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Metabolism of Indole-3-Acetic Acid by Pericarp Discs from Immature and Mature Tomato (*Lycopersicon esculentum* Mill.)[1]

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ABSTRACT

[1′-^{14}C,$^{13}C_6$]Indole-3-acetic acid was infiltrated into immature pericarp discs from fruits of tomato (*Lycopersicon esculentum* Mill., cv Moneymaker). After a 24-h incubation period the discs were extracted with methanol and the partially purified extract was analyzed by reversed-phase high-performance liquid chromatography-radiocounting. Five metabolite peaks (1–5) were detected and subsequently analyzed by combined high-performance liquid chromatography-frit-fast atom bombardment-mass spectrometry. The metabolite 4 fraction was found to contain [$^{13}C_6$]-indole-3-acetylaspartic acid, and analysis of metabolite 5 identified [$^{13}C_6$]indole-3-acetyl-β-D-glucose. The other metabolites could not be identified, but alkaline hydrolysis studies and gel permeation chromatography indicated that metabolites 1 and 3 were both amide conjugates with a molecular weight of approximately 600. Studies with radiolabeled indole-3-acetic acid, indole-3-acetylaspartic acid, and indole-3-acetyl-β-D-glucose demonstrated that in immature pericarp indole-3-acetic acid is deactivated primarily via metabolism to indole-3-acetylaspartic acid, which is further converted to metabolites 1, 2, and 3. In mature, pink pericarp discs, indole-3-acetic acid is converted more extensively to its glucosyl conjugate. Conjugation of indole-3-acetic acid to indole-3-acetylaspartic acid appears to be dependent upon protein synthesis because it is inhibited by cycloheximide. In contrast, cycloheximide has little effect on the further conversion of indole-3-acetylaspartic acid to metabolites 1, 2, and 3.

There is evidence that auxins act as ripening regulators in both climacteric (4, 10) and nonclimacteric fruit (11), and it has been proposed that IAA is an inhibitor of fruit ripening, which has to be inactivated for the process to advance (10). Such hypotheses remain a topic of some controversy, and other than bioassay-based estimates of questionable accuracy, there are relatively few data in the literature concerning changes in endogenous IAA levels during ripening. On the basis of time-course studies during the ripening of apple fruits in which IAA and ethylene levels were monitored, it has been suggested that an increase in IAA may initiate the autocatalytic production of ethylene in climacteric fruit (14).

The size of IAA pools in plant tissues is regulated by the relative rates of biosynthesis, inactivation, and transport. IAA can be inactivated by several routes, including conjugation with sugars and amino acids (6) and catabolism via decarboxylative and nondecarboxylative pathways (19, 20). Catabolism results in a deactivation of IAA, whereas synthesis and hydrolysis of conjugates may participate in the homeostatic control of free IAA pools (6).

It has been observed that there is an increase in the activity of enzymes associated with IAA decarboxylation, namely, peroxidase and IAA oxidase, during the ripening of certain fruits, and it has been suggested that this may result in a lowering of endogenous IAA levels (9, 21). These reports are based on in vitro studies of crude homogenates and tissue extracts, and caution should be exercised in relating such data to the in situ conditions within the fruit tissues. There is an increasing body of evidence indicating that oxidative decarboxylation accounts for only a relatively small proportion of IAA catabolism and that pathways of nondecarboxylative metabolism have a higher capacity to deactive IAA (8, 13, 18, 22, 23). Pathways of nondecarboxylative IAA metabolism in ripening fruit tissues have received relatively little attention, although it has recently been demonstrated that more than 80 to 90% of [1′-^{14}C]IAA taken up by tomato pericarp discs is metabolized in this manner (5). In this report, we describe studies in which HPLC-RC[2] and HPLC-MS were used to investigate the metabolism of isotopically labeled IAA by tomato pericarp tissues.

MATERIALS AND METHODS

Plant Material

Fruits of tomato (*Lycopersicon esculentum* Mill. cv Moneymaker) were grown in a greenhouse and selected according

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[2] Abbreviations: RC, radiocounting; FAB, fast atom bombardment; IAAsp, indole-3-acetylaspartic acid; IAGluc, indole-3-acetyl-β-D-glucose; $[MH]^+$, molecular ion.

to the method of Lyons and Pratt (12). Fruits were picked at two stages: green fruit at approximately 80% maturity and mature pink fruit.

Isotopically Labeled Substrates

[1′-^{14}C]IAA (specific activity 2.04 GBq mmol^{-1}) and [^{3}H]-IAA (999 GBq mmol^{-1}) were purchased from Amersham International (Amersham, Buckinghamshire, UK), and [1′-^{14}C]IAA (222 MBq mmol^{-1}) was obtained from Sigma (St. Louis, MO), and [$^{13}C_6$]IAA was from Cambridge Isotope Laboratories (Cambridge, UK). In some instances, the specific activity of the radiolabeled IAA was adjusted by the addition of a cold carrier before use.

