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A Novel Metabolic Pathway for Indole-3-Acetic Acid in Apical Shoots of *Populus tremula* (L.) × *Populus tremuloides* (Michx.)[1]

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Metabolism of indole-3-acetic acid (IAA) in apical shoots of *Populus tremula* (L.) × *Populus tremuloides* (Michx.) was investigated by feeding a mixture of [^{12}C]IAA, [$^{13}C_6$]IAA, and [1′-^{14}C]IAA through the base of the excised stem. HPLC of methanolic plant extracts revealed eight major radiolabeled metabolites after a 24-h incubation period. Comparison between feeds with [5-^{3}H]IAA and [1′-^{14}C]IAA showed that all detectable metabolites were non-decarboxylative products. The purified radiolabeled HPLC fractions were screened by frit-fast atom bombardment liquid chromatography-mass spectrometry for compounds with characteristic fragment pairs originating from the application with ^{12}C and ^{13}C isotopes. Samples of interest were further characterized by gas chromatography-mass spectrometry. Using this procedure, oxindole-3-acetic acid (OxIAA), indole-3-acetyl-*N*-aspartic acid (IAAsp), oxindole-3-acetyl-*N*-aspartic acid (OxIAAsp), and ring-hydroxylated oxindole-3-acetic acid were all identified as IAA metabolites. Furthermore, a novel metabolic pathway from IAA via IAAsp and OxIAAsp to OxIAA was established on the basis of refeeding experiments with the different IAA metabolites.

IAA is an important plant growth regulator that in the vegetative shoot is involved in the control of many processes, such as induction and maintenance of vascular tissues, apical dominance, abscission, and responses to light and gravity (see Osborne, 1989; Steeves and Sussex, 1989; Cline, 1994). A strict control of the concentration and distribution of IAA within the plant is crucial for normal patterns of growth. Imbalances in IAA homeostasis lead to aberrant growth, which, for example, is displayed in transgenic plants overproducing IAA (Klee et al., 1987; Sitbon et al., 1992). Inactivation of IAA by catabolism and/or conjugation is an important process in the control of IAA homeostasis (see Sembdner et al., 1980; Cohen and Bandurski, 1982; Reinecke and Bandurski, 1987; Sitbon et al., 1993).

Early studies on IAA metabolism were focused mainly on in vitro peroxidase-catalyzed decarboxylative metabolism, and this pathway is now well characterized (see Sandberg et al., 1987a). However, recent in vivo metabolism experiments employing both 1′- and 2′-^{14}C-labeled IAA have demonstrated the importance of nondecarboxylative IAA metabolism in many plant tissues (e.g. Davies, 1973; Nonhebel et al., 1983, 1985b; Wiese and Grambow, 1986; Ernstsen et al., 1987; Monteiro et al., 1988). In *Zea mays*, nondecarboxylative metabolism of IAA to 7-Gluc-OxIAA via OxIAA and 7-OH-OxIAA has been established (Reinecke and Bandurski, 1981, 1983; Nonhebel and Bandurski, 1984; Nonhebel et al., 1985a; Lewer, 1987; Lewer and Bandurski, 1987). Formation of OxIAA as the major IAA metabolite and its presence as an endogenous constituent was also demonstrated in germinating *Pinus sylvestris* seeds (Ernstsen et al., 1987). In addition, OxIAA, DiOxIAA, and their 5-hydroxy derivatives have been isolated and characterized in rice bran (Kinashi et al., 1976).

The amide conjugate IAAsp is probably the most common product formed after feeds with exogenous IAA (e.g. Andreae and Good, 1955; Cohen and Bandurski, 1982), and IAAsp has also been demonstrated as an endogenous compound in several plant species (Andersson and Sandberg, 1982; Cohen, 1982; Cohen and Baldi, 1983; Cohen and Ernstsen, 1991; Nordström and Eliasson, 1991; Sitbon et al., 1993). Many IAA amide conjugates can be hydrolyzed to release free IAA. Like IAA ester conjugates in *Zea mays*, amide conjugates are considered to act as storage forms of IAA (Cohen and Bandurski, 1982; Bialek and Cohen, 1992). However, recent studies in various plant materials have demonstrated that IAAsp is the first step in IAA catabolism rather than a storage form of IAA. In *Vicia*, IAAsp is the precursor of the major IAA metabolites DiOxIAAsp and 3-Gluc-OxIAAsp (Tsurumi and Wada, 1986). In *Populus tremula*, IAAsp and OxIAAsp are the major metabolites obtained after feeding with IAA (Plüss et al., 1989). In tomato pericarp and *Dalbergia dolichopetala* seeds, IAAsp is the major IAA catabolite, which is further metabolized to various OxIAA derivatives (Monteiro

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Abbreviations: DiOxIAA, 3-hydroxy-oxindole-3-acetic acid; DiOxIAAsp, 3-hydroxy-oxindole-3-acetyl-*N*-aspartic acid; frit-FAB, frit-fast atom bombardment; 7-Gluc-OxIAA, 7-(*O*-β-D-glucosyl)-oxindole-3-acetic acid; 3-Gluc-OxIAAsp, 3-(*O*-β-D-glucosyl)-oxindole-3-acetyl-*N*-aspartic acid; HPLC-RC, HPLC-radiocounting; IAAsp, indole-3-acetyl-*N*-aspartic acid; LC-MS, liquid chromatography-mass spectrometry; Me, methyl; NBS, *N*-bromosuccinimide; 5-OH-OxIAA, 5-hydroxy-oxindole-3-acetic acid; 7-OH-OxIAA, 7-hydroxy-oxindole-3-acetic acid; OxIAA, oxindole-3-acetic acid; OxIAAsp, oxindole-3-acetyl-*N*-Asp; PVPP, polyvinylpolypyrrolidone; TMS, trimethylsilyl.

et al., 1988; Catalá et al., 1992; Östin et al., 1992b; Riov and Bangerth, 1992). Furthermore, in *Glycine max* seedlings IAAsp was metabolized to compounds other than IAA or IAA conjugates (Cohen and Baldi, 1983).

