Plant Physiol. (1986) 80, 14–19
0032-0889/86/80/0014/06/$01.00/0

$^{13}C_6$-[Benzene Ring]-Indole-3-Acetic Acid

A NEW INTERNAL STANDARD FOR QUANTITATIVE MASS SPECTRAL ANALYSIS OF INDOLE-3-ACETIC ACID IN PLANTS

Received for publication April 1, 1985 and in revised form August 12, 1985

JERRY D. COHEN*, BRUCE G. BALDI[1], AND JANET PERNISE SLOVIN
United States Department of Agriculture, Agricultural Research Service, Plant Hormone Laboratory, Beltsville Agricultural Research Center-West, Beltsville, Maryland 20705

ABSTRACT

Indole-3-acetic acid (IAA) labeled with ^{13}C in the six carbons of the benzene ring is described for use as an internal standard for quantitative mass spectral analysis of IAA by gas chromatography/selected ion monitoring. [$^{13}C_6$]IAA was compared to the available deuterium labeled compounds and shown to offer the advantages of nonexchangeability of the isotope label, high isotopic enrichment, and chromatographic properties identical to that of the unlabeled compound. The utility of [$^{13}C_6$]IAA for measurement of endogenous IAA levels was demonstrated by analysis of IAA in *Lemna gibba* G-3.

Techniques for identification and quantitative analysis of endogenous plant hormones are important for studies of the physiology and biochemistry of these regulatory messengers of higher plants. The use of combined GC-MS for both identification and quantification has proven to be the most accurate and certain method for plant hormone analysis. Even MS has its limitations and a major difficulty in the application of GC-MS to plant hormone analysis has been the limited availability of the appropriate stable isotope labeled 'heavy' internal standards.

Various internal standards have been utilized for the analysis of IAA. For example, 5-methyl-IAA (26), indole-3-butyric acid (20), and indole-3-propionic acid (24) as well as ^{3}H and ^{14}C labeled IAA have been used to correct for losses. A double internal standard GC method utilizing [^{14}C]indole-3-butyric acid and [^{14}C]IAA has also been used to determine the levels of IAA in plants (12). Most recent reports of quantitative GC-MS analysis of IAA have utilized deuterium labeled IAA as an internal standard. IAA-2,2-d_2 has been used as an internal standard by several groups (6, 14, 16), although, with this compound problems of deuterium exchange can lead to erroneous results as described in detail by Magnus *et al.* (15) and by Caruso and Zeisler (7). To avoid such problems and to have a compound greater than three mass units heavier than naturally occurring IAA, so as to avoid the normal isotope cluster, Magnus *et al.* (15) synthesized two ring labeled compounds. The most effective of the two compounds described was 4,5,6,7-d_4-IAA, and this internal standard has been used for a number of investigations (5, 12, 13, 19, 23). Probably due to the recent commercial availability of 2,4,5,6,7-d_5-IAA (described by Magnus *et al.* [15] several groups have adopted this internal standard for the quantitative GC-MS analysis of IAA (1, 2, 21). However, the slight exchange of the indole-2 deuterium renders this compound less desirable for studies in which acidic conditions are used or where harsh alkaline conditions are employed for hydrolysis of amide-linked IAA conjugates (2, 15).

For our studies of the levels of IAA and IAA conjugates in plant tissues it was desirable to have an internal standard that contained stable isotopes in structural positions covalently linked within the molecule. The use of ^{13}C to label the six carbons of the benzene ring of IAA provides such an internal standard. The [$^{13}C_6$]IAA internal standard has been used to measure precisely the IAA levels in the duckweed *Lemna gibba* G-3. *Lemna* is one of the few higher plants that can be used intact for pulse chase experiments under sterile conditions (28). This makes it a particularly suitable organism for studying indole metabolism.

MATERIALS AND METHODS

Apparatus. All mass spectra and GC-SIM[2]-MS were obtained on a Hewlett-Packard[3] 5992A GC-MS. The instrument was modified as follows: HP18740B capillary inlet system, direct capillary connection, improved ion source, Edwards E2M2 roughing pump, and a 'B' type diffusion pump. SIM analysis was done using a 4 ion program dwelling on each ion for 400 ms. For methyl esters using [$^{13}C_6$]IAA as the internal standard, the ions at m/z 130, 136, 189, and 195 were monitored. The GC-MS was operated for SIM using underresolved peaks ('fat peak monitoring') and a window size of 0.30 atomic mass unit. All work was performed on WCOT fused silica columns. Two types were used: a 12 meter cross-linked methyl silicone column with a film thickness of 0.33 μm and an internal diameter of 0.20 mm (Hewlett-Packard 19091-60312) and an 11 meter mid-polarity bonded phase column of CP Sil 19 CB with a film thickness of 0.18 μm and an internal diameter of 0.31 mm (Chrompack 7732-214620). The injector was at 250°C and He at 1 ml/min was the carrier gas. With the Hewlett-Packard column the oven was at 160°C for 2 min followed by temperature programming at 10°C/min. The Chrompack column was held at 140°C for 2 min followed by temperature programming at 5°C/min. Injections were made in the splitless mode with the column vented for the first 1.5 min.