Incubation Conditions

Tomato fruits were surface sterilized with a 0.2% sodium hypochlorite solution before being washed with sterile distilled water. Pericarp discs were cut from the equatorial part of the fruit with an 8 mm i.d. stainless steel cork borer. Discs were rinsed with distilled water and blotted, and triplicates weighing 0.7 to 1.0 g were vacuum infiltrated at 40 kPa for 5 min in 1.3 or 2 mL of aqueous incubating medium containing 20 mM citrate-phosphate buffer, pH 4.5, 50 μg mL^{-1} of chloramphenicol, and isotopically labeled IAA. After the vacuum was released, discs were kept in the infiltration solution for 20 min before being rinsed in 20 mL of buffer solution for 1 min. After the pericarp discs were rinsed, they were incubated in darkness at 25°C in 25-mL containers through which humidified ethylene-free air was circulated at a flow rate of 22 mL min^{-1}. The duration of the incubation periods used in individual experiments is indicated in "Results."

Extraction and Purification

After incubation, the triplicate pericarp discs were homogenized in methanol (approximately 4 mL g^{-1} fresh weight) containing 100 mg L^{-1} of butylated hydroxytoluene and extracted for 20 h at 4°C. The homogenate was centrifuged for 10 min at 3000*g* and the pellet reextracted under the same conditions. The two methanolic supernatants were combined and reduced to the aqueous phase, which was adjusted to pH 2.7 with acetic acid and passed through a C_{18} Sep-Pak (Waters Associates, Milford, MA). IAA metabolites were then eluted with 5 mL of methanol containing 0.1 M acetic acid. The methanolic eluate was reduced to dryness in vacuo, redissolved in 10% methanol in 50 mM acetic acid, and filtered through a 0.45-μm membrane (Waters Associates) before analysis by HPLC-RC.

HPLC

Reversed-phase HPLC was carried out on a Waters 721 liquid chromatograph using a 150- × 3.9-mm i.d. 5-μm Nova-Pack C_{18} column. The column was eluted at a flow rate of 1 mL min^{-1} with an 18-min linear gradient of 10 to 50% methanol in 0.1 M acetic acid. Mobile phase emerging from the column was directed to a Trace II 7150 radioactivity monitor (Packard Instruments Co., Downers Grove, IL), fitted with a 500-μL flow cell and operating in the homogeneous mode. The scintillant was Ready-Flow III (Beckman Instruments, Irvine, CA), which was used with a 4:1 (v/v) scintillant to eluant ratio.

Identification of IAA Metabolites

Incubation, Extraction, and Preparative HPLC

Two large-scale incubations were carried out in which 32 immature green pericarp discs (approximately 9 g) were infiltrated in 16 mL of (a) 260 μM [1′-^{14}C]IAA (400 kBq, 94 MBq mmol^{-1}) and (b) 181 μM IAA comprising 400 μg of [$^{13}C_6$]IAA and 100 μg of [1′-^{14}C]IAA (127 kBq, 44 MBq mmol^{-1}). After a 24-h incubation, each sample of 32 discs was extracted with methanol as described previously. The methanolic extracts were reduced to the aqueous phase in vacuo, and 10 mL of 50 mM phosphate buffer, pH 8.0, was added before the extract was run through a 30- × 10-mm i.d. polyvinylpolypyrrolidone column, which was further eluted with 12 mL of the same buffer. The eluent from the column was adjusted to pH 3.5 with 1 M hydrochloric acid and passed through a C_{18} Sep-Pak, which was then eluted with 5 mL of methanol containing 0.1 M acetic acid to remove the compounds of interest.

The methanolic eluate was reduced to dryness in vacuo and redissolved in 10% methanol in 50 mM acetic acid before reversed-phase HPLC. The HPLC conditions were the same as those described above except that a radioactivity monitor was not used. Instead, successive fractions were collected and aliquots analyzed for radioactivity by liquid scintillation counting. Fractions corresponding to individual metabolite peaks were combined and reduced to dryness in vacuo in a SpeedVac Concentrator (Savant Instruments, Inc., Hicksville, NY).

Alkaline Hydrolysis

Aliquots from HPLC-purified peaks, corresponding to the most abundant [1′-^{14}C]IAA metabolites, were stirred in 1 N sodium hydroxide for 1 h at room temperature (20–25°C). Under these mild conditions, ester, but not amide, conjugates of IAA are hydrolyzed (2). At the end of the incubation period, samples were neutralized with glacial acetic acid and analyzed by HPLC-RC. Aliquots of metabolite peaks that did not hydrolyze under these conditions were dried, resuspended in 1 mL of 7 N sodium hydroxide, and incubated under nitrogen at 100°C for 7 h. This stronger treatment results in the hydrolysis of amide IAA conjugates (2). After strong alkaline hydrolysis, samples were cooled, adjusted to pH 3.0 with phosphoric acid, and partitioned three times in equal volumes of ethyl acetate. The ethyl acetate extracts were combined, treated with anhydrous sodium sulfate to remove water, filtered, and reduced to dryness in vacuo before being analyzed by HPLC-RC.

