labelled with 14 C at a specific radioactivity of 0.1 μ Ci/ μ mol, was incubated with 3 μ mol of UDP-glucose and 0.2 ml each of enzyme stages III-A and III-B preparations in a total volume of 0.5 ml. Incubation was for 1 h and was terminated with propan-2-ol. The amount of esters formed was determined by DEAE-Sephadex chromatography and PA-28 h.p.l.c. as described above. Allowance was made for differing retention times due to acyl polarity.

Sugar nucleotide specificity. Nucleoside diphosphate sugar (2 μ mol) was incubated with 1 μ mol of [14 C]IAA-OH (0.1 μ Ci/ μ mol) and enzyme preparations as above. Incubation was for 10 min and was terminated with propan-2-ol. The total reaction volume was 0.5 ml. Possible ester formation was measured as non-ionic radioactivity passing through the DEAE-Sephadex column and was zero in all cases other than incubations with UDP-glucose.

Specificity for alcohol or amino acid acceptors for IAA-myo-inositol synthase. The reaction mixture contained [14 C]IAA-OH (0.1 μ Ci/ μ mol; 1 μ mol), UDP-glucose (2 μ mol), enzyme stage III-A and III-B preparations (0.2 ml of each) and 5 μ mol of the desired acyl acceptor in a total volume 0.5 ml. The *myo*-inositol galactoside and arabinoside were obtained by ammoniolysis of the naturally occurring IAA esters isolated from corn kernels and were used for the reaction as a mixture of glycosides (Ueda & Bandurski, 1974). The amount of glycosides used for the enzymic reaction was calculated

from the amount of IAA esters used for ammonio-lysis and from the efficiency of cleavage. Incubation was for 1 h and was terminated with propan-2-ol. The reaction products were determined by gel filtration through DEAE-Sephadex, PA-28 h.p.l.c. and t.l.c. with solvent A.

Results and discussion

Substitution of UDP-glucose for UTP plus Mg^{2+}

Indications of the involvement of UDP-glucose in the synthesis of both IAA-glucose and IAA-*myo*-inositol were the facts that UTP was the most active nucleoside triphosphate tested (compared with CTP, ATP and GTP) and that UDP-glucose substituted for UTP. Further, Mg^{2+} was required when UTP was utilized, but was not required in the presence of UDP-glucose (Table 1).

Reciprocal relationship between IAA-glucose and IAA-*myo*-inositol formation

Enzyme stage II preparation (0.3 ml), [^{14}C]IAA-OH (10 μ Ci/ μ mol; 0.002 μ mol) and UDP-glucose (1 μ mol) were incubated and assayed as described above. Some tubes also contained *myo*-inositol (5 μ mol). In the tubes without *myo*-inositol only IAA-glucose was formed (14000 d.p.m.), and no IAA-*myo*-inositol could be detected. When *myo*-inositol was added to the incubation mixture, most of the radioactivity (23000 d.p.m.) was found in the IAA-*myo*-inositol fractions, and only 2600 d.p.m. was in IAA-glucose. Thus there is an inverse relationship between the amounts of IAA-glucose and IAA-*myo*-inositol formed. The formation of more IAA-*myo*-inositol than IAA-glucose disappearing would be due to the energetically favourable transfer of the IAA moiety from the 1-*O*-position of glucose to a secondary hydroxy group of *myo*-inositol, thus displacing the equilibrium of the first reaction to the right.

Reaction mechanism for the formation of IAA-glucose and IAA-*myo*-inositol

Knowledge that both IAA-glucose and IAA-*myo*-

inositol were being formed, that UDP-glucose could substitute for UTP and Mg^{2+} in the reactions leading to IAA-*myo*-inositol and IAA-glucose, that the enzyme was making UDP-glucose, that the enzyme was not making UDP-*myo*-inositol or glucosyl-*myo*-inositol and that there was a reciprocal relationship between the amounts of IAA-glucose and IAA-*myo*-inositol formed indicated strongly that the reaction mechanism was:

Formation of IAA-glucose and IAA-*myo*-inositol as a function of time

The formation of esters of IAA-OH as a function of time was bimodal (Fig. 1). When total ester

Fig. 1. Bimodality of synthesis of esters of IAA-OH as a function of time

The incubation conditions were as described in the text. *Z. mays* enzyme stage III-A and stage III-B preparations were used. ▼, Total ester formation; ●, IAA-glucose formation; ■, IAA-*myo*-inositol formation. Each point is the mean $\pm$ s.d. for the replicates.