This study was undertaken to elucidate decarboxylative and nondecarboxylative metabolism of IAA and to identify metabolites after feeding with a mixture of [^{12}C]IAA, [$^{13}C_6$]IAA, and [1′-^{14}C]IAA to apical shoots of *Populus tremula* × *Populus tremuloides*. Radiolabeled HPLC fractions of the plant extract were subjected to frit-FAB LC-MS and GC-MS for the identification of IAA metabolites. IAAsp, OxIAAsp, OxIAA, and ring-hydroxylated OxIAA were identified as IAA metabolites, and a metabolic route leading from IAA to IAAsp, OxIAAsp, and OxIAA was established on the basis of refeeding experiments.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Hybrid aspen, *Populus tremula* (L.) × *Populus tremuloides* (Michx.), clone T89, was cultured in vitro on half-strength Murashige and Skoog basal medium (Sigma) as described by Nilsson et al. (1992). The rooted, approximately 10-cm-tall shoots were potted in mineral wool (Esskron, Malmö, Sweden) and transferred to a controlled environment chamber with a photon flux density of 240 μmol m^{-2} s^{-1} from Osram HQI-TS 400 W/DH metal halogen lamps, a temperature of 19/10°C (day/night), a photoperiod of 18 h, and an RH of approximately 70%. The plants were watered with a complete nutrient solution (Ingestad, 1970) containing 100 mg nitrogen/L. The use of mineral wool as a support allowed us to replace the nutrient solution daily by ample watering, thereby maintaining stable and optimal availability of nutrients. Apical shoots from 50-cm-tall trees were used for metabolism experiments. The shoots were approximately 10 cm tall and had 10 developing leaves longer than 1 cm and a weight of approximately 3 g.

Chemicals

[1′-^{14}C]IAA (2.05 GBq/mmol) and [5-^{3}H]IAA (851 GBq/mmol) were purchased from Amersham International (Buckinghamshire, UK) and [$^{13}C_6$]IAA was purchased from Cambridge Isotope Laboratories (Woburn, MA).

[$^{13}C_6$]IAAsp and [1′-^{14}C]IAAsp were synthesized according to Cohen (1981) from [$^{13}C_6$]IAA and [1′-^{14}C]IAA, respectively. The substrate conferring the Asp moiety (Asp-bis-*t*-butyl ester HCl) was in the L conformation. The conformation of the resulting IAAsp isotopes was not determined.

[1′-^{14}C]OxIAA was synthesized by the method of Hinman and Bauman (1964) with the following modifications. NBS dissolved in *t*-butanol was added to [1′-^{14}C]IAA (2.05 GBq/mmol) dissolved in *t*-butanol in a molar ratio of 1:2 (NBS:IAA). The reaction mixture was concentrated to 20 μL, placed on a TLC plate (Kieselgel 60 F254, 10 × 5 cm; Merck, Darmstadt, Germany) and eluted with butanon:ethyl acetate:ethanol:water (3:5:1:1, v/v) (Ehman, 1977). The fraction corresponding to the retention time of OxIAA was collected.

A mixture of [^{12}C]OxIAAsp, [$^{13}C_6$]OxIAAsp, and [1′-$^{14}C_1$]-OxIAAsp was obtained after feeding [^{12}C]IAA, [$^{13}C_6$]IAA, and [1′-$^{14}C_1$]IAA to hybrid aspen shoots (see "Results" for purification and identification of metabolite number 2).

The identity and purity of the synthesized substrates was confirmed by HPLC and MS. The OxIAAsp standard contained two diastereomers from which identical LC-MS spectra were obtained. The two diastereomers are formed due to epimerization at C-3 in the indole ring (see Plüss et al., 1989).

Feeding Conditions, Extraction, and Purification

Metabolism of IAA

Metabolism of IAA was investigated by applying a mixture of 10 μg of [^{12}C]IAA, 10 μg of [$^{13}C_6$]IAA, and 1.2 μg of [1′-^{14}C]IAA to each of 10 apical shoots. Each shoot was placed in 200 μL of aqueous solution containing the IAA mixture. After approximately 1 h, when the solution had been absorbed into the transpiration stream, the shoots were transferred to water for a further 23 h. After this period they were frozen in liquid N_2 and stored at −70°C.

The plant material was homogenized with an Ultra Turrax in 100 mL of 80% methanol containing 0.02% of the antioxidant diethyldithiocarbamate. The methanolic homogenate was stirred for 1 h at 4°C before being filtered through cellulose powder (CAMAG, Muttenz, Switzerland). The methanolic extract was then reduced to the aqueous phase at 40°C under reduced pressure in a rotary evaporator. The sample was buffered with 0.05 M phosphate buffer, pH 7.0, slurried with insoluble PVPP (0.3 g/g fresh weight), and filtered. The filtrate was adjusted to pH 2.7 with 1 M HCl and passed through a 10-g Bond Elute C_{18} cartridge (Varian, Harbor City, CA), which was eluted stepwise in 10% increments with 15-mL volumes of 10 to 100% methanol in 1% aqueous acetic acid. Fractions containing radioactivity were combined and reduced to the aqueous phase in vacuo. Phosphate buffer (0.5 M, pH 7) was added to the aqueous residue to give a final phosphate buffer concentration of 0.05 M and a total volume of 50 mL. The sample was then partitioned twice against 25 mL of diethyl ether. The aqueous phase was adjusted to pH 2.7 with 1 M HCl and partitioned three times against 10 mL of diethyl ether. The aqueous phase was then lyophilized and the acidic ether extracts were combined and reduced to dryness under a stream of N_2. The aqueous phase and the acidic ether phase were each dissolved in 10% methanol in 1% aqueous acetic acid and divided into 15 and 7 200-μL subsamples, respectively, each of which was subjected to gradient elution, ion-suppression HPLC. Appropriate radiolabeled fractions, as specified in "Results," were collected and corresponding fractions from the different subsamples were pooled. Radiolabeled fractions from the aqueous phase were further purified by isocratic, ion-suppression HPLC, before and after methylation with ethereal diazomethane (Schlenk and Gellerman, 1960). Radiolabeled HPLC fractions collected from the acidic ether phase were methylated. Homogeneity of the radiolabeled peaks in all fractions was investigated by subjecting unmethylated sample aliquots to gradient elution, ion-pair HPLC. The

purified and methylated fractions were subjected to MS for identification of IAA metabolites.