Gel Permeation Chromatography

Aliquots of individual metabolites of [1′-^{14}C]IAA were dried under nitrogen, dissolved in 2 mL of 50 mM phosphate buffer, pH 7.0, and applied to the top of a 350- × 30-mm

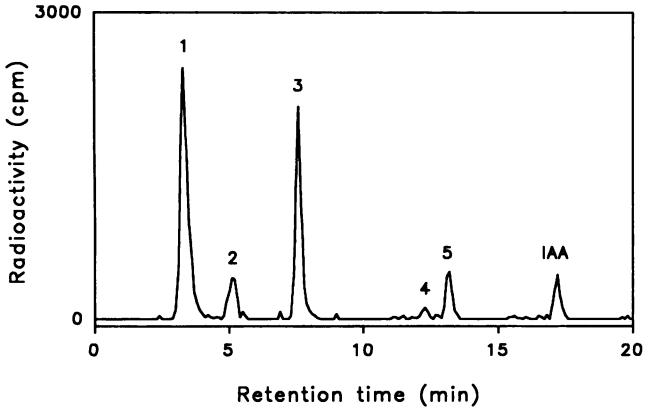

Figure 1. Gradient elution reversed-phase HPLC-RC of an aliquot of a partially purified methanolic extract from immature tomato pericarp discs following a 10-h incubation with 400 kBq of [1′-^{14}C]IAA (2.04 GBq mmol^{-1}).

Sephadex G-15 column. The column was eluted with the same buffer at a flow rate of 27 mL h^{-1}. Successive 5-mL fractions were collected and analyzed for radioactivity by liquid scintillation counting. IAA (M_r 175), IAAsp (M_r 290), and peptide GSH (M_r 600) were included as internal mol wt markers in all samples and were detected with an absorbance monitor operating at 280 nm.

HPLC-MS

The partially purified IAA metabolites were analyzed by capillary HPLC-MS using equipment described by Östin et al. (16). The liquid chromatograph (Waters Associates) consisted of a M680 gradient controller and two M510 pumps, with micropump heads, which were programmed to produce a mobile phase flow rate of 250 μL min^{-1}. To obtain a flow rate of 4 μL min^{-1} through the analytical capillary column, a preinjection split was used to divert most of the solvent, via a tee, to a 120- × 2.1-mm i.d. 5-μm Nucleosil C_{18} "balance" column (Macherey-Nagel, Düren, Germany). The other outlet of the tee was linked to a Rheodyne 7520 injection valve (200-nL loop) (Cotti, CA), which was coupled directly to a 250- × 0.32-mm i.d. capillary HPLC column packed with 5 μm C_{18} (LC Packings, Amsterdam, The Netherlands). The HPLC solvents were methanol and water containing 1% acetic acid together with a 1% glycerol matrix. The mobile phase gradient was 0 to 8 min, 30% methanol; 8 to 38 min, 30 to 80% methanol.

The capillary HPLC column was connected, via a fused silica capillary tubing (1 m × 50 μm i.d.), to a frit-FAB-HPLC-MS interface (JEOL Ltd., Tokyo, Japan), which was attached to the ion source of a double-focusing JEOL JMS SX 102 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 mass spectrometer acceleration voltage was 8 kV, and the slits were set to provide a resolution of 1000. Positive ion FAB was acquired at a rate of 3 s per scan with a cyclic time of 3.2 s for a mass range of 50 to 800 atomic mass units.

RESULTS

Identification of IAA Metabolites

Gradient elution reversed-phase HPLC-RC analysis of a partially purified methanolic extract from 9 g of tomato pericarp discs incubated with 400 kBq of [1′-^{14}C]IAA (94.2 MBq mmol^{-1}) indicated the presence of five metabolite peaks, all of which eluted before IAA. As shown in Figure 1, the metabolites were designated, in order of decreasing polarity, 1 to 5. A similar range of metabolites was detected following incubation of pericarp discs with a mixture of 127 kBq of [1′-^{14}C]IAA (44 MBq mmol^{-1}) and 2.29 μmol of [$^{13}C_6$]IAA. Both extracts were fractionated by HPLC, and the individual ^{14}C- and ^{13}C,^{14}C-metabolite peaks were collected, dried, and analyzed further.

Aliquots of major metabolites derived from [1′-^{14}C]IAA were subjected to mild alkaline conditions that result in the hydrolysis of IAA ester conjugates. If this was without effect, they were exposed to strong alkaline conditions that hydrolyze IAA amide conjugates (2). Metabolite 1 was hydrolyzed under strong but not mild alkaline conditions, suggesting that it is an amide conjugate. Analysis of the hydrolyzed reaction mixture by HPLC-RC showed that metabolite 1 had been converted not to IAA but to a component at the same retention time as metabolite 4 (Fig. 2A). Metabolite 3 was similarly hydrolyzed only under strong alkaline conditions, and the reaction mixture contained radiolabeled peaks at both the IAA and metabolite 4 retention times (Fig. 2B). Metabolite 4 was also hydrolyzed only under strong alkaline conditions, indicating that it, too, was an amide conjugate, and IAA was the sole radiolabeled component remaining in the reaction mixture (Fig. 2C). This implies that the product(s) that accumulate following strong alkaline hydrolysis of me-

