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Quantification of Free Plus Conjugated Indoleacetic Acid in Arabidopsis Requires Correction for the Nonenzymatic Conversion of Indolic Nitriles[1]

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The genetic advantages to the use of *Arabidopsis thaliana* mutants for the study of auxin metabolism previously have been partially offset by the complexity of indolic metabolism in this plant and by the lack of proper methods. To address some of these problems, we developed isotopic labeling methods to determine amounts and examine the metabolism of indolic compounds in Arabidopsis. Isolation and identification of endogenous indole-3-acetonitrile (IAN; a possible precursor of the auxin indole-3-acetic acid [IAA]) was carried out under mild conditions, thus proving its natural occurrence. We describe here the synthesis of $^{13}C_1$-labeled IAN and its utility in the gas chromatography-mass spectrometry quantification of endogenous IAN levels. We also quantified the nonenzymatic conversion of IAN to IAA under conditions used to hydrolyze IAA conjugates. $^{13}C_1$-Labeled IAN was used to assess the contribution of IAN to measured IAA following hydrolysis of IAA conjugates. We studied the stability and breakdown of the indolic glucosinolate glucobrassicin, which is known to be present in Arabidopsis. This is potentially an important concern when using Arabidopsis for studies of indolic biochemistry, since the levels of indolic auxins and auxin precursors are well below the levels of the indolic glucosinolates. We found that under conditions of extraction and base hydrolysis, formation of IAA from glucobrassicin was negligible.

The study of plant hormone biogenesis and metabolism has been enhanced greatly by the application of genetic techniques, especially the use of metabolic mutants (Normanly et al., 1995). Various plants have been used for such biochemical genetic studies and these include maize, pea, tobacco, and *Lemna gibba*. The use of Arabidopsis for such studies has significant advantages because of the simplicity of its genome, short life cycle, and the existence of an impressive number of mutant plants and information concerning specific genes. For studies of auxin metabolism, however, the advantages associated with Arabidopsis previously have been offset by the complexity of indolic metabolism in members of the Cruciferae. Additionally, there has been a lack of sufficient knowledge and methodology for undertaking detailed investigations of levels and metabolic transformation of indolic auxins and auxin precursors in these plants.

Several genera within the Cruciferae are noted for the abundance of indolic compounds that they produce. For example, *Isatis* sp. are noted for production of the indolic blue dye indigo (Stowe et al., 1968), and *Brassica* spp. contain a variety of glucosinolates, which are thiocyanates originally referred to as "mustard oil glucosides" (Ettlinger et al., 1961). These compounds are responsible for much of the characteristic flavors and odors associated with cabbage, broccoli, and their relatives. Among the glucosinolates found in cruciferous plants are the indolic glucosinolates glucobrassicin and neoglucobrassicin (Gmelin, 1964). Arabidopsis contains significant amounts of these compounds (Haughn et al., 1991), and thus the stability of glucobrassicin and its breakdown products are potentially important concerns when using Arabidopsis for studies of indolic biochemistry, in light of the fact that levels of indolic auxins and auxin precursors (usually present at about 10 pmol/mg; Normanly et al., 1993) are well below those of the indolic glucosinolates (which are present at approximately 500 pmol/mg; Haughn et al., 1991). In addition, there are reports that cruciferous plants contain free IAN (Kutacek and Kefeli, 1968), although there has been some debate as to the origin of IAN found in plant extracts, since it is thought to be liberated from glucobrassicin during extraction under mildly acidic conditions (Underhill, 1980).

In this report we describe methods that we have developed to study amounts and isotopic labeling of indolic compounds in Arabidopsis to allow the utility of this excellent genetic system to be fully applied to studies of auxin biochemistry. Some of these methods were utilized but not described in exact detail in a previous report (Normanly et al., 1993).

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Abbreviations: IAN, indole-3-acetonitrile; SIM, selected ion monitoring; SPE, solid phase extraction.

MATERIALS AND METHODS

Hydrolysis of IAN

Twenty milliliters of a 1.0 mg/mL methanolic solution of IAN (no. 4035, Calbiochem[2]) and 20 mL of a 0.5 mg/mL isopropanol solution of [$^{13}C_6$]IAA (synthesized as described by Cohen et al., 1986) were added to each of six Teflon 30-mL vials (Tuf-tainers, no. 14030, Pierce). Two of the containers were set aside for the analysis of free IAA in the commercial IAN. Another set of two containers were treated exactly as would be done for the analysis of free plus esterified IAA (Cohen et al., 1986). The solvents were removed using a stream of dry nitrogen, and 1 mL of 1 N NaOH was added. These samples were capped and incubated for 1 h at room temperature (25°C). The final set of containers was treated as would normally be done for the analysis of total IAA (Cohen et al., 1986). Specifically, 5 mL of a freshly made 7 N NaOH solution were added, and the containers were capped with Teflon-lined septa and incubated at 100°C for 3 h with a slow O_2-free nitrogen purge (Bialek and Cohen, 1989). Following incubation, all four containers were neutralized with phosphoric acid, and the pH was reduced to 4.0. The contents were applied to a C_{18} SPE column (no. 7020–03, J.T. Baker) that had been preconditioned with methanol followed by water. After application of the solutions from the Teflon vials, the columns were washed with 4 mL of water and finally the IAA was eluted using 4 mL of methanol. The samples were evaporated to dryness, resuspended in 100 μL of methanol, and methylated using ethereal diazomethane (Cohen, 1984). Samples were resuspended in ethyl acetate and analyzed by GC-SIM-MS as previously described (Normanly et al., 1993).