Metabolism of IAAsp, OxIAAsp, OxIAA, and [5-^{3}H]IAA

To investigate the metabolism of (a) IAAsp, (b) OxIAAsp, and (c) OxIAA and (d) to detect any decarboxylative IAA metabolism, the following substrates were applied to single apical shoots: (a) a mixture of 2 μg of [$^{13}C_6$]IAAsp and 314 ng (220,000 dpm) of [1′-^{14}C]IAAsp; (b) a mixture of 2.3 μg of [^{12}C]OxIAAsp, 2.3 μg of [$^{13}C_6$]OxIAAsp, and 271 ng (190,000 dpm) of [1′-^{14}C]OxIAAsp; (c) 286 ng (205,000 dpm) of [1′-^{14}C]OxIAA; and (d) a mixture of 10 μg of [^{12}C]IAA, 10 μg of [$^{13}C_6$]IAA, and [5-^{3}H]IAA (1.1×10^6 dpm). The feeding conditions were as described above. Due to the smaller sample size the purification procedure in these experiments was modified as follows. The tissue was homogenized in liquid N_2 with a mortar and pestle and extracted in 10 mL of 80% methanol. After slurrying with PVPP the extract was passed through a 3-g C_{18} Bond Elute cartridge and eluted with 3 mL of 80% methanol. The eluate was reduced to dryness in a Speed-Vac concentrator and dissolved in an appropriate HPLC mobile phase. A sample aliquot was analyzed on gradient elution, ion-pair HPLC and the remainder was analyzed by gradient elution, ion-suppression HPLC. Radiolabeled fractions from the application of IAAsp and OxIAAsp were collected and methylated. Methylated OxIAAsp metabolite fractions were additionally purified by isocratic ion-suppression HPLC before MS analysis. All the different experiments were repeated at least three times.

In control experiments, breakdown during purification and HPLC analysis was investigated by adding 5×10^5 dpm [1′-^{14}C]IAA or 2×10^5 dpm [1′-^{14}C]IAAsp to methanolic plant extracts that were treated as described above. To investigate breakdown during methylation and repeated dryings, standard solutions of IAA, IAAsp, and OxIAA were methylated with diazomethane, dissolved in HPLC solvent, repeatedly dried in a Speed-Vac concentrator, and subjected to LC-MS.

HPLC

The Waters HPLC (Millipore, Milford, MA) consisted of a M680 gradient controller and two M510 pumps. Samples were introduced by a Waters 712 WISP onto a 10 cm × 8 mm i.d. 4-μm Nova-Pak C_{18} cartridge fitted in a Waters RCM 8 × 10 module. The solvent was delivered at a flow rate of 1 mL min^{-1}, either as a 10 to 50% methanol gradient over 28 min or isocratically with an appropriate methanol concentration for optimal separation conditions. Ion-suppression reversed-phase HPLC utilized a mobile phase in 1% acetic acid. For ion-pair reversed-phase HPLC, the mobile phase was buffered with 0.05 M phosphate buffer, pH 7, containing 0.02 M tetrabutylammonium hydrogen sulfate as a counterion (Sandberg et al., 1981). Column eluate was directed to a radioactivity monitor (Reeve Analytical Ltd., Glasgow, Scotland); model 9701 with a 200-μL heterogeneous flow cell packed with cerium-activated lithium glass scintillator (counting efficiency 30–40%) or model 1208 Betacord LKB-Wallac with a homogeneous 500-μL flow cell (counting efficiency 10–15%) for analysis of tritiated metabolites (see Sandberg et al., 1987b).

MS

Capillary HPLC-MS

The capillary HPLC-MS system used for identification of the metabolites has been described in detail elsewhere (Östin et al., 1992b). The liquid chromatograph consisted of a M680 gradient controller and two M510 pumps with micro pump heads (Waters) delivering a mobile phase containing 1% glycerol as a matrix, at a flow rate of 300 μL min^{-1}. To obtain a flow rate of approximately 5 μL min^{-1} through the analytical capillary column, a preinjection split was generated by diverting most of the solvent, via a tee (Valco, Houston, TX), through a 120 × 2.1 mm i.d. HPLC Nucleosil C_{18} 3-μm balance column (Macherey-Nagel, Düren, Germany). The other outlet of the tee was linked to a Rheodyne (Cotati, CA) 7520 injection valve with a 500-nL loop, which was coupled directly to a 300 × 0.32 mm i.d. capillary HPLC column (Fusica-C packed with C_{18}, 5 μm; LC Packings, Amsterdam, The Netherlands). The mobile phase gradient was 0 to 8 min, 30% methanol in 1% acetic acid; 8 to 40 min, 30 to 80% methanol in 1% acetic acid. The eluate from the capillary column was directed via fused silica capillary tubing (1 m × 50 μm i.d.) and a frit-FAB LC-MS interface (JEOL, Tokyo, Japan) to the ion source of a double-focusing JEOL JMS-SX102 mass spectrometer. The ion source temperature was 50°C, and ions were generated with a beam of 5-kV xenon atoms at an emission current of 20 mA. The acceleration voltage was 8 kV. Positive ion mass spectra were obtained at a rate of 5.0 s per scan for a mass range of m/z 10 to 2000. All spectra were background subtracted.