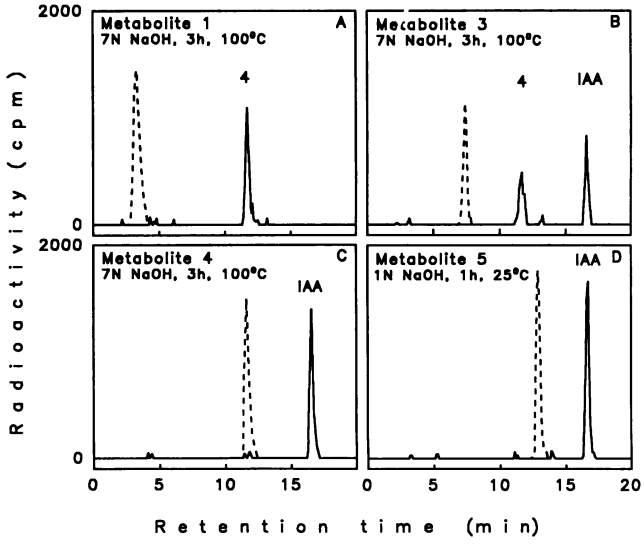

Figure 2. Gradient elution reversed-phase HPLC-RC of metabolites 1, 3, 4, and 5 (A–D, respectively) obtained from immature tomato pericarp discs following incubation with 400 kBq of [1′-^{14}C]IAA (94.2 MBq mmol^{-1}). Traces obtained before (dotted line) and after alkaline treatment (solid line) of the metabolites, under the conditions indicated.

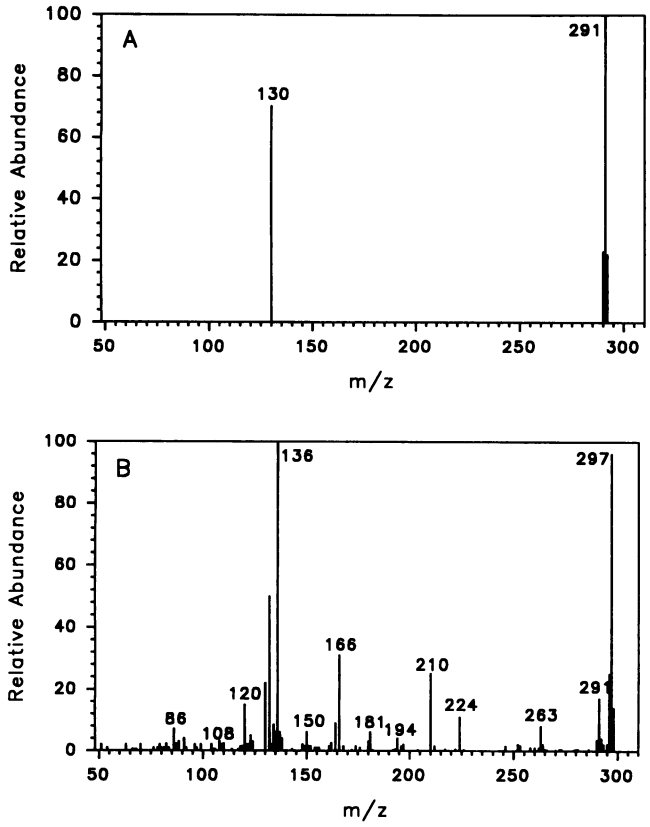

Figure 3. Frit-FAB HPLC-MS analysis of metabolite 4 extracted from tomato pericarp discs following incubation with [1′-^{14}C,$^{13}C_6$]IAA. A, Positive ion mass spectrum of IAAsp standard; B, positive ion mass spectrum obtained from the analysis of an aliquot of metabolite 4.

tabolites 1 and 3, despite their HPLC properties (Fig. 2, A and B), are not identical with metabolite 4. Metabolite 5 would appear to be an IAA ester conjugate, because it was hydrolyzed under mild alkaline conditions, undergoing complete conversion to IAA (Fig. 2D). Similar data were obtained when duplicate samples were subjected to alkaline hydrolysis.

Gel permeation chromatography was carried out on a Sephadex G-15 support with GSH (M_r 600), IAAsp (M_r 290), and IAA (M_r 175) as internal markers. Metabolite 4 had the same retention as IAAsp. Metabolites 1 and 3 both had a slightly longer retention time than the GSH marker. They would, therefore, appear to have an M_r of approximately 600.

The metabolite fractions derived from [1′-^{14}C,$^{13}C_6$]IAA were analyzed by frit-FAB-HPLC-MS. The spectrum presented in Figure 3B was obtained from the analysis of an aliquot of metabolite 4 and matches that of IAAsp (Fig. 3A) with an m/z 291 $[MH]^+$ and an m/z 130 quinolinium ion. There is clear evidence of $^{13}C_6$ labeling in the form of the major fragments at m/z 297 and 136. The lack of a significant response at m/z 293 indicates that the level of the ^{14}C label was too low to facilitate detection. Although there is a major fragment at m/z 132, this is probably due to effects associated with the substraction of the glycerol background.

A mass spectrum was obtained from HPLC-MS analysis of metabolite 5, which corresponds to that of [$^{13}C_6$]1-*O*-IAGluc (Fig. 4). The positive ion frit-FAB mass spectrum of 1-*O*-IAGluc is characterized by a $[MH]^+$ glycerol adduct at an m/z 430, 338/337 ($[MH]^+/[M]^+$), 176 fragment, and 130 quinolinium ion (Fig. 4A). The equivalent fragments were detected when metabolite 5 was analyzed, but because of the $^{13}C_6$ label, they were increased by 6 atomic mass units to m/z 436, 343, 182, and 136 (Fig. 4B). However, in the absence of standards of other IAGluc isomers, such as 2-*O*-IAGluc, 4-*O*-IAGluc, and 6-*O*-IAGluc, which may have HPLC retentions and spectral fragmentation properties similar to those of 1-*O*-IAGluc (see ref. 7), it would be premature at this juncture to reach any firm conclusions about the exact location of the linkage between [$^{13}C_6$]IAA and the glucose moiety. HPLC-MS analysis of metabolites 1, 2, and 3 did not yield any spectra recognizable as being derived from potential indoles or metabolites of IAA.