Table 1. Substitution of UDP-glucose for UTP and Mg^{2+} for the synthesis of IAA-glucose

Reaction mixtures contained: (a) *Z. mays* enzyme stage II preparation [0.3 ml (equivalent to 0.2 g of kernels, 4 mg of protein), [^{14}C]IAA-OH (10 μ Ci/ μ mol; 0.002 μ mol), ATP (4 μ mol), UTP (1 μ mol), $MgCl_2$ (5 μ mol) and *myo*-inositol (5 μ mol) in a total volume of 0.5 ml; (c) as in (a), except that 1 μ mol of UDP-glucose was substituted for ATP and UTP. Incubation was for 1 h and was terminated with 0.5 ml of propan-2-ol. Assay of esters was by DEAE-Sephadex chromatography and PA-28 h.p.l.c. and the data are the means $\pm$ s.d. for three repetitions.

Reaction mixture	Yield of IAA-glucose (d.p.m.)	Yield of IAA- <i>myo</i> -inositol (d.p.m.)
(a) IAA-OH + ATP + UTP + $MgCl_2$ + <i>myo</i> -inositol + enzyme	2650 $\pm$ 200	19 700 $\pm$ 350
(b) As in (a), but without Mg^{2+}	1300 $\pm$ 100	1600 $\pm$ 150
(c) IAA-OH + UDP-glucose + $MgCl_2$ + <i>myo</i> -inositol + enzyme	1800 $\pm$ 750	24 400 $\pm$ 500
(d) As in (c), but without $MgCl_2$	1800 $\pm$ 200	24 400 $\pm$ 950
(e) As in (c), but with boiled enzyme	Not detectable	Not detectable

Fig. 2. Separation of the *Z. mays* enzyme catalysing IAA-glucose formation from that catalysing IAA-myo-inositol formation

For experimental details see the text. The Figure shows the profile of radioactivity eluted from a PA-28 h.p.l.c. column by propan-2-ol/water (1:1, v/v) after incubation with enzyme stage III-A preparation alone (0.3 ml) (▲) and with enzyme stage III-A and stage III-B preparations (0.3 ml of each) (■). Under these conditions IAA-glucose is eluted at 8–16 ml and the IAA-myo-inositol isomers are eluted at 21–37 ml.

formation was separated into its components, IAA-glucose and IAA-myo-inositol, it could be seen that IAA-glucose was formed to a maximum amount at 20 min and then remained at nearly steady state, with only a slight decline, for almost 2 h. IAA-myo-inositol synthesis was a linear function of time for as long as the reaction was measured. This finding showed that IAA-glucose was at least potentially an intermediate in IAA-myo-inositol formation.

The rates of formation of IAA-glucose and IAA-myo-inositol were optimal at pH 7.1 and 6.9 respectively. The proximity of these maxima would permit IAA-glucose to be an intermediate in IAA-myo-inositol synthesis.

Formation of IAA-glucose

[^{14}C]IAA-OH and UDP-[^3H]glucose were incubated with enzyme. This reaction yielded a chro-

matographically homogeneous product labelled with both ^{14}C and ^3H . Ammoniolysis of this compound gave two products: one, labelled with ^3H , which migrated on t.l.c. (see the Experimental section) as D-glucose, and the second, labelled with ^{14}C , which migrated on t.l.c. as IAA-NH $_2$. Later, the experiment was repeated with unlabelled substrates and on a 500-fold-increased reaction scale. The products were purified on DEAE-Sephadex gel filtration and PA-28 h.p.l.c. The putative IAA-glucose was eluted from the PA-28 column between 9 and 16 ml. This was the same elution volume as for authentic 1-O-IAA- β -D-glucose and the same elution volume as observed for the doubly labelled compound. On t.l.c. two major Ehmann-positive spots (Ehmann, 1977), with R_F 0.57 and 0.68, and one minor spot, with R_F 0.62, were observed, and these were identical with the R_F values of authentic 4-O-IAA- β -D-glucose, authentic 2-O-IAA- β -D-glucose and authentic synthetic 1-O-IAA- β -D-glucose respectively (Keglevic, 1971; Ehmann, 1974). The pentakis(trimethylsilyl) derivatives of each of these substances yielded a molecular ion at 697 and major fragment ions at m/z 202, 217 and 361, all identical with those of an isomeric mixture of IAA-glucose esters. Thus the enzymic formation of IAA-glucose was proved.

Separation of the enzyme catalysing IAA-glucose formation from that catalysing IAA-myo-inositol formation

Incubation of [^{14}C]IAA-OH (5.0×10^3 d.p.m.; $1 \mu\text{Ci}/\mu\text{mol}$); UDP-glucose ($1 \mu\text{mol}$); myo-inositol ($5 \mu\text{mol}$) and enzyme stage III-A preparation led to the formation of $0.0082 \mu\text{mol}$ of IAA-glucose and no detectable IAA-myo-inositol (Fig. 2). Under these same conditions except for the addition of both enzyme stage III-A and III-B preparations, $0.0014 \mu\text{mol}$ of IAA-glucose and $0.0052 \mu\text{mol}$ of IAA-myo-inositol were formed. Enzyme stage III-B preparation alone formed neither IAA-glucose nor IAA-myo-inositol.