GC-MS

GC-MS was performed using a Hewlett-Packard 5890 gas chromatograph linked via a direct inlet to the JEOL JMS-SX102 mass spectrometer. The dry samples were dissolved in 25 μL of acetonitrile and silylated by adding 25 μL of bis(trimethylsilyl)trifluoroacetamide with 1% (v/v) methylchlorosilane (Pierce, Rockford, IL). The mixture was heated in a sealed vial at 70°C for 15 min, reduced to dryness, and redissolved in heptane. Samples were injected splitless at 280°C onto a 25 m × 0.25 mm i.d. methylsilicone fused silica column with a film thickness of 0.25 μm (Quadrex Co., New Haven, CT). The carrier gas was helium. The column temperature was programmed at 20°C min^{-1} from 60 to 200°C and at 4°C min^{-1} from 200 to 250°C. For analysis of OxIAAsp-Me_2-TMS_2 the temperature was programmed at 20°C min^{-1} from 60 to 260°C and held at 260°C until elution of the sample. The interface and the ion source temperatures were 280 and 250°C, respectively. Ions were generated with 70 eV at an ionization current of 300 μA. The acceleration voltage was 10 kV. Positive ion mass spectra were obtained at a rate of 1.0 s per scan for a mass range of m/z 50 to 800. The spectra were background subtracted. All mass spectrometric data were processed by a JEOL MD-7000 data system.

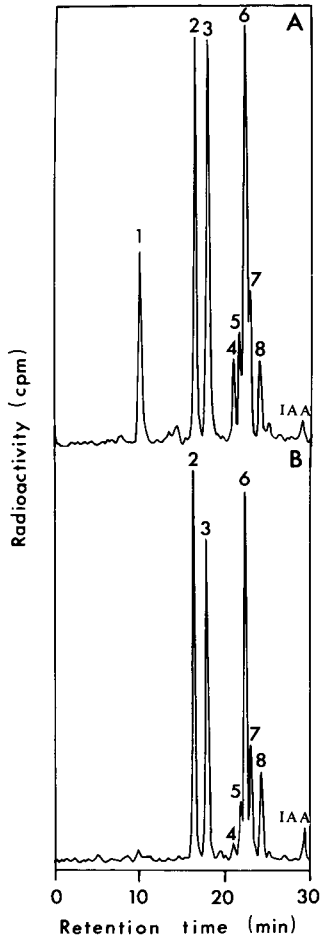

Figure 1. Gradient-elution, ion-suppression reversed-phase HPLC-RC of a crude methanolic extract of *P. tremula* × *P. tremuloides* shoots after feeding with [1′-$^{14}C_1$]IAA (A) and [5-^{3}H]IAA (B). The major metabolites are numbered from 1 to 8, and the peak corresponding to IAA is indicated.

RESULTS

Metabolism of IAA

IAA metabolism in apical shoots of hybrid aspen was investigated by feeding a mixture of 10 μg of [^{12}C]IAA, 10 μg of [$^{13}C_6$]IAA, and 1.2 μg (8 × 10^5 dpm) of [1′-$^{14}C_1$]IAA through the cut base of the stem to each of 10 apical shoots. After 24 h most of the IAA was metabolized and eight major peaks were detected after gradient elution, ion-suppression HPLC of the semi-purified methanolic extract (Fig. 1A). In feeding experiments where [1′-^{14}C]IAA was replaced with [5-^{3}H]IAA, no additional peaks were detected, indicating that the observed metabolism of IAA was not a result of decarboxylation (Fig. 1B). Instead, the most polar metabolite (metabolite 1) disappeared, suggesting that the tritium at the C-5 position was lost as a consequence of the metabolism.

After ether partitioning of the semi-purified extract, 17% of the radioactivity was in the acidic ether phase and the remaining 83% was in the aqueous phase. The neutral ether phase did not contain significant amounts of radioactivity.

Eight major radiolabeled peaks could still be detected in the aqueous phase after gradient elution, ion-suppression HPLC (Fig. 2A), and four fractions were collected that corresponded to metabolites number 1, 2, 3, and 4 to 8. The fraction containing metabolites 4 to 8 was further divided into three fractions containing metabolite 4, metabolites 5 to 7, and metabolite 8 after isocratic HPLC.

Aliquots of the purified fractions were first screened by LC-MS for substances giving a mass spectrum with characteristic fragment pairs six mass units apart, originating from the feeding with the [^{12}C]IAA and [$^{13}C_6$]IAA isotopes. After LC-MS, each fraction was further analyzed by GC-MS with a suitable derivatization and GC programming. With this technique it is possible to identify metabolites that would