Fourier transform IR spectra were obtained in KBr on a Nicolet 60SX instrument.

Reagents. Stable isotope labeled compounds were obtained as follows: Indole-3-acetic-2,2-d_2 acid (MD-1709, lot 2555-I) and

[1] Current address: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164.

[2] Abbreviation: SIM, selected ion monitoring.

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

indole-2′,4′,5′,6′,7′-d_5-3-acetic acid (custom synthesis, now available as MD-2203) from MSD Isotopes/Merck Frosst Canada, Montreal; benzene ($^{13}C_6$, 99%, No. 530758) from Kor Isotopes, Cambridge, MA; aniline (UL-^{13}C, 90%, No. C-36, lot MZ179) from Stohler Isotope Chemicals, Waltham, MA; aniline (UL-$^{13}C_6$, 99+%, No. CLM-714) from Cambridge Isotope Laboratories, Woburn, MA; and indole-4′,5′,6′,7′-d_4-3-acetic acid was a gift from Drs. V. Magnus and R. S. Bandurski (Michigan State University).

Synthesis. The synthesis of [$^{13}C_6$]IAA at 90% enrichment was by a procedure similar to that described by Robinson (27) and by Baldi *et al.* (3). A brief prior report of this synthesis has appeared (11). [^{13}C]Aniline (100 mg) was dissolved in diethyl ether and crystallized as the hydrochloride by gently bubbling with HCl gas. The aniline hydrochloride crystals were dissolved in 1 ml of 1 M HCl and then diluted to 6 ml with water. A freshly prepared solution (2 ml) of 1 M $NaNO_2$ was added dropwise at 0°C. Following the addition of the $NaNO_2$, 15 ml of 25 mM $NaHSO_3$ was rapidly added and the mixture refluxed for 2 h. After the first reflux, 2.5 ml of concentrated HCl was added and the mixture was refluxed under N_2 gas for an additional 3 h. The resulting solution was reduced in volume on a rotary evaporator and the precipitated phenylhydrazine was collected and dried *in vacuo* over P_2O_5 overnight. A mixture of 7.5 ml 37% HCl and 2.5 ml 85% H_3PO_4 was added to the dry precipitate and then 7.5 ml of pyridine containing 236 mg of 2-oxoglutaric acid was added dropwise. The resultant suspension was refluxed for 2 h, the supernatant solution removed and partitioned against chloroform (three times) at acidic pH, and the product purified by column chromatography on Sephadex LH-20 using 50% 2-propanol/water as mobile phase. Two fractions were obtained, one corresponding to authentic [$^{13}C_6$]IAA (yield, based on aniline, was 2.1%) and a second at the retention volume of 2-carboxy-indole-3-acetic acid-$^{13}C_6$ (yield, based on aniline, was 14.6%). The 2-carboxy-IAA-$^{13}C_6$ fraction was saved for later conversion to [$^{13}C_6$]IAA (15).

Two unsuccessful attempts were made to produce [$^{13}C_6$]IAA from benzene (11). The third attempt, therefore, to obtain highly enriched [$^{13}C_6$]IAA was from [U-$^{13}C_6$]aniline (99+%) at a 1 g scale (highly enriched [^{13}C]aniline has only very recently become available). The synthesis, performed by the MSU Chemical Synthesis Laboratory, yielded 380 mg, (19.6% from aniline) of a white crystalline product, melting point 160 to 162°C. The identity of the product was confirmed by IR and mass spectral analysis.

Selective Hydrolysis of Amide and Ester IAA Conjugates. To determine the levels of IAA that are present as ester or amide-linked conjugates, it is necessary to hydrolyze plant samples using basic conditions to yield free IAA from the conjugated forms. The conditions of hydrolysis we employed were essentially as reported by Bandurski and Schulze (4). We checked the specificity of the hydrolysis using the following model compounds: IAA ethyl ester (Calbiochem), IAA *t*-butyl ester (synthesized from IAA and *t*-butyl alcohol by coupling with dicyclohexylcarbodiimide in acetonitrile), IAA pentafluorobenzyl ester (synthesized as in Epstein and Cohen [13]), IAA *n*-hexyl ester (Vega Biochemicals), IAA *p*-nitrophenyl ester (a gift from R. Hangarter), indole-3-acetyl-*myo*-inositol (synthesized as in Nowacki *et al.* [22]), and IAA conjugates with the amino acids L-alanine, DL-aspartate, L-phenylalanine, glycine (Research Organics), ε-lysine (a gift from M. P. Sanger), L-leucine, β-alanine (gifts from M. Peterson) and L-glutamate (synthesized as in Cohen [9]). Using this range of different ester and amide conjugates we were able to confirm that treatment with 1 N NaOH for 1 h at room temperature resulted in the quantitative hydrolysis of all of the ester conjugates listed yet did not hydrolyze detectable levels (>0.05%) of the amide conjugates. The treatment with 7 N NaOH for 3 h at 100°C was sufficient to quantitatively hydrolyze all of the compounds listed above.