The elution volumes from Sephadex G-150 of both the IAA-glucose synthase and the IAA-myo-inositol synthase indicate that both enzymes have a molecular weight of below 100 000.

1-O-IAA- β -D-glucose as acyl donor for IAA-myo-inositol synthesis

A direct test of myo-inositol acylation by IAA-glucose was made by using 1-O-IAA- β -D-glucose as acyl donor for IAA-myo-inositol synthesis. Synthetic 1-O-IAA- β -D-glucose ($1.0 \mu\text{mol}$) was incubated with $20 \mu\text{mol}$ of inositol and 4 ml of enzyme stage III-B preparation. This resulted in the formation of $0.8 \mu\text{mol}$ of a substance with chromatographic mobilities on PA-28 h.p.l.c. and silica-gel t.l.c. identical with those of authentic synthetic IAA-myo-

Table 2. *Substrate specificity of the Z. mays enzymes IAA-glucose synthase and IAA-myo-inositol synthase*

Reaction mixtures contained: (a) 2 μ mol of the desired acid, 3 μ mol of UDP-glucose, 5 μ mol of *myo*-inositol and 0.2 ml each of enzyme stage III-A and stage III-B preparations in a total volume of 0.5 ml; (b) 2 μ mol of the indicated nucleotide, 1 μ mol of [14 C]IAA-OH (0.1 μ Ci/ μ mol) and 0.3 ml of enzyme stage III-A preparation in a total volume of 0.5 ml; (c) 1 μ mol of [14 C]IAA-OH, 1 μ mol of UDP-glucose, 5 μ mol of *myo*-inositol or aspartic acid or 15 μ mol of a mixture of galactosyl-*myo*-inositol and arabinosyl-*myo*-inositol and 0.2 ml each of enzyme stage III-A and stage III-B preparations in a total volume of 0.5 ml. Galactosyl-*myo*-inositol and arabinosyl-*myo*-inositol were obtained by ammoniolysis (15 min at 25°C) of IAA-*myo*-inositol galactoside and IAA-*myo*-inositol arabinoside that had been isolated and purified from kernels of *Zea mays* (Ueda & Bandurski, 1974). The inositol glycosides were freed of IAA-OH and IAA-NH₂ by t.l.c. (solvent A), and eluted from the plate with ethanol/water (1:1, v/v) and dried. In (a), (b) and (c) incubation was for 1 h and was terminated with propan-2-ol. In (a) assay was by DEAE-Sephadex chromatography and PA-28 h.p.l.c. In (b) the amount of IAA-glucose formed was calculated from non-ionic radioactivity after DEAE-Sephadex chromatography. In (c) all assays except that with aspartic acid were performed by DEAE-Sephadex chromatography and PA-28 h.p.l.c. In the assay testing aspartic acid as acceptor, the possible presence of IAA aspartate was checked by t.l.c. (solvent A).

Substrate	Relative activity	
	Acyl-glucose synthesis	Acyl-inositol synthesis
(a) Acyl acceptors for glucose		
IAA-OH	100	100
2,4-Dichlorophenoxyacetic acid	0	—
Indol-3-ylbutyric acid	1	0
Naphthalene-1-acetic acid	70	1
(b) Glycosyl donor for IAA-OH		
UDP-glucose	100	—
ADP-glucose	0	—
CDP-glucose	0	—
GDP-glucose	0	—
UDP-galactose	0	—
(c) Hydroxy or amine acceptor for IAA group from IAA-glucose		
<i>myo</i> -Inositol	—	100
Glycerol	—	0
Propan-2-ol	—	0
Aspartic acid	—	0
Galactosyl- <i>myo</i> -inositol	—	0
Arabinosyl- <i>myo</i> -inositol	—	0

inositol. The g.c.-m.s. analysis of the hexakis(trimethylsilyl) derivatives of the reaction products showed $M^+ = 769$ with a major fragment ions at $m/z = 202, 229, 318, 433, 507$ and 697 , all identical with those of hexakis(trimethylsilyl)-IAA-*myo*-inositol (Ehmann & Bandurski, 1974). This proved that IAA-*myo*-inositol was formed by IAA transfer from 1-*O*-IAA- β -D-glucose.