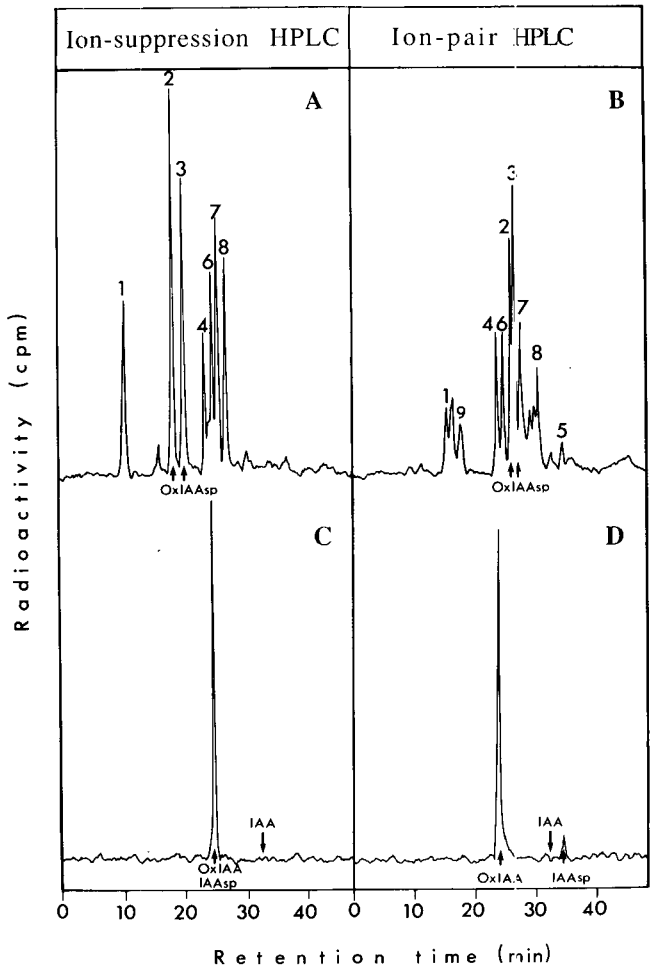

Figure 2. Gradient-elution, ion-suppression and ion-pair reversed-phase HPLC-RC of the aqueous phase (A and B) and acidic ether phase (C and D) of the methanolic extract of *P. tremula* × *P. tremuloides* shoots after feeding with [^{12}C]IAA, [$^{13}C_6$]IAA, and [1′-$^{14}C_1$]IAA. The numbering of the metabolites is as in Figure 1. The retention times of authentic OxIAAsp, IAAsp, OxIAA, and IAA are indicated in corresponding phases.

otherwise escape detection by direct GC-MS analysis because of thermolability and/or unsuitable chromatographic properties.

Metabolites 2 and 3

The radiolabeled compounds in the two fractions containing metabolites 2 and 3 had the same retention times as the synthetic OxIAAsp diastereomers in both ion-suppression and ion-pair HPLC (Fig. 2, A and B). During purification of the metabolites in fractions 2 and 3, spontaneous conversion between the two OxIAAsp stereomers was observed. One additional minor radiolabeled substance, denoted metabolite 9, was detected after both ion-pair and isocratic ion-suppression HPLC of methylated fraction number 3. LC-MS demonstrated the presence of OxIAAsp-Me_2 in both fraction 2 and 3 with a protonated molecular ion pair at m/z 335/341 (Fig. 3A). Loss of the bismethylated Asp moiety, characterized by m/z 162 and m/z 102, resulted in the formation of an oxonium ion pair (m/z 174/180). β-Cleavage of the side chain resulted in the formation of a quinolonium ion pair (m/z 146/152). The entire side chain reveals a fragment at m/z 204. The identity of metabolites 2 and 3 was further confirmed by GC-MS, which yielded a spectrum corresponding to OxIAAsp-Me_2-TMS_2 (Fig. 3B), with a molecular ion pair at m/z 478/484 and a quinolonium-TMS_2 ion pair at m/z 290/296.

Metabolites 5 and 6

The retention times of metabolites 5 and 6 corresponded to authentic IAAsp and OxIAA, respectively, on gradient elution, ion-suppression HPLC (Fig. 2). Ion-pair HPLC of the fraction containing metabolites 5 to 7 showed two metabolites corresponding to the retention times of OxIAA and IAAsp, whereas isocratic ion-suppression HPLC of the methylated fraction revealed four radiolabeled metabolites (data not shown). A cluster of three metabolites, one having a retention time identical to that of OxIAA, was collected as one sample, denoted A. The other sample, denoted B, consisted of the fourth metabolite, with a retention time identical to that of IAAsp.

In sample A, two spectra with the characteristic ion pairs six mass units apart were detected after LC-MS. One spectrum revealed the presence of OxIAA-Me with a molecular ion pair at m/z 206/212 (Fig. 3C). Fragmentation resulted in the formation of oxonium (m/z 174/180) and quinolonium (m/z 146/152) ion pairs. The identity of OxIAA was further confirmed as OxIAA-Me-TMS_2 by a GC-MS spectrum, with a molecular ion pair at m/z 349/355 and a diagnostic quinolonium-TMS_2 ion pair at m/z 290/296 (Fig. 3D).

A further LC-MS spectrum obtained of sample A, with the characteristic ion pairs, corresponded to a methylated OxIAA derivative with hydroxylation in the benzene ring giving a molecular ion pair at m/z 222/228 (Fig. 3E). Loss of a hydroxyl group as water is indicated by m/z 204/210. Fragment pairs at m/z 146/152 and m/z 162/168 correspond to quinolonium ion and its hydroxylated form, respectively. GC-MS analysis revealed two spectra with characteristic fragment pairs corresponding to hydroxylated OxIAA-Me-TMS and hydroxylated OxIAA-Me-TMS_2. The hydroxy-OxIAA-Me-TMS_2 spectrum shows the molecular ion pair at m/z 365/371 and the base peak at m/z 292/298 (Fig. 3F). The observed masses of hydroxy-OxIAA-Me-TMS (m/z 293/299, m/z 278/284, 250/256, m/z 234/240, m/z 220/226, m/z 218/224, m/z 204/210, m/z 172/178, m/z 89, m/z 75, and m/z 73) are identical to those of the methylated and silylated DiOxIAA described by Horng and Yang (1975), but are different from the available frit-FAB fragmentation pattern of DiOxIAAsp-Me_2 and 7-OH-OxIAA-Me, indicating that the hydroxyl group is not located at the C-3 or C-7 position of the benzene ring but rather at the C-4, C-5, or C-6 position.

LC-MS analysis of sample B from the fraction corresponding to metabolites 5 to 7 produced a spectrum with a molecular ion pair at 319/325, indicating the presence of IAAsp-Me_2 (Fig. 3G). The quinolinium ion is represented by m/z 130/136, the bis-methylated Asp moiety by m/z 162, and the methylated immonium ion by m/z 102. The identity of IAAsp was further confirmed by GC-MS of IAAsp-Me_2-TMS, which yielded a spectrum with a molecular ion pair at m/z 390/396 and a quinolinium-TMS ion pair at m/z 202/208 (Fig. 3H).

Metabolites 1, 4, and 8

Fractions corresponding to metabolites 1, 4, and 8 each contained one radiolabeled substance, as indicated by the detection of a single peak after ion-pair HPLC and isocratic, ion-suppression HPLC of the methylated sample (data not shown). However, no spectrum with characteristic ion pairs 6 mass units apart was obtained by LC-MS and GC-MS analysis of these fractions.

