Plant Physiol. (1980) 66, 775–781
0032-0889/80/66/0775/07/$00.50

Synthesis of 4,5,6,7 and 2,4,5,6,7 Deuterium-labeled Indole-3-Acetic Acid for Use in Mass Spectrometric Assays[1]

Received for publication January 25, 1980 and in revised form May 19, 1980

VOLKER MAGNUS[2], ROBERT S. BANDURSKI[3], AND AGA SCHULZE
Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Syntheses are described for tetra and pentadeutero indole-3-acetic acid (IAA) labeled in positions 4, 5, 6, 7 or 2, 4, 5, 6, 7 of the indole moiety. Polydeuterated IAA is proposed as an internal standard for gas chromatographic-mass spectrometric analysis of IAA by selected ion monitoring. Nanogram amounts of IAA may be assayed by monitoring the base peak of IAA at m/z = 130 (134 for d_4-IAA) and the molecular ion of the methyl ester of IAA at 189 (193 for d_4-IAA). Deuterium in positions 4, 5, 6, and 7 and, to only a slightly lesser extent, that in position 2 of IAA is retained during alkali treatment, thus permitting use of these compounds as internal standards for assay of IAA released by alkaline hydrolysis of ester and amide conjugates. The use of polydeutero internal standards separates the standards from the "isotope cluster" caused by the normal abundance of heavy isotopes and also permits use of reduced mass resolution, thus leading to a 10-fold increase in sensitivity.

Tetradeutero IAA was used as an internal standard for determining free plus ester IAA in alkaline hydrolysates of *Zea mays*, and showed exact agreement between estimates based on the molecular ion of the methyl ester and those based upon base peak. Application of the method to measuring free IAA in the upper and lower halves of geotropically stimulated *Zea* shoots showed 61 ± 4% of the free IAA to be on the lower side.

Indolylic compounds, other than tryptophan, occur in plant (*cf.* 3, 4) and in animal tissue (*cf.* 23) in μM amounts. Low tissue concentrations of the indoles and their lability (16, 22, 24) make assay by GC-SIM-MS[4], with deuterated IAA as an internal standard, an attractive procedure. A number of procedures have been described using side chain d_2-indoles as standards for assay of IAA and 5-hydroxy-IAA in plants and animals (1, 6, 10, 26). However, plants contain most of their IAA as ester or amide conjugates, conveniently assayed as free IAA, following hydrolysis of the esters by 1 N NaOH and the amide conjugates in 7 N NaOH (4). Alkaline hydrolysis precludes use of d_2-IAA with deuterium in the side chain, since these deuterium are lost during base treatment. Deuterium in positions 4, 5, 6, and 7 is not exchanged with hydrogen during alkaline hydrolysis. Even deuterium in the 2 position is only slightly exchanged. Thus, we report here a synthesis for 4,5,6,7-d_4-IAA and 2,4,5,6,7-d_5-IAA and use of these compounds as internal standards.

In addition to alkali stability, polydeuterated compounds provide additional advantages as internal standards for GC-SIM-MS. The ions generated by d_4- and d_5-IAA during MS are remote from the ions of IAA and, thus, the background caused by the normal abundances of heavy ions at 1 and 2 mass units above the IAA is eliminated. Further, providing sample purity is adequate, mass resolution may be broadened to ±1 amu (fat peak monitoring) yielding a tenfold or more increase in sensitivity.

Pentadeutero IAA, labeled in positions 2, 4, 5, 6, and 7 has recently become commercially available (Merck, Sharp and Dohme, Isotopes Division Canada, Ltd.) and, although less desirable than tetradeutero IAA, its availability will facilitate the use of the method here described.

MATERIALS AND METHODS

CHEMICALS AND APPARATUS

Apparatus. GC-SIM-MS was with a Hewlett-Packard 5985A GC/MS/COM using a 3-m × 2-mm, 3% column of SP-2250 on 80/100 Supelcoport (Supelco), a He carrier gas flow of 26 ml min^{-1}, electron impact ionization at 70 ev, the ion source 200 C, and the separator at 250 C. Some spectra were obtained with a Hitachi using a solid probe operated between ambient and 150 C. IR spectra were obtained with a Perkin Elmer model 621.