Plant Material. *Lemna gibba* L. G-3 plants in sterile culture were obtained from Dr. C. F. Cleland (Smithsonian Environmental Research Center). Plants were grown on 1 L of E medium (8) in 2 L flasks, essentially as described by Slovin and Tobin (28) except that the plants were maintained in a nonflowering condition (8 h white light, 16 h dark cycle) and the temperature in the growth chamber was 26 to 27°C. After 4 weeks, the plants were harvested, rinsed with distilled H_2O, blotted dry with soft absorbent paper, and the wet weight determined. They were then frozen in liquid N_2 and stored at −70°C until use.

Quantitative Assay of IAA. The major differences in the technique we utilize and that reported by Magnus *et al.* (15) are: use of a high resolution 5 μm C_{18}-HPLC column with high carbon loading in place of the 10 μm less highly substituted chromatographic material used in the earlier report; the use of fused silica bonded phase capillary GC columns in place of the packed column; the use of a benchtop laboratory grade GC-MS in place of the research grade instrument used previously; and, in particular, the use of the [^{13}C]IAA internal standard in place of the deuterium standard reported by Magnus *et al.* (15).

Plant material estimated to contain at least 50 ng of IAA (or of IAA plus IAA-conjugates, if the sample was to be hydrolyzed) was added to 2 ml/g fresh weight of acetone (at −50°C) containing about a 10-fold greater amount of [$^{13}C_6$]IAA (that is, 0.5 μg or more) and 50,000 dpm of [methylene-^{14}C]IAA (59 mCi/mmol, Amersham) and the tissue homogenized at −50°C for 2 min at high speed in a Waring Blendor (model 707SB-31BL47). The radiolabeled IAA serves to aid in peak detection and its mol wt of 177 separates it from the analyzed ions. It is important to note that compounds with very high enrichment of ^{14}C at a single position and minimum amounts must be used to avoid significant mass 175 IAA addition to the extract. After isotope equilibration and extraction (1.5 h or overnight) at 4°C, the residue was filtered at room temperature through Reeve Angel No. 802 paper, the residue washed with 70% acetone, and the combined acetone extracts reduced to the water phase *in vacuo*.

For measurement of total IAA, the suspension was made to 7 N with respect to NaOH. The alkaline solution was placed in a heating mantel at 100°C and a purge of water saturated nitrogen maintained throughout a 3 h hydrolysis period. For determination of free plus esterified IAA, we made the suspension to 1 N NaOH by addition of an appropriate volume of freshly prepared 5 N NaOH. Hydrolysis was for 1 h at room temperature (25°C). For the determination of free IAA the water phase was used directly.

The hydrolysates or water phase were adjusted to pH 7.0 with HCl or with NaOH and partitioned with chloroform. The aqueous phase was then adjusted to pH 2.5 and extracted three times with chloroform.

A note of caution should be made with regard to the solvent used for partitioning. Chloroform that is stored without preservative, thus containing traces of carbonyl chloride, should be avoided since it can result in total loss of the IAA. Diethyl ether is also a suitable solvent and we have used it in place of chloroform; however, peroxide contamination of ether can be equally disastrous. It is recommended that, at a minimum, one should use reagent grade chloroform containing ethanol as preservative (avoid glass distilled HPLC grade without preservative), freshly distilled diethyl ether (distilled from sodium or potassium metal in an N_2 atmosphere), or a freshly opened dated can of 'anesthesia grade' diethyl ether.

After drying over anhydrous granular sodium sulfate for 1 h, the acid chloroform phase was filtered and then evaporated *in vacuo* to dryness in a rotary evaporator with bath temperature at 45°C. The residue was redissolved in 100 μl of 50% methanol/

water (v/v). The sample was further purified by HPLC on a C_{18} reverse phase column. Columns used in this work were 4.6 mm × 250 mm Whatman Partisil ODS-3 5 μm columns packed at 600 bar with a slurry of 2.5 g of media in 25 ml of 50% ethylene glycol/methanol and methanol was the packing solvent. A guard column containing Whatman Co:Pell ODS or Whatman Pellicular ODS media was used. The solvent was 35% methanol/water plus 1% acetic acid and the flow rate was maintained at 1 ml/min. Under these conditions IAA had an elution volume of 18 ml, the plate number/meter calculated for IAA was 12,200, and the peak symmetry was 1.1.