Synthesis of Labeled IAN

The use of a heavy mass standard with a single label was selected based on ease of preparation and on the necessity of having a molecular mass, following conversion to IAA, between that of the unlabeled IAA and the [$^{13}C_6$]IAA internal standard. A small overlap by the natural isotopic content of plant-derived IAN or IAA into the M+1 ion region results in the need for some special considerations, as will be described below in "Mass Fragmentation Analysis." These considerations are especially important when the endogenous compound is present in the analyzed sample in relatively large amounts compared to the $^{13}C_1$-standard (a complete discussion of the use of a standard with one heavy atom was given by Sutter and Cohen, 1992).

The synthesis of [$^{13}C_1$]IAN was conducted via a two-step reaction sequence. Gramine methosulfate was first synthesized from 3.48 g (20 mmol) of gramine (no. G1647, Sigma) and 12.6 g (100 mmol) of dimethyl sulfate (no. D18,630–9, Aldrich) by the method of Schoepf and Thesing (1951). The second step involved the reaction between the gramine salt (280 mg, 0.9 mmol) and 60 mg (0.9 mmol) of K[^{13}C]N (99 atom %, no. CLM-297; Cambridge Isotope Laboratories, Andover, MA) by the method of Stowe (1963). Purification was accomplished by normal-phase chromatography on silica gel (Silica gel 60, 0.063–0.2 mm, no. 7734; E. Merck, Darmstadt, Germany) with benzene as the solvent, and product identity was confirmed by GC-MS of the trimethylsilyl derivative (Fig. 1A). [^{14}C]IAN was prepared similarly from 1.52 mg (5 μmol) of the gramine salt and 0.163 mg of K[^{14}C]N (2.5 μmol, 125 μCi, no. 17423H, ICN Radiochemicals).

Isolation of Glucobrassicin

Glucobrassicin was isolated from 800 g of cabbage (*Brassica oleracea* L. var *capitata*) and, in a second extraction/purification, from 160 g of kale (*Brassica oleracea* L. var *acephala*) using a slight modification of the methods of Gmelin and Virtanen (1961). Plant material was obtained from a local grocer, and individual leaves were separated and collected on ice. The material was weighed and frozen in liquid nitrogen, and the leaves were pulverized in a liquid nitrogen chilled mortar with a liquid nitrogen chilled pestle. The pulverized leaf samples (160 or 800 g) were added in 1- to 5-g quantities to rapidly boiling methanol (2.5 L for the cabbage sample and 1.5 L for kale). After all material was added, the methanol was diluted with water (430 mL for the cabbage sample and 200 mL for kale), and then the sample was filtered through S/P grade 513 qualitative paper (no. F2313–500; American Scientific Products, McGraw Park, IL) and chilled to ice temperature. The sample was then reduced in vacuo to an oily residue and purified by alumina column chromatography as described by Gmelin and Virtanen (1961). The fractions were analyzed by silica gel TLC using Ehmann's reagent (Ehmann, 1977) for detection. Fractions were pooled and reduced in vacuo, and the sample was crystallized from 90% ethanol: water as the tetramethylammonium salt. Product identity was confirmed by TLC and by IR spectra taken in potassium bromide on a 60SX Fourier transform IR instrument (Nicolet, Madison, WI). For studies of base conversion of glucobrassicin to IAA, 10 μg of glucobrassicin were added to a 10-mL Teflon vial containing 1 μg of [$^{13}C_6$]IAA in 2 mL of 1 N or 7 N NaOH and treated as described below for studies of free plus ester and total IAA.

Quantification of IAA and IAN

Arabidopsis thaliana Britton ecotype Columbia plants were grown and harvested as previously described (Normanly et al., 1993). Free, free plus ester-linked, and free, ester-linked, and amide-linked IAA were isolated as described by Chen et al. (1988) with slight modifications. Five hundred nanograms of [$^{13}C_6$]IAA per 200 mg fresh weight of tissue were added as an internal standard, and approximately 100,000 dpm of [^{3}H]IAA (25.4 mCi/mmol, no. TRK781, Amersham) were added as a radiotracer. Also, 500 ng of [$^{13}C_1$]IAN were added. After the isopropanol was removed in vacuo, one-third of the sample was set aside for isolation and quantitation of IAN. The hydrolysis and iso-