Analysis of the Acidic Ether Phase

Gradient elution, ion-suppression HPLC revealed the presence of one radiolabeled metabolite in the acidic ether phase (Fig. 2C). Gradient elution, ion-pair HPLC resolved one major and one minor metabolite, with retention times corresponding to OxIAA and IAAsp, respectively (Fig. 2D). The identity of these metabolites was confirmed by GC-MS analysis of the methylated and silylated derivatives. In addition to OxIAA and IAAsp, a considerable amount of ring-hydroxylated OxIAA was detected in this fraction by GC-MS.

Metabolism of IAAsp, OxIAAsp, and OxIAA

Metabolism of IAAsp was investigated by feeding experiments using a mixture of [^{13}C]IAAsp and [1'-^{14}C]IAAsp. According to the gradient elution, ion-suppression HPLC, IAAsp (metabolite 5) was metabolized to three major products with the same retention behavior as IAA metabolites 2, 3, and 6 (Fig. 4, A and B). The identity of metabolites 2 and 3 was confirmed as OxIAAsp by LC-MS, and GC-MS identified metabolite 6 as OxIAA. GC-MS analysis of a fraction containing metabolites 5 and 6 demonstrated also the presence of unmetabolized IAAsp and trace amounts of ring-hydroxylated OxIAA. Three minor catabolites corresponding to IAA metabolites 1, 8, and 9 were also detected by HPLC (Fig. 4, A and B).

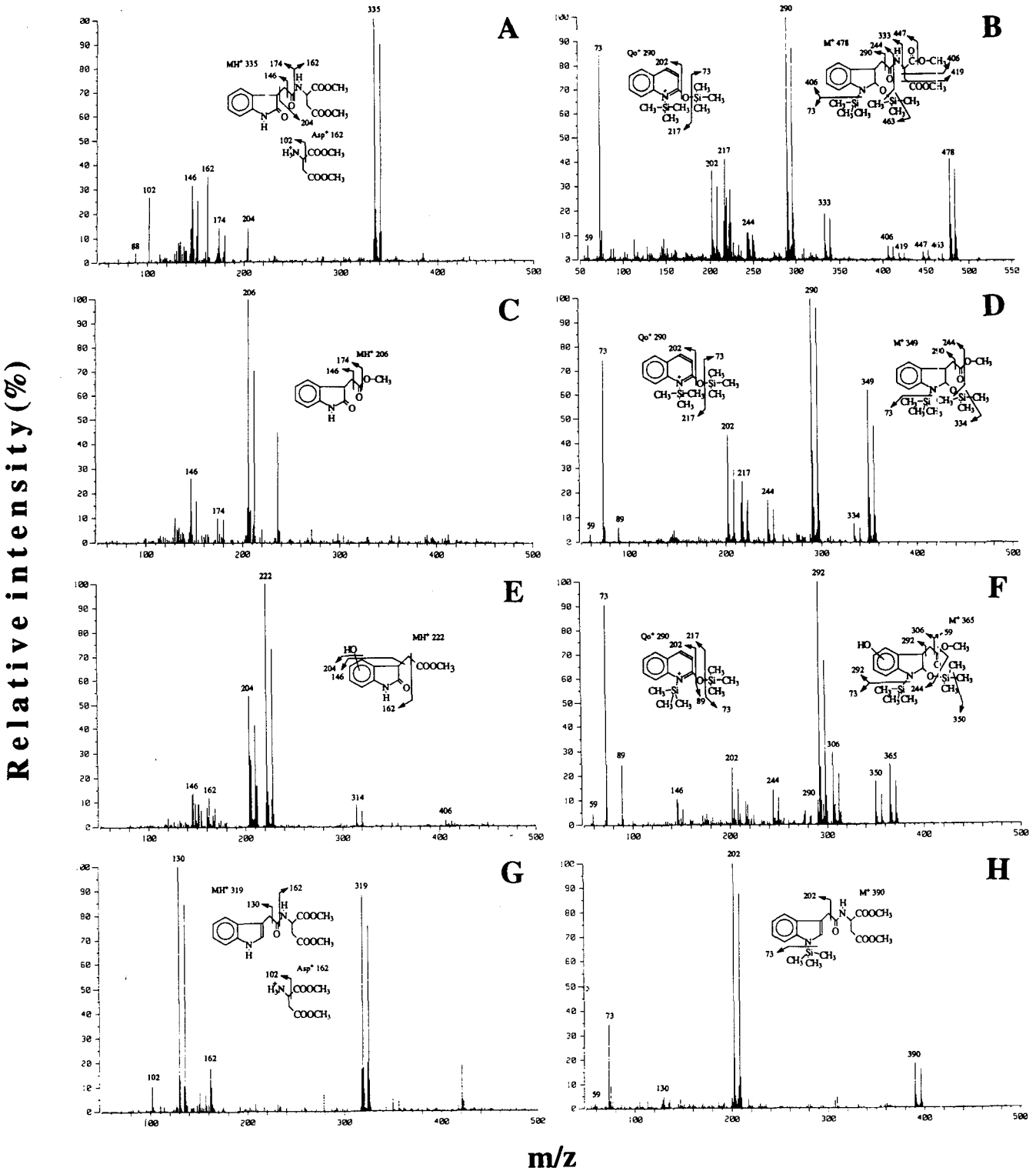

Figure 3. Mass spectra of the IAA metabolites identified in apical shoots of *P. tremula* × *P. tremuloides*. Frit-FAB LC-*MS* spectra of the methylated derivatives of OxIAAsp (A), OxIAA (C), ring-hydroxylated OxIAA (E), and IAAsp (G). GC-*MS* spectra of the methylated and silylated derivatives of OxIAAsp (B), OxIAA (D), ring-hydroxylated OxIAA (F), and IAAsp (H).