Affinity of the IAA-glucose synthetase for UDP-glucose and for IAA-OH

The K_m for UDP-glucose was 2.5 mM and V_{max} was 0.5 μ mol $\cdot$ h⁻¹, a normal K_m value for a UDP-glucose-linked enzyme. The K_m for IAA-OH was determined in the presence of 10 mM- and 20 mM-UDP-glucose with identical results. The K_m for IAA-OH was 1.08 mM and V_{max} was 0.5 μ mol $\cdot$ h⁻¹. Since the concentration of free IAA-OH in corn kernels at the stage of maturity used in the present studies would be about 0.01 mM (A. Ehmann, unpublished work), this enzyme operates *in vivo* at about 1% of its K_m . Nonetheless, the amount of

IAA-OH in a single kernel of corn would be 30 nmol, and sufficient enzyme was recovered from a single kernel to esterify 300 nmol in 1 h, so that the small amounts of free IAA-OH characteristic of mature corn kernels (Ueda & Bandurski, 1969; Epstein *et al.*, 1980) would be easily attained.

Substrate specificity of the IAA-glucose synthase and the IAA-myo-inositol synthase

Several aromatic organic acids promote plant growth and differentiation in a manner analogous to IAA-OH (cf. Thimann, 1969). Some of these were tested for their ability to cause lysis of UDP-glucose according to the general reaction:

Both IAA-OH and naphthalene-1-acetic acid were active, whereas 2,4-dichlorophenoxyacetic acid and indol-3-ylbutyric acid were not (Table 2).

Various nucleoside diphosphate sugars were tested for their susceptibility to acetolysis by IAA-OH in

the above reaction. Only UDP-glucose served as substrate; ADP-glucose, CDP-glucose, GDP-glucose and UDP-galactose were totally ineffective (Table 2).

Several alcohols and one amine were tested as acyl acceptors for the IAA group from IAA-glucose in the reactions:

Only *myo*-inositol proved to be effective, whereas glycerol, propan-2-ol, galactosyl-*myo*-inositol and arabinosyl-*myo*-inositol were ineffective alcohols and aspartic acid was an ineffective amine for forming an amide with the IAA group. The failure of the *myo*-inositol glycosides to accept the IAA group is noteworthy, since both IAA-*myo*-inositol galactoside and IAA-*myo*-inositol arabinoside are abundant in *Zea* (cf. Ueda & Bandurski, 1974). This suggests that IAA-*myo*-inositol is first formed and is then glycosylated (Corcuera *et al.*, 1982). Similarly the failure of aspartic acid to accept the IAA group from IAA-glucose, although it is a commonly occurring IAA-OH conjugate (cf. Thimann, 1969; Cohen, 1982), suggests an alternative, and as yet unknown, route for its biosynthesis.

General discussion

Agranoff & Bleasdale (1978) queried why, of the nine isomers of hexahydroxycyclohexane, only *myo*-inositol serves for phosphoinositide formation. Equally remarkable is the involvement of only *myo*-inositol in the biosynthesis of cell-wall components (cf. Loewus *et al.*, 1978) and of *myo*-inositol glycosides in glycosyl-transfer reactions, such as the biosynthesis of raffinose (Tanner, 1969), lactose (Wells & Burton, 1978) and glucan (Kemp *et al.*, 1978). In the present instance, the IAA-*myo*-inositol ester has several physiological functions (see the introduction), and the formation of IAA-*myo*-inositol glycosides portends still other functions. Of possible importance is the fact that models of IAA-*myo*-inositol may be folded to assume either a planar or a globular structure to enhance polarity or lipophilicity, and there is also the peculiar ease of acyl migration exhibited by IAA-*myo*-inositols (Labarca *et al.*, 1965; Ehmann, 1974; Ehmann & Bandurski, 1974).

Utilization of a 1-*O*-acylated sugar as an acyl donor is, to our knowledge, a unique reaction. Since publication of our prior brief report of IAA transfer from IAA-glucose to *myo*-inositol, Strack and his colleagues (Tkotz & Strack, 1980) have discovered and thoroughly studied the synthesis of 1-sinapyl-glucose and the subsequent transfer of the sinapyl moiety to L-malate. Thus this may be a more general reaction for ester synthesis.

Thermodynamically, one might expect the free energy of hydrolysis of 1-*O*-IAA- β -D-glucose to bear the same relationship to IAA-OH esterified to an alcohol as the free energy of hydrolysis of glucose 1-phosphate bears to that of glucose 6-phosphate, i.e. about 30 kJ/mol (7.2 kcal/mol) and 14 kJ/mol (3.4 kcal/mol) respectively. The enzymes used in the present study are, as yet, too impure for the study of equilibria, but an indication of the differences in bond energy between 1-*O*-IAA- β -D-glucose and IAA-*myo*-inositol is shown by the accumulation of IAA-*myo*-inositol at the expense of 1-*O*-IAA- β -D-glucose in an enzyme mixture forming both compounds.

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