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

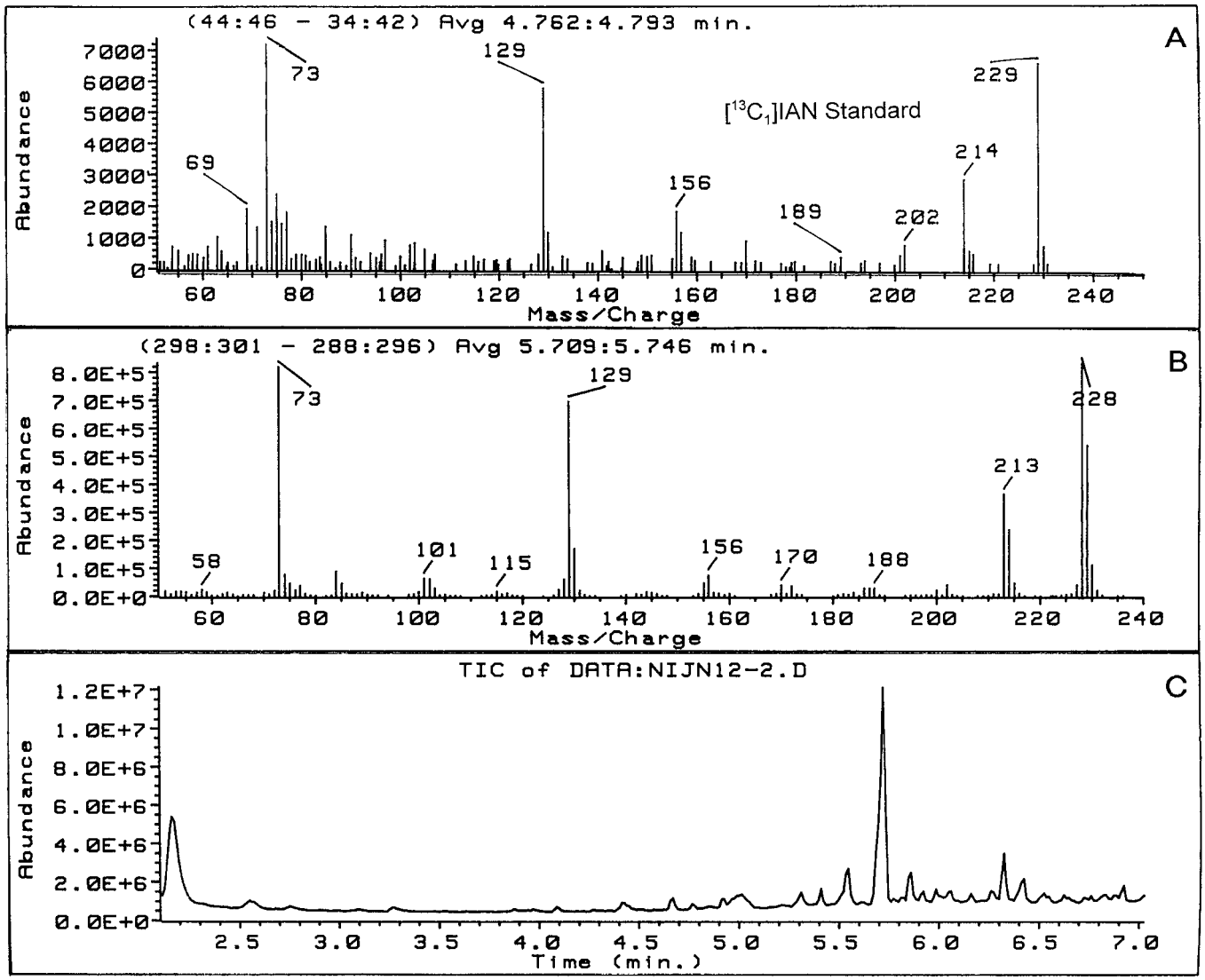

Figure 1. GC-MS analysis showing full-scan (m/z 50–250) mass spectrum of the synthesized [$^{13}C_1$]IAN after derivatization with bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (as described in "Materials and Methods") (A), full-scan (m/z 50–250) mass spectrum (B), and total ion chromatogram (C) of IAN isolated from *A. thaliana* ecotype Columbia carrying the *trp2–1* mutation. The 660-mg sample contained 650 ng of added [$^{13}C_1$]IAN, which accounts for the strong ions at m/z 229 and 214 seen in B. Ions at m/z 228 and 213 in B are due to the endogenous IAN from the Arabidopsis tissue analyzed.

lation of the free plus ester fraction were done as described by Chen et al. (1988) except that use of the amino SPE column was omitted from the protocol. The HPLC column used was an Ultracarb 5 ODS 30 (50 × 4.6 mm; Phenomenex, Torrance, CA). The mobile phase was 25% methanol plus 1% acetic acid. Those fractions with the highest disintegrations per minute were evaporated to dryness in vacuo and resuspended in 100 μL of methanol. IAA-containing samples were methylated prior to GC-SIM-MS analysis by adding 500 μL of a bright yellow solution of ethereal diazomethane and incubating at room temperature for 5 min (Cohen, 1984). The solvent and residual diazomethane were evaporated using a stream of N_2 gas, and the samples were resuspended in 30 μL of ethyl acetate.

For IAN isolation, the fraction that was set aside from the IAA isolation was brought to 2 mL with water and passed over an SPE amino column (500 mg, no. 188–1050; J&W Scientific, Folsom, CA) as described by Chen et al. (1988). The nonretained fraction was collected and applied to a C_{18} SPE column (Prep-Sep SPE, 300 mg, no. P453, Fisher Scientific) previously conditioned with methanol and water. The IAN was eluted from this column using 2.5 mL of acetonitrile, evaporated to dryness, resuspended in 50% methanol, and injected onto the HPLC column system described above for IAA purification. Fractions collected were determined by retention time in comparison to that of an authentic standard run under identical conditions. Immediately prior to GC-SIM-MS analysis, samples were

evaporated to dryness, resuspended in 30 μL of bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (no. 3-3154, Supelco, Bellefonte, PA), and incubated at 45°C for 30 min.