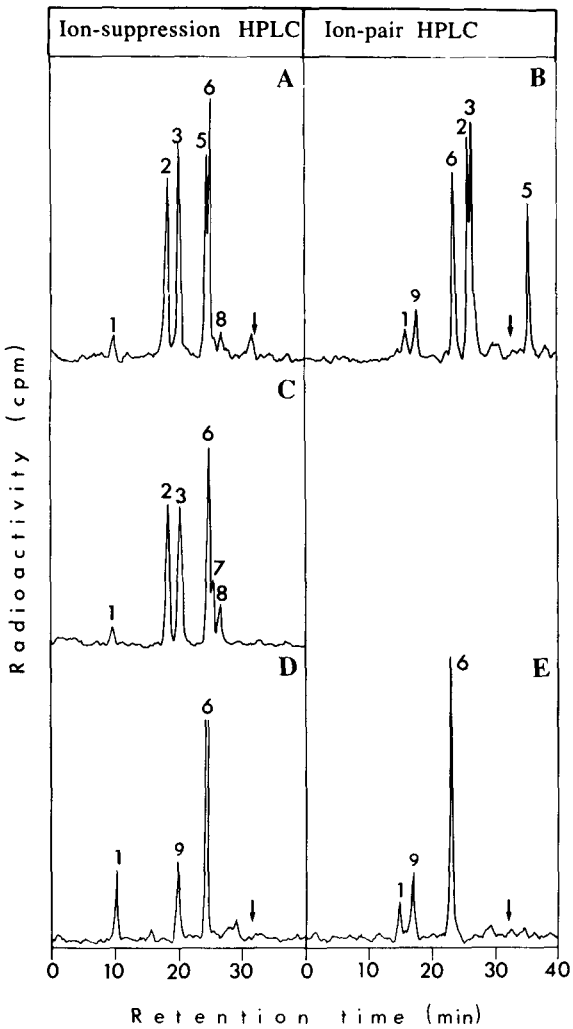

Figure 4. Gradient-elution, ion-suppression and ion-pair reversed-phase HPLC-RC of a crude methanolic extract of *P. tremula* × *P. tremuloides* after feeding with a mixture of [^{13}C]IAAsp and [1′-^{14}C]-IAAsp (A and B), a mixture of [^{12}C]OxIAAsp, [$^{13}C_6$]OxIAAsp, and [1′-^{14}C]OxIAAsp (C), or with [1′-^{14}C]OxIAA (D and E). The numbering of the metabolites is as in Figure 1. The retention time of authentic IAA is indicated by the arrows.

Purified IAA metabolite 2, identified as OxIAAsp, was applied to apical shoots to study the metabolism of OxIAAsp. Gradient-elution, ion-suppression HPLC of the methanolic shoot extract revealed the epimerization of the applied OxIAAsp to two different diastereomers (Fig. 4C, peaks 2 and 3). The major metabolite, peak 6, showed the characteristic GC-MS fragmentation pattern of OxIAA. In the OxIAA fraction trace amounts of ring-hydroxylated OxIAA were also detected by GC-MS. Three minor catabolites corresponding to IAA metabolites 1, 7, and 8 were also detected by HPLC (Fig. 4C).

Feeding experiments with [1′-^{14}C]OxIAA showed that the metabolism of this substance (metabolite 6) was slow. Only two minor compounds, with the same retention time as the IAA metabolites 1 and 9, were detected after gradient-elution, ion-suppression and ion-pair HPLC (Fig. 4, D and E). Neither metabolite was identified by GC-MS or LC-MS.

In control experiments, when either [1′-^{14}C]IAA or [1′-^{14}C]IAAsp was added to a methanolic plant extract, no significant breakdown of either substance was detected by HPLC. Furthermore, no spontaneous conversion of standard solutions of IAA, OxIAA, and IAAsp was observed by LC-MS after methylation and repeated reduction to dryness in a Speed-Vac concentrator. However, long storage of an OxIAA-Me standard in acidic solution resulted in the formation of a hydroxylated OxIAA with a GC-MS spectrum identical to that of the ring-hydroxylated OxIAA metabolite that was identified in this study.

DISCUSSION

Reversed-phase HPLC showed that labeled IAA was metabolized to a number of polar metabolites in the apical shoot of hybrid aspen (Figs. 1 and 2). Feeding experiments with [1′-^{14}C]IAA and [5-^{3}H]IAA indicated that the 1′-carboxyl group was retained by all the major IAA metabolites. The observed metabolism was thus not a consequence of decarboxylative degradation (Fig. 1). Identification of the metabolites was facilitated by including equal amounts of [^{12}C]IAA and [$^{13}C_6$]IAA in the substrate, so that the resulting metabolites could be recognized by having a mass spectrum with characteristic m/z and m/z + 6 fragment pairs. Screening for such spectra was initially done by LC-MS, followed by GC-MS after appropriate derivatization and temperature programming. With this strategy it was possible to identify IAAsp, OxIAAsp, OxIAA, and ring-hydroxylated OxIAA as IAA metabolites (Fig. 3).

Both IAAsp and OxIAA can be considered as primary metabolites of IAA. Refeeding experiments with IAAsp resulted in the formation of OxIAAsp and OxIAA as the major products without any sign of free IAA (Fig. 4, A and B), indicating that IAAsp was first oxidized to OxIAAsp and then hydrolyzed to OxIAA. Further evidence for this metabolism was obtained by feeding with OxIAAsp that was converted mainly to OxIAA (Fig. 4C). When OxIAA was applied to the *Populus* shoot it was slowly metabolized to two minor products, metabolites 1 and 9 (Fig. 4, D and E), that were also present after feeds with IAA, IAAsp, and OxIAAsp (Figs. 2B and 4). The identities of metabolites 1 and 9 could not be established, but the disappearance of the radioactivity in metabolite 1 after the incubation with [5-^{3}H]IAA (Fig. 1B) is suggestive of a hydroxylation of the benzene ring at the C-5 position (Daly and Witkop, 1967), or alternatively at the C-4 or C-6 position (Guroff et al., 1967). A similar loss of C-5 tritium occurs when [5-^{3}H]7-Gluc-OxIAA is metabolized by *Zea mays* (Lewer and Bandurski, 1987). Other fractions with radiolabel in which no MS spectra with characteristic fragment pairs could be detected included metabolites 4, 7, and 8. Metabolite 4 was detected only after feeding with IAA, suggesting that it was not a part of the IAAsp or OxIAA metabolism (Fig. 4). Metabolites 7 and 8 were detected both after IAAsp and OxIAAsp feedings, but not after application of OxIAA, suggesting that they are products from OxIAAsp

(Fig. 4). Taken together, the results indicate a novel metabolic route from IAA via IAAsp and OxIAAsp to OxIAA (Fig. 5). However, direct formation of OxIAA from IAA cannot be excluded.