The IAA containing fraction (located by counting radioactivity in the fractions) was reduced to dryness. This was done in a custom modified 'short path' version of a Heidolph microrotary evaporator using −20°C cooling and a two-stage vacuum pump. The dry sample was resuspended in 100 μl methanol and methylated with diazomethane as previously described (10). The methylated sample, resuspended in ethyl acetate, was analyzed immediately by GC-SIM-MS. Ions at m/z 136 and 195 were monitored for the base peak and molecular ion, respectively, of the $[^{13}C_6]$IAA internal standard and ions at m/z 130 and 189, the base peak and molecular ion of the plant IAA, were also monitored. The ratio of 130:136 was used to calculate the endogenous content of IAA and the ratio of 189:195 was used for confirmation. This procedure was preferred over an average of the two ratios because of the better ion statistics for the more abundant base peak (quinolinium ion); however, differences of more than 5% were considered an indication of an invalid analysis. The most common reason for such an invalid run was traced to the inability of the GC-MS data system to integrate low abundance peaks accurately. This problem could often be overcome by manual integration of the peak data.

RESULTS AND DISCUSSION

Quantitative Analysis. The amount of free IAA in *Lemna gibba* G-3 was calculated from the data in Figure 1 using a modification of the isotope dilution equation:

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

where Y is the amount of free IAA in the tissue; C_i is the initial percentage of m/z 136 relative to 130 + 136, or 100%; C_f is the percentage of m/z 136 relative to the total ion current at m/z 130 and 136 found by GC-SIM-MS, or 75.6%; X is the amount of $[^{13}C_6]$IAA added, or 1.16 μg; and R is the ratio of the fraction of endogenous IAA that has a peak at m/z 130 to the fraction of the internal standard that is fully substituted and has an ion at m/z 136. These were empirically determined to be 0.89 and 0.79, respectively, therefore $R = 1.13$. Thus, in this sample from 50 g of *Lemna gibba* the IAA content was:

$$\left(\frac{100}{75.6} - 1\right) \frac{1.16\ \mu g}{1.13} = 0.331\ \mu g/50\ g \text{ fresh weight}$$

In a pooled analysis of 382 g of *Lemna* divided into six fractions and analyzed as paired samples (two samples for free IAA, two for free + ester, and two for total IAA), the replicate samples were within 4% of each other. The maximum deviation was in the free IAA determination due to the low amount of material in the sample.

Isotope Enrichment. $[^{13}C_6]$IAA at two levels of enrichment was produced from aniline. The advantage of the high enrichment level is clearly shown in the mass spectra of the synthetic compounds (Fig. 2). The IAA obtained from aniline at 90% enrichment has only 39% of the total ion abundance at the $m + 6$ peak, the remainder was in the $m + 8$ (1%), $m + 7$ (3%), $m + 5$ (32%), $m + 4$ (14%), $m + 3$ (9%), and $m + 2$ (2%) region. The

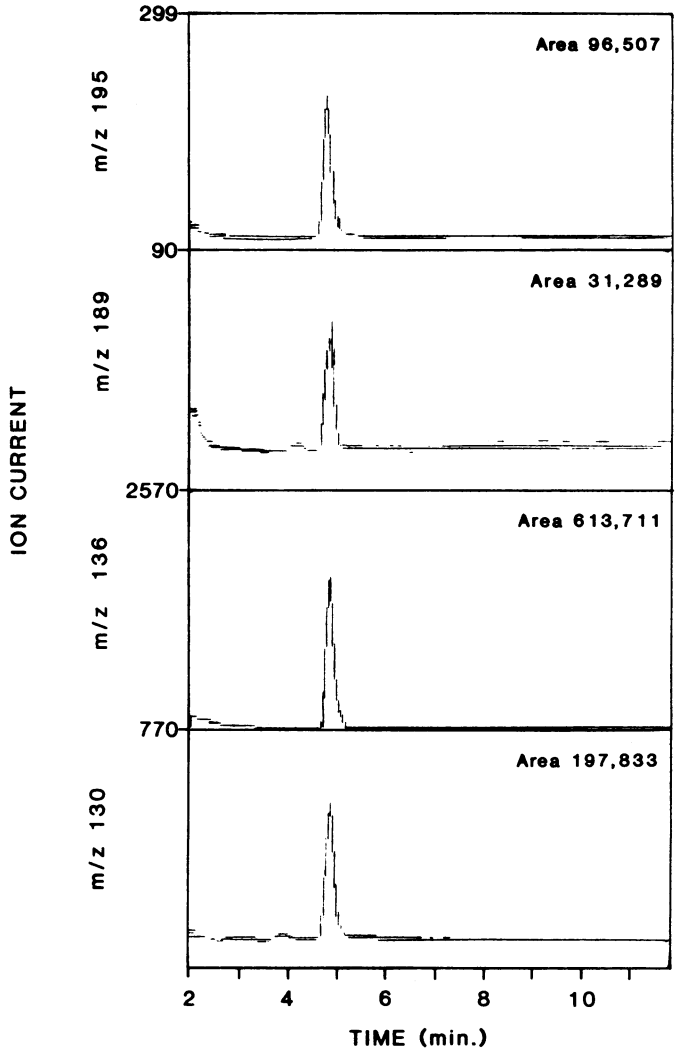

FIG. 1. Selected ion chromatogram of methylated HPLC purified sample of free IAA from *Lemna gibba* G-3. The ions at m/z 130 and 189 are from the quinolinium ion and molecular ion, respectively, of the methyl ester of the endogenous IAA. Ions at m/z 136 and 195 are the corresponding ions from the $[^{13}C_6]$IAA internal standard. The chromatogram was obtained on an 11 meter fused silica column of CP Sil 19 CB.