Mass Fragmentation Analysis

Under the conditions used to isolate total IAA, IAN is converted to IAA (Table I). Under the conditions used to isolate ester-linked IAA, 3% of the IAN present is converted to IAA. Therefore, the IAA quantified in a sample that has been treated in either manner will comprise IAA that is generated from endogenous IAN during extraction and hydrolysis. [$^{13}C_1$]IAN added to the sample upon extraction can be used to distinguish between the endogenous IAA and that derived from IAN. Plant extracts containing [$^{13}C_1$]IAN and [$^{13}C_6$]IAA were divided into two fractions. From one fraction IAN was isolated under conditions of neutral pH, which does not result in the conversion of IAN to IAA, and the ratio of [$^{13}C_1$]IAN to unlabeled IAN was determined by GC-SIM-MS (Fig. 2). The ratio of labeled to unlabeled trimethylsilyl-IAN was determined by calculating the abundance of m/z 228 (unlabeled) and m/z 229 ($^{13}C_1$-labeled IAN) after correction for the natural abundance of heavy and light atoms. Natural abundances of heavy atoms were determined experimentally: (a) for the ion at m/z 228, the natural abundance of (m+1)/z was 0.182, and (b) for the ion at m/z 229, the natural abundance of (m−1)/z was 0.088. Thus, isotopic ratios could be corrected for natural abundance using the following set of equations (where *RI* = relative ion intensity):

$$RI'_{228} = RI_{228} + RI_{228}(0.182) - RI_{229}(0.088) \quad (1)$$

and

$$RI'_{229} = RI_{229} - RI_{228}(0.182) + RI_{229}(0.182) + RI_{229}(0.088). \quad (2)$$

Thus, the IAN correction factor (CF) is given by:

$$CF_{IAN} = RI'_{228}/RI'_{229}. \quad (3)$$

Once the correction factor for IAN is empirically determined, it can be used to determine how much of the IAA has been derived from IAN and how much IAA is endogenous to the plant in the form of IAA or IAA conjugates.

IAA (either total or ester linked) was isolated from the other fraction. The IAA obtained comprised [$^{13}C_1$]IAA (from [$^{13}C_1$]IAN), [$^{13}C_6$]IAA (the added standard), and unlabeled IAA (which was a mixture of IAA derived from unlabeled IAN and endogenous IAA). The methylated $^{13}C_1$-labeled IAA yielded ions (Fig. 2) at m/z 190 and 130. The unlabeled methylated IAA from both sources yielded ions of m/z 189 and 130, and the methylated $^{13}C_6$-labeled IAA yielded ions at m/z 136 and 195. The calculation was done in a similar way as was done for IAN values. First, the ion intensities at m/z 189, 190, and 195 were corrected for natural abundance using the known natural abundance values (Silverstein et al., 1974). The natural abundance of (m+1)/z for m/z = 189 is 0.1148 and the (m−1)/z for ion at m/z 190 is 0.088 and for (m+1)/z for m/z 195 it is 0.059. Based on these values, then:

$$RI'_{189} = RI_{189} + RI_{189}(0.1148) - RI_{190}(0.088) \quad (4)$$

$$RI'_{190} = RI_{190} - RI_{189}(0.1148) + RI_{190}(0.1148) + RI_{190}(0.088) \quad (5)$$

and

$$RI'_{195} = RI_{195} + RI_{195}(0.059). \quad (6)$$

Next, the *RI*″ value at m/z 189 representing endogenous IAA was determined by subtracting the RI_{189} that was due to IAN breakdown as determined using CF_{IAN}, as follows:

$$RI''_{189} = RI'_{189} - CF_{IAN}(RI'_{190}). \quad (7)$$

After these calculations, it was possible to accurately measure the endogenous free plus ester and total IAA values using the isotope dilution equation:

$$Y = \left(\frac{C_i}{C_f} - 1\right) \times X, \quad (8)$$

where C_i is the fraction of *RI* at m/z 195 to the *RI* sum of m/z 189 plus m/z 195 in the internal standard, C_f is the calculated ratio for the isolated IAA sample, *X* is the amount of internal standard added (in ng/mg sample), and *Y* is the endogenous IAA in the sample (also in ng/mg sample). In this case the internal standard initially contained no ion intensity at m/z 189 and C_i = 1; thus, the isotope dilution equation simplifies to:

$$Y = \left(\frac{RI''_{189} + RI'_{195}}{RI'_{195}} - 1\right) \times X. \quad (9)$$

Table I. *The conversion of IAN to IAA by conditions of alkaline hydrolysis commonly used to study IAA and IAA conjugates*

Analysis was by GC-SIM-MS using [$^{13}C_6$]IAA as the internal standard. Values were calculated using the isotope dilution equation and are the averages ± SD of two samples.

Conditions	Percentage of Unlabeled IAA Found following Treatment Relative to the IAN Supplied
Untreated	<0.1[a]
1 N NaOH, 1 h, 25°C	3.1 ± 0.1
7 N NaOH, 3 h, 100°C	99.8 ± 0.2

[a] Below level of detection.