Reagents. Reagent sources were as follows: Pentadeutero aniline, D_2O, and α-acetyldiethylglutarate (Aldrich); C_2H_5OD, D_3PO_4, and 35% DCl in D_2O (Merck, Sharp and Dohme); and IAA (Sigma). Other solvents and reagents were of reagent grade and not further purified. β-Chloropropionitrile was prepared from acrylonitrile and dry HCl (27, 36) and condensed with ethyl acetoacetate (20) to form the ethyl ester of α-acetyl-γ-cyano butyric acid. An ethereal solution of diazomethane was freshly prepared by a microscale method (35).

Analytical Methods. TLC was on silica gel 60 (Merck, Darmstadt), and plates were developed with methylene chloride-acetone, 100:5 (v/v) for neutral compounds, or with ethylacetate-2-propanol-25% aqueous ammonia, 45:35:20 (v/v) for acidic compounds. Indole detection was with the Ehmann reagent (11) with 2-carboxy-IAA, yielding a blue spot, whereas its ester yielded a red spot.

^{1}H-NMR spectra were obtained with a Bruker WH-180 Fourier transform NMR-spectrometer, at 180 MHz, using acetone-d_6 as solvent and tetramethylsilane as an internal standard. In analyses for small aromatic proton peaks in deuterated compounds, quadrature detection was not used so as to avoid mirror images of signals from the aliphatic region of the spectra. These small peaks were integrated manually by enlarging the plot and cutting out and weighing the peaks and a reference peak, small enough to be treated in this manner but intense enough to be accurately inte-

[1] This work was supported by the Metabolic Biology Section of the National Science Foundation, PCM 76-12356 and PCM 790-4637, and is Journal Article 9245 from the Michigan Agricultural Experiment Station.

[2] Present Address: Ruder Boskovic Institute, 41001 Zagreb, Croatia, Yugoslavia.

[3] To whom reprint requests should be directed.

[4] Abbreviations: GC-SIM-MS, gas chromatography-selected ion monitoring-mass spectrometry; mp, melting point; NMR, nuclear magnetic resonance.

grated by the electronic program used for signals of the nondeuterated part of the molecule.

SYNTHESIS OF POLYDEUTERATED IAA

IAA Methylene-d_2. IAA (100 mg) was dissolved in 2 ml 14% (w/w) KOH in H_2O, then evaporated to dryness, and this procedure was repeated twice using 1.2 ml of D_2O. The residue remaining after each evaporation was dried *in vacuo*. Finally, the IAA was dissolved in 2 ml D_2O, sealed into an ampule, and heated to 120 C to complete the deuterium exchange (18). This procedure obviates the use of expensive KOD which is also readily contaminated by atmospheric HOH (2). The resultant d_2-IAA was purified by ether extraction (5 × 5 ml with the ether discarded), then diluted with water to 15 ml, acidified to pH 2.8, and the IAA methylene-d_2 extracted into ether (5 × 50 ml) to yield, after evaporation of the solvent, 93.4 mg of white crystals, mp 169.5 to 171 C.

α-Keto-γ-cyano Butyric Acid Ethyl Ester Pentadeuterophenylhydrazone. Aniline-2,3,4,5,6-d_5, 0.46 ml, was diazotized with 2 ml 20% (w/w) aqueous sodium nitrite, in 4.25 ml 4 N HCl using the general precautions recommended by Pütter (31) and modifying procedures so as to minimize deuterium loss (13, 21, 34). The aniline and the nitrite solution were added intermittently from syringes in aliquots of about 0.03 and 0.13 ml, respectively, to the well stirred HCl, always maintaining a slight excess of nitrite. The diazonium salt solution was then added to a freshly prepared solution of α-acetyl-γ-cyano butyric acid ethyl ester, 0.875 ml, in 5 ml 95% ethanol and 5 ml 20% (w/w) aqueous NaOH (17, 20, 30). No homogeneous condensation product could be obtained using α-acetyl-γ-glutaric acid diethyl ester (21). The factors having large effects on the purity of the final product were maintenance of the reaction temperature at −15 ± 3 C throughout, maintaining a pH of 14 in the solution, and using a reaction time of 15 to 20 min for the dropwise addition of the diazonium solution with an additional 10 minutes of stirring. The resulting suspension was neutralized, filtered, and the bright orange residue washed with water and dried to give 1.16 g of α-keto-γ-cyano butyric acid ethyl ester anti-pentadeuterophenylhydrazone, mp 144 to 151 C with sintering above 140 C. Recrystallization from methanol, for NMR analysis, gave orange-red prisms, mp 157 to 159 C. The deuterium content in mol % in the benzene ring, determined for seven samples of 20 mg/ml and 25 scans was 99.36 ± 0.113.