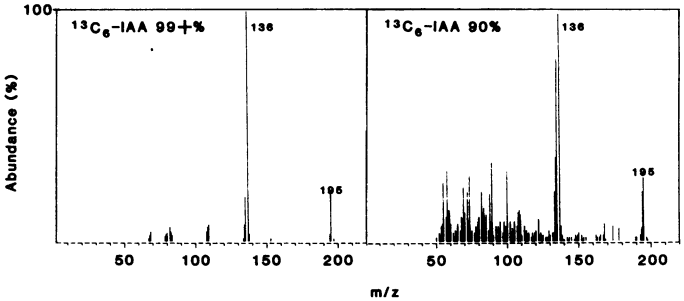

FIG. 2. Mass spectra of methyl esters of $[^{13}C_6]$IAA prepared from aniline of 99+% and 90% enrichment (according to supplier). Improved clustering of isotopes at the higher enrichment is indicated by the decrease in the ions at 134 and 135 relative to 136 and, also, 193 and 194 relative to 195.

IAA produced from 99+% enrichment aniline shows better clustering of the ions toward the $m + 6$ region with 78.6% at the $m + 6$ region, 3% at $m + 7$, 13.4% at $m + 5$, and 5% at $m + 4$. These values are somewhat lower than what would be predicted from theory. For the 90% enrichment the calculated abundance should be given by:

$$M_a = E^n = 0.90^6 = 0.53^4$$

where M_a is the abundance of the ion of the fully substituted isotope, E is the enrichment, and n is the number of substituted positions. This equation is derived from the more complex form used for calculation of isotopic abundance for each partially or totally enriched species (from the expansion of the binomial[4]: $[E_1 + E_2]^n$). The $m + 7$ and $m + 8$ abundance are due to the natural occurrence of ^{13}C in the other carbons of the molecule and, also, a small amount is due to the natural occurrence of deuterium, ^{15}N, and ^{18}O. Thus, for the 90% enrichment compound the sum of $m + 8$, $m + 7$, and $m + 6$ should be 53% but was found to be only 43%. Similarly, the 99+% compound should have had an abundance of 94% but was determined to be only 81.6%. These values would correspond to average enrichment levels of 86.9 and 96.7% for the compounds produced from the 90 and 99% aniline, respectively. This difference of 2 to 3% between the theory and the actual data could be due to an isotope effect (18) on the loss of a proton during fragmentation, or the actual enrichment could have been less than that indicated by both suppliers. Contamination during synthesis would lead to major m/z 130 and 189 ions, and this was not observed. Regardless of these considerations, the compound produced from the 99% aniline produced minimal fragmentation at lower masses with the methyl ester being completely devoid of ions at 130, 131, 132, 189, 191, and 192, and thus the product is an excellent internal standard for quantitative GC-SIM-MS.

Comparison to Deuterium Labeled IAA Standards. The [$^{13}C_6$] IAA standard has the advantage of the assurance of no isotope exchange under the conditions of sample preparation and purification. In addition, its high mass relative to the naturally occurring IAA avoids overlap between the internal standard and the endogenous compound.

IAA with a single substitution of deuterium or ^{13}C have been produced for various purposes (1, 17, 25); however, their extensive overlap with the isotope cluster from the naturally occurring IAA makes them of little value for quantitative analysis by GC-SIM-MS. Similarly, the compound with deuterium substitutions on the methylene carbon shows significant isotope overlap and, in addition, undergoes rapid isotope exchange under those conditions used for IAA isolation, derivatization, and analysis (7, 15). For example, 7 N NaOH hydrolysis such as is employed to release IAA from amide conjugates, results in major loss of deuterium from the methylene carbon (Table I). Although methylene-d_2-IAA has been used in a number of investigations for quantitative GC-SIM-MS, large errors have been reported with its use (7) and it cannot be recommended for such studies.

Polydeutero-IAA has been recommended for IAA analysis because it avoids the problem of isotope overlap, ring deuterium labels are much less prone to exchange than are those in the side chain, and it is relatively economical to obtain (deutero-aniline, for example, is available for about 5% of the cost of [^{13}C]aniline) (1, 2, 15). 4,5,6,7-d_4-IAA is an excellent standard for such use; however, it is not available from commerical sources. Considering the time, deuterated solvents, and special equipment required to produce this compound, it is doubtful that it can be synthesized for less than the cost of the highly enriched [^{13}C]IAA compound we have utilized. Use of the ^{13}C label eliminates the need to use special sealed containers, to develop special procedures, and to use isotopically enriched solvents and other reagents during synthesis. Thus, although the cost of the initial labeled compound is higher, the synthesis is considerably less complex.