Isolation and Identification of IAN by Full-Scan GC-MS

A. thaliana ecotype Columbia carrying the *trp2-1* mutation has been described (Last et al., 1991). Seeds were surface sterilized (Last and Fink, 1988) and sown on plant nutrient medium with 0.5% Suc (Haughn and Somerville, 1986) solidified with 0.75% bacteriological grade agar (ICN). Plates were wrapped with Micropore tape (no. 1530-1, 3M) and incubated under continuous illumination (cool-white, 40-W fluorescent bulbs) with a light intensity of 100 to 160 μE m^{-2} at room temperature for 16 d. Tissue was harvested, weighed, immediately frozen in liquid N_2, and then stored at −80°C. [$^{13}C_1$]IAN (650 ng) in isopropanol was added to 1 mL of ice-cold extraction buffer in an

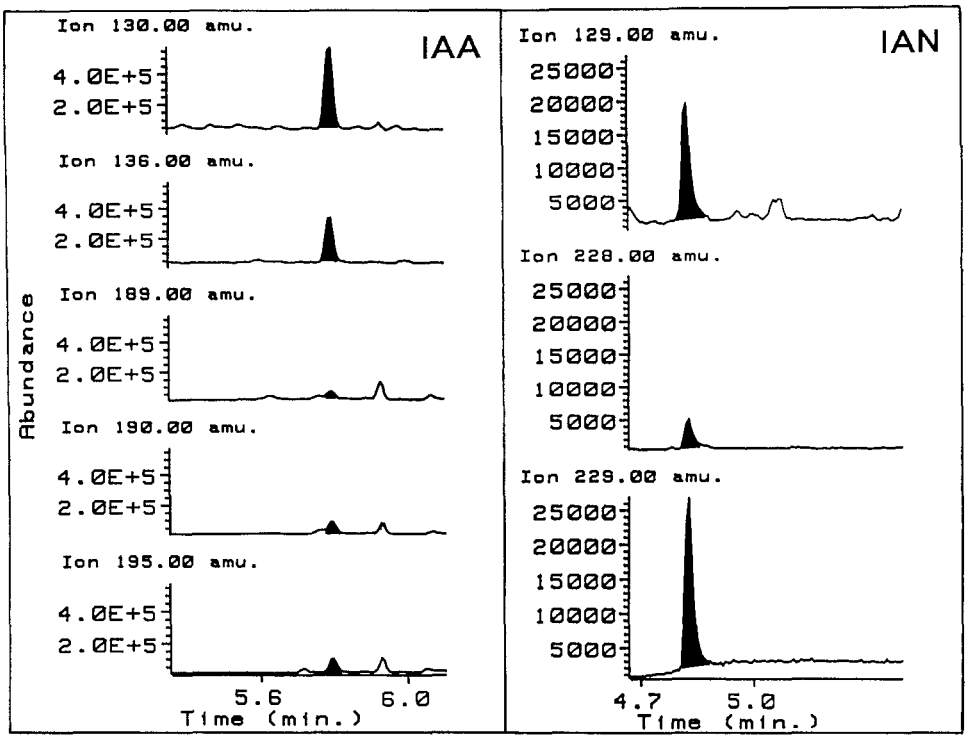

Figure 2. Selected ion chromatograms for samples of IAA (left) and IAN (right) isolated from Arabidopsis. Selected ion chromatograms are from a sample extract that contained both [$^{13}C_6$]IAA- and [$^{13}C_1$]IAN-added internal standards. For IAA analysis (as the methyl esters; left) the extract was subjected to 7 N NaOH hydrolysis at 100°C for 3 h. Ions at m/z 136 and 195 were from [$^{13}C_6$]IAA methyl ester, and the ion at m/z 190 was predominately from [$^{13}C_1$]IAA methyl ester derived following hydrolysis from the added [$^{13}C_1$]IAN. The ion at m/z 189 is mainly from methyl ester of unlabeled plant-derived IAA plus IAA generated from plant IAN. The ion at m/z 130 comes from the methyl ester of unlabeled plant-derived IAA and IAN as well as from [$^{13}C_1$]IAN. Ions at m/z 129, 228, and 229 shown on the right are from purified and derivatized IAN. Ion at m/z 229 is the molecular ion for trimethylsilyl-[$^{13}C_1$]IAN and corresponds to the m/z 228 molecular ion for the unlabeled trimethylsilyl-IAN. The ion at m/z 129 is formed by both labeled and unlabeled IAN, since the side chain is lost during fragmentation. It is possible to determine the amount of IAN in the plant based on the isotope dilution equation using proper correction factors (as described in "Materials and Methods"). With knowledge of the level of IAN, it is then possible, using the equations described in "Materials and Methods," to calculate accurately the level of total IAA in the sample.

ice-cold mortar. Approximately 660 mg of frozen tissue was added and ground with an ice-cold pestle. The isotope was allowed to equilibrate in the extract for 1 h at 4°C, and then the extract was centrifuged in a clinical centrifuge to pellet the cell debris. After isopropanol was removed from the supernatant in vacuo, the sample was brought to 2 mL with water and applied to an SPE amino column (PrepSep, 300 mg, no. P456, Fisher Scientific) that had been preconditioned as described by Chen et al. (1988). The nonretained fraction containing the IAN was collected and subjected to SPE C_{18} purification, HPLC fractionation, silanization, and full-scan GC-MS analysis using conditions as described in Normanly et al. (1993). The ions monitored were from m/z 50 to 250 (Fig. 1, B and C).

RESULTS AND DISCUSSION

Plant IAA is present as the free acid and also conjugated to a variety of compounds (Cohen and Bandurski, 1982). These conjugated forms can be classified as esterified and amide-bound forms based on their relative stability under basic conditions. Several workers have determined the conditions necessary for quantitative hydrolysis of such conjugates (Bandurski and Schulze, 1977; Cohen and Bandurski, 1982; Cohen et al., 1986). Thus, treatment with 1 N NaOH for 1 h at room temperature will result in the quantitative hydrolysis of ester forms with no measurable hydrolysis of amide conjugates (Baldi et al., 1989). Amide forms require more rigorous hydrolysis and are cleaved quantitatively by 7 N NaOH for 3 h at 100°C (Bialek and Cohen, 1989). Acidic hydrolysis is usually avoided because of the lability of the indole ring at low pH. At higher pH the indole ring is relatively stable (Bandurski and Schulze, 1974), but problems of oxidative degradation in crude extracts and plant residues have dictated the use of oxygen-free nitrogen as a purge gas during strong basic hydrolysis (Bialek and Cohen, 1989). These convenient methods for evaluation of IAA and conjugated IAA in plants were devised using a variety of different plants and confirmed using direct analysis of conjugates in comparison to the hydrolysis techniques (Epstein et al., 1986).