Effects of Incubation Time, Substrate Concentration, and Cycloheximide on IAA Metabolism

A time-course study of the metabolism of [1′-^{14}C]IAA by green and mature tomato pericarp discs was conducted. The

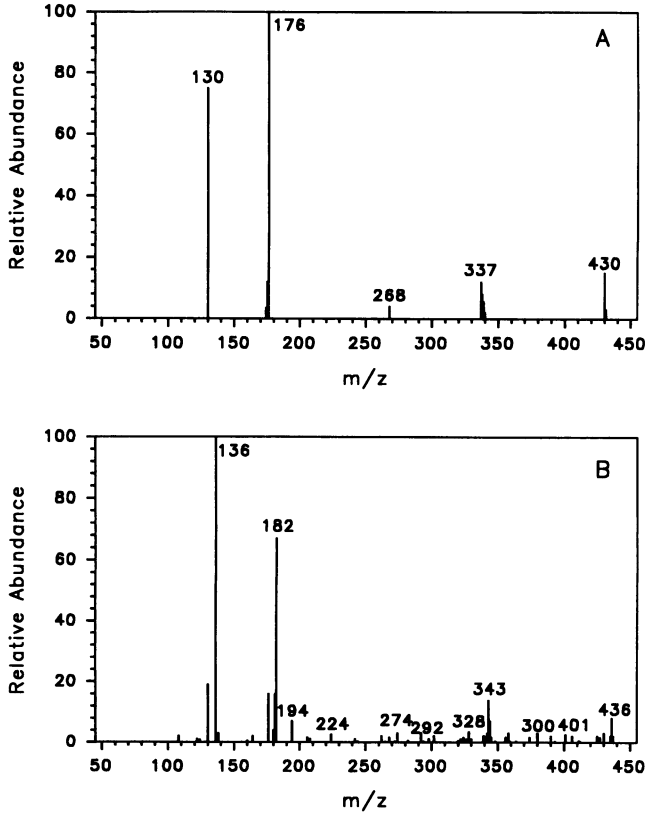

Figure 4. Frit-FAB HPLC-MS analysis of metabolite 5 extracted from tomato pericarp discs following incubation with [1′-^{14}C,$^{13}C_6$]IAA. A, Positive ion mass spectrum of 1-*O*-IAGluc standard; B, positive ion mass spectrum obtained from the analysis of an aliquot of metabolite 5.

Table I. *Effect of Incubation Time on the Metabolism of [1′-^{14}C]IAA by Immature and Mature Pink Tomato Pericarp Discs*

Discs infiltrated in a 2-mL aqueous solution containing 13 kBq of [1′-^{14}C]IAA (2.0 GBq mmol^{-1}). At the end of the incubation period, discs were extracted with methanol, and methanolic extracts were analyzed by HPLC-RC. Radioactivity associated with IAA and metabolites expressed as percentage of total radioactivity in the extract ± SD (n = 4).

Maturity of Discs	Incubation Time	Percentage of Radioactivity in Extract					
		IAA	Metabolite 1	Metabolite 2	Metabolite 3	IAAsp	IAGluc
	h						
	2	75.5 ± 12.0	11.5 ± 7.0	3.7 ± 3.3	3.2 ± 1.8	1.1 ± 1.0	5.3 ± 0.4
	5	41.3 ± 16.6	31.1 ± 8.0	4.9 ± 2.9	13.9 ± 4.4	1.5 ± 0.1	7.4 ± 1.6
	10	7.6 ± 2.3	51.0 ± 4.2	4.8 ± 2.6	28.9 ± 3.0	2.1 ± 1.1	5.7 ± 2.6
Immature	22	0.7 ± 1.0	55.3 ± 1.7	5.1 ± 0.4	30.5 ± 4.2	2.3 ± 0.7	7.1 ± 3.2
	32	0.4 ± 0.6	54.5 ± 6.8	4.4 ± 2.8	34.1 ± 4.7	3.3 ± 2.3	3.8 ± 1.1
	48	0.0 ± 0.0	58.7 ± 1.9	3.8 ± 1.8	34.0 ± 4.1	2.1 ± 0.9	0.8 ± 1.0
	2	66.7 ± 2.3	3.1 ± 1.0	0.4 ± 0.5	0.7 ± 0.9	0.4 ± 0.5	29.1 ± 3.8
	5	56.3 ± 1.1	11.1 ± 5.6	0.5 ± 0.6	4.4 ± 1.9	0.3 ± 0.4	27.0 ± 7.1
	10	25.3 ± 15.0	24.2 ± 5.4	4.7 ± 1.0	11.1 ± 3.9	2.1 ± 1.3	32.4 ± 3.4
Mature pink	22	5.6 ± 7.9	41.0 ± 0.6	6.1 ± 1.3	24.5 ± 2.8	2.2 ± 1.3	20.6 ± 7.0
	32	1.5 ± 2.1	47.6 ± 6.5	7.5 ± 2.1	23.7 ± 0.5	2.5 ± 1.2	16.7 ± 6.8
	48	0.0 ± 0.0	50.0 ± 9.9	7.3 ± 1.1	29.4 ± 3.6	2.1 ± 0.5	13.8 ± 2.6

data obtained, which are presented in Table I, indicate that all the labeled IAA taken up by both green and mature pericarp discs was fully metabolized within 22 to 48 h, although the rate of metabolism was more rapid in the immature tissue. In the immature green discs, metabolites 1 and 3 accumulated to the greatest extent, and there were relatively smaller amounts of metabolite 2 and IAGluc, with only trace quantities of IAAsp being detected. In general, the kinetics of metabolite accumulation were similar in mature discs with the notable exception of IAGluc, which accumulated in proportionally much larger amounts than it did in immature discs.