2,4,5,6,7-d_5-IAA is now commercially available and is a reasonable internal standard for many uses. However, the deuterium at the indole-2 position has produced some difficulties during derivatization (2) when acidic conditions were employed and Magnus *et al.* (15) reported a small amount of exchange following treatment with 7 N KOH at 100°C for 3 h. We have also found a small amount of deuterium exchange following strong base treatment of 2,4,5,6,7-d_5-IAA (Table I). It is, therefore, probably a reasonable internal standard for analysis of free IAA levels in tissues only when mild conditions are employed during isolation and for derivatization.

One major limitation on the use of polydeutero-IAA internal standards is the chromatographic fractionation that occurs during purification using high resolution capillary GC columns (Fig. 3). This fractionation is not the result of a separation based on only the mass difference of the deutero-IAA and the unlabeled compound since [$^{13}C_6$]IAA does not fractionate. Thus, the deuterium labeling imparts some physical difference in the molecule that changes its chromatographic properties. This difference in the chromatographic properties of d_5-IAA and unlabeled IAA allowed a complete separation during reverse phase HPLC (10% methanol/water plus 1% acetic acid on a Waters C_{18} μBondapak column). However, under these same conditions [$^{13}C_6$]IAA did not separate from unlabeled IAA (T. J. Wodzicki and R. P. Pharis, personal communication). Fractional separations such as this can lead to enrichment in one or the other isotopic form during purification if fairly broad fractions are not collected. However, collection of larger fractions or monitoring ions over a broader time period would seem to preclude full utilization of the separation potential of these high resolution systems. [$^{13}C_6$] IAA thus has the advantage over the polydeuterium labeled IAA standards in that we have not been able to separate it from naturally occurring IAA by any of the chromatographic methods we have employed in our laboratory and it does not fractionate in the HPLC system noted above.

The use of [$^{13}C_6$]IAA as an internal standard for IAA analysis by GC-SIM-MS should be a significant improvement in the application of MS techniques for precise measurement of endogenous IAA levels in plant tissues. This method is invaluable for studies of hormonal responses in plants and provides a reference for evaluation of simpler, faster, or more sensitive, but less chemically rigorous, techniques such as chromatographic or immunological methods (12, 13, 19, 23). The levels of IAA in members of the Lemnacea have been described in early work by Thimann *et al.* (29) who used bioassays to show that auxin was present in bound and free forms in *Lemna minor* and, in later work, by Witztum *et al.* (30), who used a spectrofluorometric method that indicated that there was greater than 2 μg free IAA per g fresh weight of *Spirodela oligorhiza*. Our finding of 6.62 ng of free IAA per g fresh weight of *Lemna gibba* grown under conditions similar to those in the study by Witztum *et al.* (30) suggests either that there is a significant problem with the spectrofluorometric technique that they employed, that there is dramatic species heterogeneity within this taxonomic family, or that members of this family undergo dramatic changes in their IAA levels during the culture cycle.

[4] This equation does not take into account the contribution of the naturally occurring stable isotopes from other parts of the molecule that may be present in a partly labeled species (*i.e.* $^{13}C_5$ or $^{13}C_4$-IAA). Depending on the convention used to define 'enrichment,' the equation used could be considered a slight underestimate of the expected enrichment. Using a more complex expansion that takes this into account, the calculated enrichments for the two compounds produced would be 86 and 96.5%.

Table I. *Effect of Treatment with 7 N NaOH at 100°C for 3 h on Mixtures of Deuterium or ^{13}C-labeled IAA and Unlabeled IAA as Measured by the Ratio of the Quinolinium Ions at Their Apparent Mass*

Values given are the mean ± SE of the ratios obtained ($n = 6$), except for the values in footnote b that report the mean of duplicate determinations.

Labeled Compound	Ions m/z	Ratio Before Alkali Treatment	Ratio After 7 N NaOH Treatment[a]
d_2-methylene-IAA	130:132	1.468 ± 0.010	47.375 ± 7.281[b]
4,5,6,7-d_4-IAA	130:134	0.898 ± 0.018	0.906 ± 0.036
2,4,5,6,7-d_5-IAA	130:135	1.275 ± 0.030	1.480 ± 0.067
$^{13}C_6$-IAA	130:136	0.979 ± 0.013	0.976 ± 0.015

[a] Treatment was in 7 N NaOH *in vacuo* in a sealed, N_2 flushed ignition tube, incubated for 3 h at 100°C. [b] After treatment of the d_2-IAA/IAA mixture for 3 h in 7 N NaOH at 25°C the ratio was 2.017; after incubation with Dowex 1 (hydroxide form) for 3 h at 25°C the ratio was 1.621; and with DEAE-Sephadex (acetate) for 3 h at 25°C the ratio was 1.564.