Ring-hydroxylated OxIAA was identified in both the acidic ether phase and the aqueous phase after feeds with IAA, IAAsp, and OxIAAsp. It was always found in the HPLC fractions containing OxIAA, which is unexpected considering the presumed difference in the polarity of these compounds on reversed-phase HPLC. Furthermore, in the acidic ether phase obtained after IAA application, the abundance of ring-hydroxylated OxIAA detected by GC-MS was relatively high, whereas, in marked contrast, it was not observed when the sample had been analyzed earlier by HPLC (Fig. 2, C and D, and data not shown). These facts, together with a previous report on the breakdown of OxIAA to DiOxIAA (Lewer and Bandurski, 1987), indicate that spontaneous hydroxylation of OxIAA is occurring after HPLC. We also observed nonenzymatic hydroxylation of methylated OxIAA after a long period of storage in acidic solution. Our control experiments did not, however, show any spontaneous conversion, and hence it cannot be excluded that the detected ring-hydroxylated OxIAA originated from enzymatic conversion.

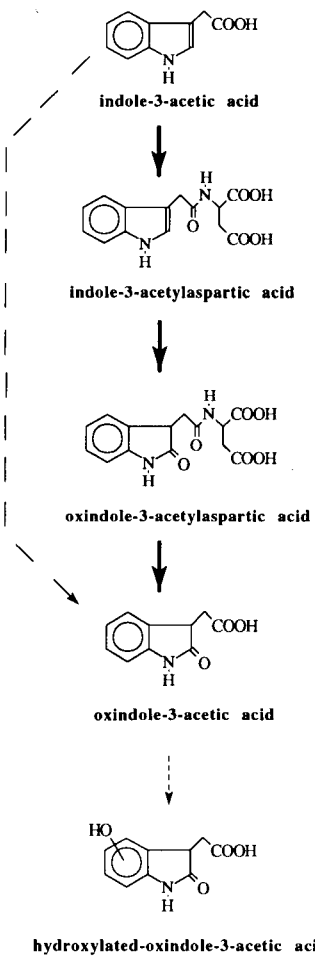

Figure 5. Proposed metabolic pathway for IAA in the apical shoots of *P. tremula* × *P. tremuloides*. The solid arrows indicate the catabolic pathway established by the refeeding studies, and the dashed arrows indicate suggested catabolic events.

Our observation that IAAsp is metabolized by oxidation, rather than by hydrolysis to release free IAA, agrees with findings in other species where formation of IAAsp is the first step in the irreversible deactivation pathway of IAA metabolism (Cohen and Baldi, 1983; Tsurumi and Wada, 1986; Monteiro et al., 1988; Catalá et al., 1992; Riov and Bangerth, 1992; Nilsson et al., 1993). Thus, although amide conjugates seem to have a role as IAA storage compounds during seed germination (Sandberg et al., 1987b; Bialek and Cohen, 1992) and to have biological activity in many auxin bioassays, a general role for amide conjugates in maintaining IAA homeostasis by reversible hydrolysis might be an oversimplification. In the case of IAAsp, its biological activity in different auxin bioassays is variable, indicating that its hydrolysis to free IAA is species and tissue specific (see Feung et al., 1977; Hangarter et al., 1980; Bialek et al., 1983). However, conclusive evidence for formation of more than trace amounts of free IAA from IAAsp is still wanting.

It has been observed repeatedly that IAAsp accumulates when increasing concentrations of IAA are applied to plant tissues, suggesting that further metabolism of IAAsp is a rate-limiting step in IAA catabolism (Tsurumi and Wada, 1980; Catalá et al., 1992; Riov and Bangerth, 1992). Accumulation of IAAsp could also be a result of an induction of acylaspartate synthetase by IAA (Venis, 1972), and it has been argued that formation of IAAsp results from applications with unphysiologically high IAA concentrations and is therefore of limited importance in intact tissues. On the other hand there are several identifications of endogenous IAAsp, which imply that the conversion of IAA to IAAsp is of physiological relevance in vivo (Andersson and Sandberg, 1982; Cohen, 1982; Cohen and Baldi, 1983; Cohen and Ernstsen, 1991; Nordström and Eliasson, 1991; Sitbon et al., 1993).

The metabolic route from IAA to OxIAAsp via IAAsp observed in our study agrees with results from *P. tremula* by Plüss et al. (1989). They did not, however, observe further metabolism of OxIAAsp to OxIAA, which may be due to the use of slightly different experimental systems. Our finding that OxIAA is involved in IAAsp metabolism differs also from results of other studies on IAAsp metabolism in which the oxidation of IAAsp at the C-2 position is usually followed by hydroxylations and glucosylations (Tsurumi and Wada, 1986; Riov and Bangerth, 1992; Östin et al., 1992a). Formation of OxIAA has previously been demonstrated only by direct oxidation of IAA (Reinecke and Bandurski, 1981; Ernstsen et al., 1987), and the identifications of OxIAA have been confined to seed material or immature tissues (Reinecke and Bandurski, 1983; Ernstsen et al., 1987; Lewer, 1987).

The different pathways of IAA degradation that have been observed suggest a differentially regulated metabolism in different species as well as in different tissues and developmental stages of a plant. Future studies involving the endogenous occurrence and localization of the IAA metabolites identified in the *Populus* shoot are necessary to establish the physiological relevance of the observed metabolic pathway.

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