The effect of substrate dose and cycloheximide on the metabolism of [^{3}H]IAA was investigated using green pericarp discs and a 10-h incubation period. The data obtained are presented in Table II. Infiltration of pericarp with [^{3}H]IAA in the range 0.3 to 30 μM was associated with an increase in IAA uptake that was more or less proportional to the concentration of the substrate. Increasing substrate concentration resulted in an increase in the proportion of unmetabolized IAA and a decrease in the relative amounts of metabolites 1 and 3. At the highest substrate dose, there was a marked increase in the accumulation of IAAsp. The relatively small amounts of IAGluc and decarboxylative products that were detected appeared not to be influenced by IAA concentration. When cycloheximide was included in the infiltration medium, there was a very marked increase in the level of unmetabolized IAA and a concomitant reduction in the accumulation of metabolites 1, 2, and 3 and IAAsp at the end of the 10-h incubation period. In contrast, the proportions of IAGluc and decarboxylated products were affected little by the presence of cycloheximide.

Table II. *Effect of Substrate Concentration and Cycloheximide on [^{3}H]IAA Metabolism by Immature Tomato Pericarp Discs*

Discs infiltrated in a 2-mL aqueous solution containing 80 kBq of [^{3}H]IAA diluted with either 0.3, 3.0, or 30 μM IAA in the presence and absence of 25 μg mL^{-1} of cycloheximide. After a 10-h incubation period, discs were extracted with methanol, and the methanolic extracts were analyzed by HPLC-RC. Radioactivity associated with IAA and metabolites expressed as percentage of total radioactivity in the extract. IAA uptake calculated from the total radioactivity extracted and the specific activity of the [^{3}H]IAA in the infiltration medium. Decarboxylative products estimated from the radioactivity associated with HPLC peaks that were not detected after incubation of pericarp discs with [1′-^{14}C]IAA. Similar data were obtained in two replicate experiments.

Cycloheximide	IAA in the Infiltration Solution	IAA Uptake	Percentage of Radioactivity in Extract						
			IAA	Metabolite 1	Metabolite 2	Metabolite 3	IAAsp	IAGluc	Decarboxylated products
	μM	*nmol g*$^{-1}$							
	0.3	0.14	15.9	41.4	3.0	27.3	1.1	3.3	7.1
Without	3.0	1.30	24.8	37.7	3.3	19.6	1.3	3.6	6.9
	30.0	12.50	44.0	19.1	2.7	10.9	11.5	4.0	6.3
	0.3	0.18	85.3	2.1	0.5	2.3	1.8	3.7	4.8
With	3.0	1.64	82.4	0.4	0.3	1.9	0.3	5.0	8.3
	30.0	14.50	91.5	0.1	0.2	0.2	0.3	3.8	3.7

Table III. *Effect of Incubation Time and Cycloheximide on the Metabolism of [^{14}C]IAAsp by Immature Tomato Pericarp Discs*

Discs infiltrated in a 1.3-mL aqueous solution containing 10 kBq of [^{14}C]IAAsp with and without 25 μg mL^{-1} of cycloheximide. At the end of the incubation period, discs were extracted with methanol, and the methanolic extracts were analyzed by HPLC-RC. Radioactivity associated with IAAsp and metabolites expressed as percentage of total radioactivity in the extract. Similar data were obtained in a duplicate experiment.

Cycloheximide	Incubation Time	Percentage of Radioactivity in Extract					
		IAAsp	Metabolite 1	Metabolite 2	Metabolite 3	IAA	IAGluc
	h						
Without	5	64.3	23.3	5.5	6.9	0.0	0.0
	10	46.6	32.6	5.8	9.4	0.0	0.0
	20	13.7	50.9	7.7	27.7	0.0	0.0
With	10	58.5	25.5	5.8	9.4	0.0	0.0

Metabolism of [^{14}C]IAAsp and [^{14}C]IAGluc

The partially purified HPLC fractions containing [^{14}C]-IAAsp and [^{14}C]IAGluc, produced by pericarp discs from [1′-^{14}C]IAA as described above, were refed to the tomato tissue in a preliminary attempt to obtain insight into the metabolic relationships of the various IAA metabolites.

A time-course study of green pericarp discs and 5-, 10-, and 20-h incubation periods showed that [^{14}C]IAAsp was converted to metabolites, 1, 2, and 3, and IAA and IAGluc did not accumulate in detectable quantities (Table III). Initially, the decline in [^{14}C]IAAsp was associated primarily with the accumulation of metabolite 1, but after 20 h a substantial amount of metabolite 3 was also present. The data, thus, indicate that metabolites 1, 2, and 3 originate from IAAsp, although the actual metabolic sequence is as yet unclear. In contrast to its inhibitory effects on the metabolism of [^{3}H]-IAA (Table II), cycloheximide had no great influence on the metabolism of [^{14}C]IAAsp (Table III).