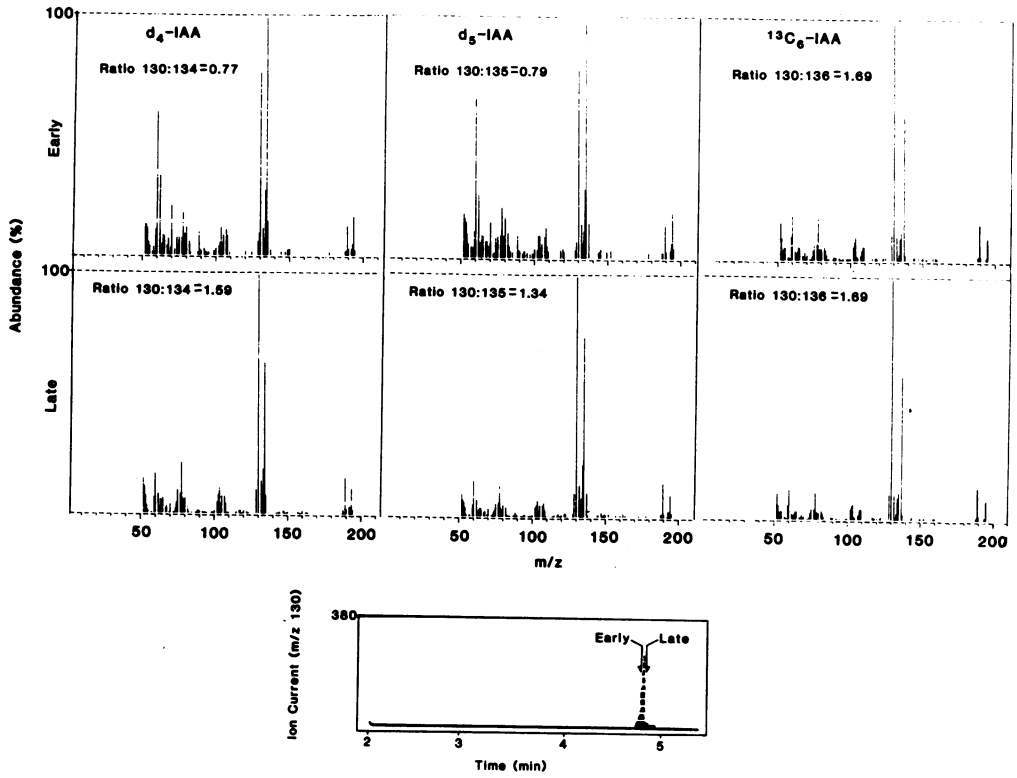

FIG. 3. Mass spectra of mixtures of unlabeled and heavy isotope labeled IAA methyl esters obtained by GC-MS. The top row of spectra ('Early') were from the first full scan in which the m/z of the quinolinium ion was base peak and which also showed a molecular ion. The lower spectra ('Late') were from the last full scan meeting the same criteria. The change in the ratio of the quinolinium ions for the mixtures containing the deuterium labeled IAA is a measure of the fractionation occurring on the GC column. GC-MS analysis was on a 12 meter Hewlett-Packard methyl silicone fused silica column.

The method described in this report was designed for routine laboratory analysis utilizing a benchtop GC-MS available for approximatley the cost of two GCs. Even more sensitive analytical procedures have been developed utilizing instruments with improved ion transfer characteristics and by utilizing instruments capable of the analysis of negative ions generated by chemical ionization of a halogen substituted IAA derivative (13; L. Rivier, personal communication). [$^{13}C_6$]IAA should also be well suited for use with these techniques and should improve the reliability of a variety of isolation and assay techniques used for quantitative GC-MS analysis of IAA.

Acknowledgments—We thank Drs. W. D. Munslow and D. G. Farnum of the MSU Synthesis Laboratory for their work on the chemical synthesis. We also thank Mr. Mark Almquist of Hewlett Packard for his expert assistance in retrofitting the HP 5992 GC-MS and Dr. J. G. Buta, Plant Hormone Laboratory, for advice on chemical and spectral methods. Dr. Volker Magnus, Michigan State University, was especially helpful in his analysis of an early version of the manuscript, for which we are most grateful. Finally, we thank Drs. R. P. Pharis and T. J. Wodzicki, University of Calgary, for sharing with us their data on HPLC of isotope labeled and unlabeled IAA.