Deuterated 2-Carboxy-IAA Diethyl Ester. DCl gas was generated from 25 ml purified (29) benzoyl chloride which was dried over Linde 4-A molecular sieve and then reacted with 1.7 ml of D_2O (9). The resultant DCl was transferred, with a slow stream of N_2, through a water-cooled reflux condenser, then to a trap cooled with dry ice-ethanol, and then to a second trap cooled with liquid N_2, as described (15). For reasons of safety, the apparatus was not evacuated but rather flushed with N_2, and the inlet and outlet for the liquid N_2 trap was reversed, to prevent clogging of the narrow inner tube by solid DCl. On completion of DCl generation, the trap containing solid DCl was isolated from the rest of the apparatus, and judicious thawing permitted slow evaporation of the DCl through all-glass connections into a solution of 835 mg α-keto-γ-cyano butyric acid ethyl ester pentadeuterophenylhydrazone in 10 ml 95% C_2H_4OD–5% D_2O and 0.2 ml of D_2O. These proportions are critical (13). The rate of DCl introduction was sufficient to cause an instantaneous change of the color of the reaction mixture to dark violet, and an increase in temperature to 60 C within 5 min. The rate of DCl introduction was then diminished to 1 to 2 bubbles/s, and the solution refluxed for 2 h. NH_4Cl separation occurred after 20 to 30 min. The brown suspension was poured into 100 ml ice-cold 10% (w/w) aqueous potassium carbonate and the slightly alkaline solution extracted five times with 150 ml chloroform. Removal of solvent and drying yielded 831 mg of a dark red tar. The crude product, dissolved in methylene chloride, was chromatographed on an 80- × 2-cm column containing 90 g silica gel 60 (Merck, Darmstadt), 0.063 to 0.2 mm, equilibrated and packed in the same solvent. Methylene chloride (900 ml) was used as the first eluent, and the column further eluted with 202 ml methylene chloride-ether, 100:1; 204 ml methylene chloride-ether, 100:2; and 210 ml of methylene chloride-ether, 100:5. Deuterated 2-carboxy-IAA diethyl ester was eluted with 220 ml methylene chloride-ether, 100:10. Evaporation of the solvent gave 369 mg, 33%, of yellow crystals, mp 83 to 84 C. Recrystallization, for NMR analysis, from water-ethanol, yielded long yellow needles, mp 84 C. The deuterium content in the side chain methylene group determined for three independently prepared samples, with c = 10 to 15 mg/ml and 200 scans was: 0.70, 0.76, and 0.71 equivalents. The protium content (in mol %) in specified positions of the indole ring, and for three samples (two determinations/sample) was: H-4, 0.59 ± 0.10; H-7, 2.91 ± 0.70; H-6, 0.66 ± 0.21; H-5, 3.70 ± 1.10.

Deuterated 2-Carboxy-IAA. Deuterated 2-carboxy-IAA diethyl ester (122.5 mg) and 0.5 g of KOH were dissolved in 10 ml absolute ethanol, and the solution was stored in darkness at 25 C for 48 h (14). Water (40 ml) was added to dissolve the precipitate formed and the solution extracted with 3- × 50-ml portions of ether. The aqueous phase was acidified to pH 2.8, extracted with 3 × 75 ml of ether which, on evaporation, yielded 103 mg yellow crystals. These were dissolved in 1 ml 50% aqueous 2-propanol and chromatographed on a 28- × 1.8-cm column of Sephadex LH-20, equilibrated with and eluted with the same solvent. Off-white crystals (96.5 mg; 98.5%) of deuterated 2-carboxy-IAA, mp 233 to 237 C (decomposition) were obtained.

4,5,6,7-Tetradeutero-IAA. Deuterated 2-carboxy-IAA, 96 mg, was suspended in 0.43 ml 1 M KOH and 2 ml water, and heated at 200 C for 2 h in a sealed tube inserted in a steel bomb, containing water for pressure compensation (7). The resultant dark brown suspension was diluted with water to 5 ml and extracted with 4 × 4 ml ether, and the ether discarded. The slightly yellow aqueous phase was diluted to 20 ml, acidified to pH 2.8, and extracted with 4 × 50 ml ether, which, on drying and evaporation, yielded 50 mg 4,5,6,7-tetradeutero-IAA as yellow crystals, mp 165 to 167 C (sintering above 161 C) which darkened on standing. Quantitative TLC, using a series of standards and visual comparison of the spots appearing after spraying with Ehmann's reagent (11), indicated the presence of about 2% of deuterated 2-carboxy-IAA as an impurity. After 2 recrystallizations from D_2O, NMR analysis (c = 10 mg/ml, 200 scans) gave the following protium content, in mol %: H-4, 0.77; H-7, 3.52; H-6, 1.43; H-5, 5.57.