In the classic work of Stowe (1963) on the preparation of radiolabeled IAA, the conversion of the IAN intermediate

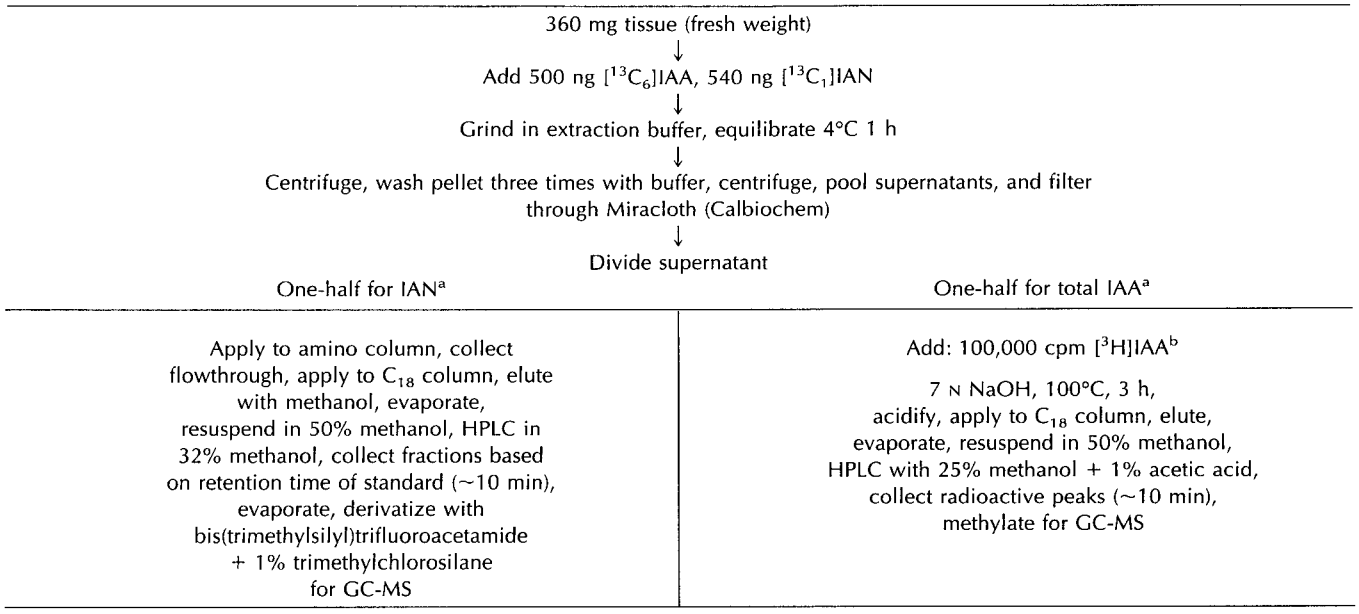

Figure 3. Flow diagram of the method used for isolation of IAA and IAN from Arabidopsis to obtain the raw data required for the calculation of the quantities of each compound present in the plant material using the equations described in "Materials and Methods." A representative example of the data obtained and the calculated values are given in Table II. [a] Sample can also be divided one-fourth for IAN and three-fourths for free, ester-linked, and total IAA. [b] If it is desired to determine free, ester-linked, and total IAA, sample can be divided again here into three separate samples.

to IAA was accomplished by basic hydrolysis using conditions similar to those used for amide conjugate hydrolysis (Bialek and Cohen, 1989). Thus, it was important to understand at least three aspects of indolic chemistry of Arabidopsis. First, we needed to know whether Arabidopsis contained IAN that could be converted to IAA under hydrolysis conditions and, if so, to what extent this conversion would occur during the two hydrolysis conditions utilized to measure ester and amide conjugates by hydrolysis. Second, we needed to know whether Arabidopsis contained compounds that, under conditions used for IAA analysis, could be converted to IAN and thus yield IAA. Finally, it was important to develop methods that allowed the accurate measurement of IAA, free plus ester IAA, and total IAA and IAN in Arabidopsis extracts.

Previously, we used isotope dilution analysis with [$^{13}C_1$]IAN to quantitate IAN in Arabidopsis (Normanly et al., 1993) using SIM GC-MS. IAN occurs as a native compound in Arabidopsis in the range of hundreds of nanograms per gram fresh weight, depending on growth conditions. In this study, we isolated IAN from the *trp2-1* mutant of Arabidopsis, which accumulates higher levels of IAN, and confirmed its identity by full-scan GC-MS, thus showing definitively that IAN is an endogenous component of the indolic pool in Arabidopsis (Fig. 1B) and fully confirming the tentative identification provided by the SIM technique.