The accumulation of metabolites following infiltration of green and mature pericarp discs with [^{14}C]IAGluc is summarized in Table IV. After an 8-h incubation period, relatively little of the substrate remained in green pericarp discs, and radioactivity was associated mainly with metabolites 1 and 3 together with small amounts of metabolite 2, IAAsp, and IAA. When mature, rather than green, pericarp discs were used, there was a much greater recovery of the [^{14}C]IAGluc substrate, and, in addition, there was a marked increase in the relative amount of IAA and a corresponding reduction in the degree of accumulation of metabolites 1 and 3. When these data are considered together with the results of the [^{14}C]IAAsp metabolism study (Table III), they suggest that IAGluc, unlike IAAsp, is readily hydrolyzed to release free IAA, which is then converted to IAAsp and on to metabolites 1, 2 and 3. Based on both the level of recovery of the [^{14}C]-IAGluc substrate and the relative amounts of radioactivity associated with IAA, IAAsp, and the metabolites 1 to 3, this pathway appears to be more active in green than in mature tomato pericarp tissue.

DISCUSSION

Green tomato pericarp discs converted isotopically labeled IAA, via nondecarboxylative pathways, to metabolites 1 to 5, which were resolved when samples were analyzed by reversed-phase HPLC-RC (Fig. 1). Studies of the properties of metabolite 2 were not feasible because of the relatively low levels of radioactivity that accumulated. However, following feedings with [$^{13}C_6$]IAA, metabolites 4 and 5 were identified by positive ion frit-FAB HPLC-MS as [$^{13}C_6$]IAAsp and [$^{13}C_6$]IAGluc, respectively. HPLC-MS analysis of metabolites 1 and 3 did not provide any definitive identifications of either native IAA derivatives or metabolites originating from [$^{13}C_6$]IAA. However, ^{14}C-labeled samples of both metabolites were hydrolyzed by strong but not mild alkaline treatment, which implies that they are IAA-amide conjugates. Gel permeation chromatography indicated that metabolites 1 and 3 both had a mol wt of approximately 600. This is in excess of the mol wt of IAA-amino acid conjugates but well below the mol wt of 3600 estimated for putative IAA-polypeptide conjugates isolated from *Phaseolus vulgaris* (3).

The data obtained demonstrate that in tomato pericarp IAA undergoes conjugation to yield both IAGluc and IAAsp and that IAAsp is further converted, via an as yet undeter-

Table IV. *Metabolism of [^{14}C]IAGluc by Immature and Mature Pink Tomato Pericarp Discs*

Discs were infiltrated in a 1.3-mL aqueous solution containing 7.5 kBq of [^{14}C]IAGluc. After an 8-h incubation period, discs were extracted with methanol, and methanolic extracts were analyzed by HPLC-RC. Radioactivity associated with IAGluc and metabolites expressed as percentage of total radioactivity in extract. Similar data were obtained in a duplicate experiment.

Maturity of Discs	Percentage of Radioactivity in Extract					
	IAGluc	Metabolite 1	Metabolite 2	Metabolite 3	IAA	IAAsp
Immature	2.5	51.2	4.2	26.5	6.2	9.4
Mature pink	24.3	17.2	1.6	7.7	39.4	10.3

mined metabolic sequence, to metabolites 1, 2, and 3. Conjugation of [^{3}H]IAA to IAAsp appears to be dependent upon protein synthesis, because it is inhibited by cycloheximide (Table I). In contrast, cycloheximide had relatively little effect on the conversion of [^{14}C]IAAsp to metabolites 1, 2, and 3 (Table III).

It has been suggested that IAA conjugates participate in homeostatic control of free IAA levels. This hypothesis implies that IAA conjugation is a reversible process and that IAA can be released by hydrolysis of IAA conjugates (1). In tomato pericarp, IAAsp does not appear to be involved in such a process; rather, it is the first step in an irreversible deactivation pathway leading to the formation of the unidentified polar compounds, metabolites 1 to 3. This is in keeping with data obtained from studies of *Vicia faba* (22, 23), *Dalbergia dolichopetala* (13, 15), and *Populus tremula* (17), in which IAAsp is not hydrolyzed to release free IAA but, instead, is oxidized to yield oxindole, dioxindole, and, in some instances, glucosyl derivatives.

In contrast to IAAsp, IAGluc is readily hydrolyzed by tomato pericarp, releasing free IAA. This is in keeping with IAGluc formation being a reversible process that constitutes a shuttle mechanism that can facilitate rapid adjustments in the size of the free IAA pool. Examination of Tables I and IV shows, first, that there is relatively little accumulation of IAGluc from [^{14}C]IAA in green tomato pericarp and, second, that applied [^{14}C]IAGluc is readily hydrolyzed, releasing free IAA, which is extensively metabolized via IAAsp to metabolites 1 and 3. In mature pericarp, [^{14}C]IAA is converted more extensively to IAGluc, and, in turn, much less [^{14}C]IAGluc is metabolized through IAA to IAAsp, and metabolites 1 and 3, than in the immature tissues. It, therefore, seems that in immature pericarp IAA is deactivated primarily via conversion to IAAsp and related metabolites, whereas in mature tissues there is more emphasis on conversion to the potential storage product, IAGluc.