LITERATURE CITED

1. ALLEN JRF, J-E REBEAUD, L RIVIER, P-E PILET 1982 Quantification of indole-3-acetic acid by GC/MS using deuterium labelled internal standards. *In* H-L Schmidt, H Forstel, K Heinzinger, eds, Stable Isotopes. Elsevier, Amsterdam, pp 529–534
2. ALLEN JRF, L RIVIER, P-E PILET 1982 Quantification of indol-3-yl acetic acid in pea and maize seedlings by gas chromatography-mass spectrometry. Phytochemistry 21: 525–530
3. BALDI BG, JP SLOVIN, JD COHEN 1985 Synthesis of ^{14}C-labeled halogen substituted indole-3-acetic acids. J Labelled Compd Radiopharm 22: 279–285
4. BANDURSKI RS, A SCHULZE 1977 Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiol 60: 211–213
5. BANDURSKI RS, A SCHULZE 1983 Gravitational effects on plant growth hormone concentration. Adv Space Res 3: 229–235
6. CARUSO JL, RG SMITH, LM SMITH, T-Y CHENG, GD DAVES 1978 Analysis of indole-3-acetic acid in Douglas fir using a deuterium analog and combined gas chromatography-mass spectrometry. Plant Physiol 62: 841–845
7. CARUSO JL, CS ZEISLER 1983 Indole-3-acetic acid in Douglas fir: a reappraisal. Phytochemistry 22: 589–590
8. CLELAND CF, WR BRIGGS 1967 Flowering responses of the long-day plant *Lemna gibba* G3. Plant Physiol 42: 1553–1561
9. COHEN JD 1981 Synthesis of ^{14}C-labeled indole-3-acetylaspartic acid. J Labelled Compd Radiopharm 18: 1393–1396
10. COHEN JD 1984 Convenient apparatus for the generation of small amounts of diazomethane. J Chromatogr 303: 193–196
11. COHEN JD, BG BALDI 1983 Synthesis of $^{13}C_6$-benzene ring labeled indole-3-acetic acid for use in mass spectral analysis. Plant Physiol 72: S–115
12. COHEN JD, A SCHULZE 1981 Double-standard isotope dilution assay I. Quan-

titative assay of indole-3-acetic acid. Anal Biochem 112: 249–257
13. EPSTEIN E, JD COHEN 1981 Microscale preparation of pentafluorobenzyl esters: electron-capture gas chromatographic detection of indole-3-acetic acid from plants. J Chromatogr 209: 413–420
14. IINO M, DJ CARR 1982 Estimation of free, conjugated, and diffusable indole-3-acetic acid in etiolated maize shoots by the indolo-α-pyrone fluorescence method. Plant Physiol 69: 950–956
15. MAGNUS V, RS BANDURSKI, A SCHULZE 1980 Synthesis of 4,5,6,7 and 2,4,5,6,7 deuterium-labeled indole-3-acetic acid for use in mass spectrometric assays. Plant Physiol 66: 775–781
16. MCDOUGALL J, JR HILLMAN 1978 Analysis of indole-3-acetic acid using GC-MS techniques. *In* JR Hillman, ed, Isolation of Plant Growth Substances. Cambridge University Press, Cambridge, pp 1–25
17. MELHADO LL, CJ PEARCE, M D'ALARCAO, NJ LEONARD 1982 Specifically deuterated and tritiated auxins. Phytochemistry 21: 2879–2885
18. MILLARD BJ 1978 Quantitative Mass Spectrometry. Heyden, London
19. MOMONOKI YS, A SCHULZE, RS BANDURSKI 1983 Effect of deseeding on the indole-3-acetic acid content of shoots and roots of *Zea mays* seedlings. Plant Physiol 72: 526–529
20. NISHIO M, S ZUSHI, T ISHII, T FURUYA, K SYONO 1976 Mass fragmentographic determination of indole-3-acetic acid in callus tissues of *Panax ginseng* and *Nicotiana tabacum*. Chem Pharm Bull 24: 2038–2042
21. NOMA M, N KOIKE, M SANO, N KAWASHIMA 1984 Endogenous indole-3-acetic acid in the stem of tobacco in relation to flower neoformation as measured by mass spectroscopic assay. Plant Physiol 75: 257–260
22. NOWACKI J, JD COHEN, RS BANDURSKI 1978 Synthesis of ^{14}C-indole-3-acetyl-*myo*-inositol. J Labelled Compd Radiopharm 15: 325–329
23. PENGELLY WL, PJ HALL, A SCHULZE, RS BANDURSKI 1982 Distribution of free and ester indole-3-acetic acid in cortex and stele of the *Zea mays* mesocotyl. Plant Physiol 69: 1304–1307
24. PRZYBYLLOK T, W NAGL 1977 Auxin concentration in the embryo and suspensors of *Tropaeolum majus* as determined by mass fragmentography (single ion detection). Z Pflanzenphysiol 83: 463–465
25. REINECKE DM 1981 The catabolism of indole-3-acetic acid in *Zea mays* endosperm. MS thesis. Michigan State University, East Lansing
26. RIVIER L, P-E PILET 1974 Indolyl-3-acetic acid in cap and apex of maize roots: Indentification and quantification by mass fragmentography. Planta 120: 107–112
27. ROBINSON JR 1957 The synthesis of 3-indoleacetic acid-^{14}C labelled in the benzene ring. Can J Chem 35: 1570–1571
28. SLOVIN JP, EM TOBIN 1982 Synthesis and turnover of the light-harvesting chlorophyll a/b-protein in *Lemna gibba* grown with intermittent red light: possible translational control. Planta 154: 465–472
29. THIMANN KV, F SKOOG, AC BYER 1942 The extraction of auxin from plant tissues II. Am J Bot 29: 598–606
30. WITZTUM A, O KEREN, Z EVEN-CHEN 1978 The effect of ultraviolet radiation and sucrose on IAA levels in *Spirodela oligorhiza*. Ann Bot 42: 595–598