As shown in Table I, mild base hydrolysis, such as that used for ester hydrolysis, results in about a 3% conversion of IAN to IAA. This level of conversion might be significant if the levels of IAN greatly exceed the amount of free plus ester IAA in the plant material. The conversion of IAN to IAA is essentially quantitative under the more severe basic hydrolysis used to release free IAA from amide-bonded forms (Table I; Fig. 3). Thus, the nonenzymatic conversion of IAN to IAA must be taken into account when measuring conjugated IAA levels in IAN-containing plants such as Arabidopsis (Fig. 3; Table II). This level of conversion will be significant if the level of IAN begins to reach levels similar to that of free plus ester IAA or total IAA in the plant material (Table II; Normanly et al., 1993). Therefore, an essential component of the process is to first determine the endogenous levels of IAN under mild conditions (so as to avoid hydrolysis; Fig. 3) and then to subtract this amount (using the correction factor described in "Materials

Table II. *Example of the isolation of IAA and IAN from Arabidopsis showing the data obtained from one representative sample*

Note that without the IAN correction factor, the value for total IAA would be 1.37 ng/mg or a 78% overestimate. Similar calculations for samples from the *trp 2-1* mutant yielded a total IAA level of 23.03 ng/mg with the IAN correction and 28.98 ng/mg without it (a 21% difference).

Ion	Peak Area	Peak Area Corrected for Natural Abundance	IAN Correction Factor	ng/mg
228	1,351.66	1,328.97	0.37	0.56 (IAN)
229	3,053.34	3,631.74		
189	82,916.45	84,345.99		0.77 (total IAA)
190	91,923.55	101,046.83		
195	80,460.45	85,207.62		

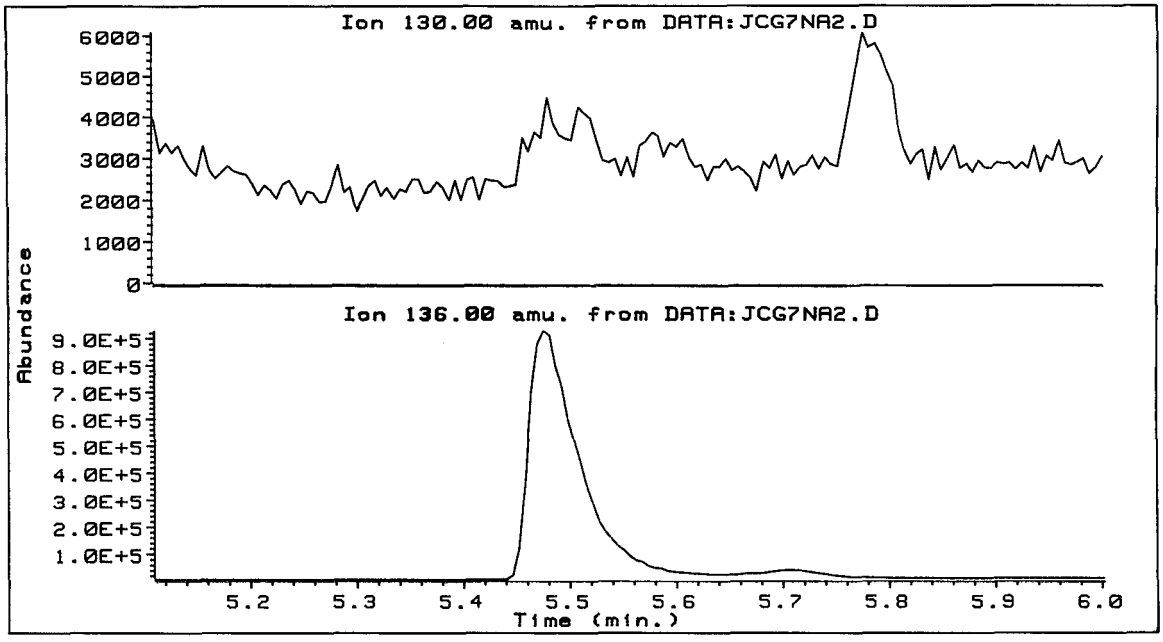

Figure 4. Selected ion chromatograms from IAA and [$^{13}C_6$]IAA methyl esters analyzed by GC-SIM-MS. Sample was prepared for analysis following treating a 10-μg standard of glucobrassicin and 1 μg of [$^{13}C_6$]IAA internal standard to 7 N NaOH hydrolysis for 3 h at 100°C. Note the difference in *Y* axis scales, which differ by a factor of 150.

and Methods") from the IAA measured in the portion of the sample that underwent base hydrolysis (Table II). For example, the superroot (*sur*) and rooty (*rty*) mutants of Arabidopsis were reported to have increased levels of IAA (Boerjan et al., 1995; King et al., 1995). These determinations were made without quantifying endogenous IAN levels, so the actual levels of total IAA in these mutants may be substantially different from those reported.

The presence of very large amounts of the glucosinolate glucobrassicin could have profound consequences for analytical studies, since the early chemical literature (Gmelin, 1964) indicated that glucobrassicin was both acid and base labile and that IAN was one of several breakdown products produced under such conditions. To assess the extent of glucobrassicin degradation under basic conditions, the compound was isolated and tested using isotope dilution techniques following base treatment under conditions used for IAA conjugate hydrolysis (Fig. 4). Although a slight change in the baseline close to the signal-to-noise level of the chromatogram is noted in the m/z 130 ion at the retention time of IAA (as shown by the heavily overloaded m/z 136 peak), this would account for less than 0.03% conversion of the added glucobrassicin in unlabeled IAA. These data indicate that glucobrassicin conversion to IAA during basic hydrolysis using the conditions used for IAA studies does not occur at measurable levels. Based on the lability of IAN under the conditions of hydrolysis, this result implies that the hydrolytic conversion of glucobrassicin to IAN also did not occur.