LITERATURE CITED

1. **Bandurski RS** (1984) Metabolism of indole-3-acetic acid. *In* A Crozier, JR Hillman, eds, The Biosynthesis and Metabolism of Plant Hormones, Society for Experimental Biology Seminar Series No. 23. Cambridge University, Cambridge, UK, pp 183–200
2. **Bandurski RS, Schulze A** (1977) Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol **60:** 211–213
3. **Bialek K, Cohen JD** (1986) Isolation and partial characterization of the major amide-linked conjugate of indole-3-acetic acid from *Phaseolus vulgaris*. Plant Physiol **80:** 99–104
4. **Brady CJ** (1987) Fruit ripening. Annu Rev Plant Physiol **37:** 1–15
5. **Catalá C** (1991) Estudio del metabolismo del ácido indol-3-acético en el pericarpo del tomate durante la maduración. Formación de conjugados y catabolismo oxidativo. PhD thesis. University of Valencia, Valencia, Spain
6. **Cohen JD, Bandurski JS** (1982) Chemistry and physiology of the bound auxins. Annu Rev Plant Physiol **33:** 401–430
7. **Ehman A** (1974) Identification of 2-*O*-(indole-3-acetyl)-D-glucopyranose, 4-*O*-(indole-3-acetyl)-D-glucopyranose and 6-*O*-(indole-3-acetyl)-D-glucopyranose from kernels of *Zea mays* by gas-liquid chromatography-mass spectrometry. Carbohydr Res **34:** 99–114
8. **Ernsten A, Sandberg G, Lundström K** (1987) Identification of oxindole-3-acetic acid, and metabolic conversion of indole-3-acetic acid to oxindole-3-acetic acid in *Pinus sylvestris* seeds. Planta **172:** 47–52
9. **Frenkel C** (1972) Involvement of peroxidase and indole-3-acetic acid oxidase isozymes from pear, tomato and blueberry fruit in ripening. Plant Physiol **49:** 757–763
10. **Frenkel C** (1975) Oxidative turnover of auxins in relation to the onset of ripening in Barlet pear. Plant Physiol **55:** 480–484
11. **Given NK, Venis MA, Grierson D** (1988) Hormonal regulation of ripening in the strawberry, a non-climacteric fruit. Planta **174:** 402–406
12. **Lyons JM, Pratt HK** (1964) Effect of stage of maturity and ethylene treatment on respiration and ripening of tomato fruits. Proc Am Hortic Soc **84:** 491–499
13. **Monteiro AM, Crozier A, Sandberg G** (1988) The biosynthesis and conjugation of indole-3-acetic acid in germinating seed and seedlings of *Dalbergia dolichopetala*. Planta **174:** 561–568
14. **Mousdale DM, Knee M** (1981) Indolyl-3-acetic acid and ethylene levels in ripening apple fruits. J Exp Bot **32:** 753–758
15. **Östin A, Monteiro AM, Crozier A, Jensen E, Sandberg G** (1992) Analysis of indole-3-acetic acid metabolites from *Dalbergia dolichopetala* by high performance liquid chromatography-mass spectrometry. Plant Physiol **100:** 63–68
16. **Östin A, Moritz T, Sandberg G** (1992) Liquid chromatography-mass spectrometry of conjugates and oxidative metabolites of indole-3-acetic acid. Biol Mass Spectrom **21:** 292–298
17. **Plüss R, Jenny T, Meier H** (1989) IAA-induced adventitious root formation in green wood cuttings of *Populus tremula* and formation of 2-indolone-3-acetylaspartic acid, a new metabolite of exogenously applied indole-3-acetic acid. Physiol Plant **75:** 89–96
18. **Reinecke DM, Bandurski RS** (1981) Metabolic conversion of ^{14}C-indole-3-acetic acid to ^{14}C-oxindole-3-acetic acid. Biochem Biophys Res Commun **103:** 429–433
19. **Sandberg G, Crozier A, Ernstsen A** (1987) Indole-3-acetic acid and related compounds. *In* L Rivier, A Crozier, eds, The Principles and Practice of Plant Hormone Analysis, Vol 2. Academic Press, London, UK, pp 169–301
20. **Sembdner G, Gross D, Liebisch HW, Schneider G** (1980) Biosynthesis and metabolism of plant hormones. *In* J MacMillan, ed, Hormonal Regulation of Development. I. Molecular Aspects of Plant Hormones. Encyclopedia of Plant Physiology, New Series, Vol 9. Springer-Verlag, Berlin, Germany, pp 281–444
21. **Thomas RL, Jen JJ, Morr CV** (1981) Changes in soluble and bound peroxidase-IAA oxidase during tomato fruit development. J Food Sci **47:** 158–161
22. **Tsurumi S, Wada S** (1985) Identification of 3-(*O*-β-glucosyl)-2-indolone-3-acetyl aspartic acid as a new indole-3-acetic acid metabolite in *Vicia* seedling. Plant Physiol **79:** 667–671
23. **Tsurumi S, Wada S** (1986) Dioxindole-3-acetic acid conjugate formation from indoleacetylaspartic acid in *Vicia* seedlings. Plant Cell Physiol **27:** 1513–1522