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LITERATURE CITED

Baldi BG, Maher BR, Cohen JD (1989) Hydrolysis of indole-3-acetic acid esters exposed to mild alkaline conditions. Plant Physiol **91:** 9–12

Bandurski RS, Schulze A (1974) Concentration of indole-3-acetic acid and its esters in *Avena* and *Zea*. Plant Physiol **54:** 257–262

Bandurski RS, Schulze A (1977) Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol **60:** 211–213

Bialek K, Cohen JD (1989) Quantitation of indoleacetic acid conjugates in bean seeds by direct tissue hydrolysis. Plant Physiol **90:** 398–400

Boerjan W, Cervera M-T, Delarue M, Beeckman T, Dewitte W, Bellini C, Caboche M, Van Onckelen H, Van Montagu M, Inze D (1995) *superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. Plant Cell **7:** 1405–1419

Chen K-H, Miller AN, Patterson GW, Cohen JD (1988) A rapid and simple procedure for purification of indole-3-acetic acid prior to GC-SIM-MS analysis. Plant Physiol **86:** 822–825

Cohen JD (1984) Convenient apparatus for the generation of small amounts of diazomethane. J Chromatogr **303:** 193–196

Cohen JD, Baldi BG, Slovin JP (1986) $^{13}C_6$-[Benzene ring]-indole-3-acetic acid. A new internal standard for quantitative mass spectral analysis of indole-3-acetic acid in plants. Plant Physiol **80:** 14–19

Cohen JD, Bandurski RS (1982) Chemistry and physiology of the bound auxins. Annu Rev Plant Physiol **33:** 403–430

Ehmann A (1977) The Van Urk-Salkowski reagent—a sensitive and specific chromagenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. J Chromatogr **132:** 267–276

Epstein E, Baldi BG, Cohen JD (1986) Identification of indole-3-acetylglutamate from seeds of *Glycine max* L. Plant Physiol **80:** 256–258

Ettlinger MG, Dateo GP, Harrison BW, Mabry TJ, Thompson CP (1961) Vitamin C as a coenzyme: the hydrolysis of mustard oil glucosides. Proc Natl Acad Sci USA **47:** 1924–1935

Gmelin R (1964) Occurrence, isolation and properties of glucobrassicin and neoglucobrassicin. *In* JP Nitsch, ed, Régulateurs Naturels de la Croissance Végétale. Édition du Centre National de la Recherche Scientifique, Paris, pp 159–167

Gmelin R, Virtanen AI (1961) Glucobrassicin der precursor von SCN^-, 3-indolylacetonitril und ascorbigen in *Brassica oleracea* species. Ann Acad Sci Feen Ser A **107:** 1–25

Haughn GW, Davin L, Giblin M, Underhill EW (1991) Biochemical genetics of plant secondary metabolites in *Arabidopsis thaliana*. Plant Physiol **97:** 217–226

Haughn GW, Somerville C (1986) Sulfonylurea-resistant mutants of *Arabidposis thaliana*. Mol Gen Genet **204:** 430–434

King JJ, Stimart DP, Fisher RH, Bleecker AB (1995) A mutation altering auxin homeostasis and plant morphology in *Arabidopsis*. Plant Cell **7:** 2023–2037

Kutacek M, Kefeli VI (1968) The present knowledge of indole compounds in plants of the *Brassicacea* familiy. *In* R Wightman, G Setterfield, eds, Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa, Ontario, Canada, pp 127–152

Last RL, Bissinger PH, Mahoney DJ, Radwanski ER, Fink GR (1991) Tryptophan mutants in *Arabidopsis*: the consequences of duplicated tryptophan synthase β genes. Plant Cell **3:** 345–358

Last RL, Fink GR (1988) Tryptophan-requiring mutants of the plant *Arabidopsis thaliana*. Science **240:** 257–264

Normanly J, Cohen JD, Fink GR (1993) *Arabidopsis thaliana* auxothrops reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. Proc Natl Acad Sci USA **90:** 10355–10359

Normanly J, Slovin JP, Cohen JD (1995) Rethinking auxin biosynthesis and metabolism. Plant Physiol **107:** 323–329

Schoepf C, Thesing J (1951) Die Umsetzung quartaerer Salze von Mannich-Basen mit tertiaeren Aminen. Angew Chem **63:** 377–378

Silverstein RM, Bassler GC, Morrill TC (1974) Spectrometric Identification of Organic Compounds, Ed 3. John Wiley & Sons, New York

Stowe BB (1963) Synthesis of high specific activity ^{14}C-carbonyl indoleacetic acid and of ^{14}C-nitrile indoleacetonitrile. Anal Biochem **5:** 107–115

Stowe BB, Vendrell M, Epstein E (1968) Separation and identification of indoles of maize and woad. *In* R Wightman, G Setterfield, eds, Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa, Ontario, Canada, pp 173–182

Sutter EG, Cohen JD (1992) Measurement of indolebutyric acid in plant tissues by isotope dilution gas chromatography-mass spectrometry analysis. Plant Physiol **99:** 1719–1722

Underhill EW (1980) Glucoinolates. *In* EA Bell, BV Charlwood, eds, Secondary Plant Products, Encyclopedia of Plant Physiology, New Series, Vol 8. Springer-Verlag, Berlin, pp 493–511