2,4,5,6,7-Pentadeutero-IAA. A solution of α-keto-γ-cyano butyric acid ethyl ester pentadeuterophenylhydrazone, 500 mg, in 15 ml of redistilled, dry pyridine was cautiously mixed with 20 ml 35% DCl in D_2O, and 5 ml 85% D_3PO_4. The solution was refluxed for 1 h (34) and then added dropwise to 100 ml cold 30% (w/w) aqueous potassium carbonate. The neutral to slightly alkaline solution was extracted with 6 × 100 ml ether and the ether discarded. In a typical experiment, using unlabeled reactants, evaporation of this extract gave 22 mg of a tarry residue, from which 4.9 mg of indole-3-acetonitrile was isolated by preparative TLC using methylene chloride-benzene, 1:1, and multiple development. Identity was established by chromatography and by the IR spectrum. The aqueous phase, remaining after ether extraction, was acidified to pH 2.8 and extracted with 5 × 200 ml ether. After evaporation of the ether, the residue was dissolved in 0.5 ml 50% aqueous 2-propanol and chromatographed on a 28- × 1.8-cm column of Sephadex LH-20 which had been equilibrated with and was eluted with the same solvent. About 97 mg (28%) of brown crystals, mp 150 to 155 C were obtained representing IAA deuterated in the indole ring and, partially, in the side chain methylene group and slightly contaminated with deuterated 2-carboxy-IAA.

An aliquot of this sample (26 mg) was dissolved in 2 ml 14% (w/w) aqueous KOH and heated, in a sealed tube, at 120 C, for 3 h. The alkaline solution was extracted with 4 × 5 ml ether and the ether discarded. The remaining solution was diluted to 10 ml, acidified to pH 2.8, and the ether phase, after evaporation, yielded 25 mg (96%) 2,4,5,6,7-pentadeutero-IAA, as reddish crystals containing traces of 2-carboxy-IAA as an impurity (Fig. 1).

GAS CHROMATOGRAPHIC SELECTED ION MONITORING-MASS SPECTROMETRIC ASSAY

Quantitative Assay of Free and Esterified IAA in Corn (*Z. mays*) Seedlings. Plants contain most of their IAA as ester or amide conjugates (3, 4). Early quantitative studies utilized a [^{14}C]IAA isotope dilution assay and required a week for a single assay and at least a kg of tissue. The following assay is applicable to 10 g corn shoot tissue, and four samples can be assayed in 1 week.

To 75 ml of acetone in a homogenizer jar, 45,000 dpm (0.06 μg) [^{14}C]IAA, 3.1 μg d_4-IAA, and 10 g fresh corn shoot tissue were added and the tissue homogenized. Conditions for seedling growth were as previously described (3). The [^{14}C]IAA facilitates column peak location and recovery determinations. The homogenate was left overnight for isotope equilibration, then filtered, the residue reextracted with 70% acetone-water, and the acetone extracts combined and reduced in volume to 10 ml. For measurement of free plus ester IAA (4), the suspension was made 1 N in alkali by addition of 2 N NaOH, reacted for 1 h at 25 C, then acidified with 2 M phosphoric acid to pH 2.5 and extracted twice with diethyl ether.

The acid ether fraction was evaporated to dryness at 12 mm Hg in a flash evaporator using a bath temperature of 50 C; the residue dissolved in 0.2 ml 50% (v/v) aqueous ethanol and applied to a 2.5-ml bed volume DEAE Sephadex (acetate form) column. After washing with 20 ml 50% ethanol, the column was eluted with a linear gradient of 50% aqueous ethanol (100 ml in the mixing flask) and 50% aqueous ethanol containing 5% glacial acetic acid (100 ml in the reservoir). Elution of IAA was at 25 to 33 ml as determined by ^{14}C monitoring with an over-all ^{14}C recovery for the extraction, partitioning, and column elution of 55%. The [^{14}C]IAA-containing tubes were pooled, evaporated to dryness at 12 mm Hg, and in a bath temperature of 50 C, dissolved in 100 μl 50% aqueous ethanol, and applied to a 4.6-mm × 25-cm C_{18}-reverse phase Partisil-10 ODS (Whatman) column. Elution with 30% ethanol in water, containing 1% acetic acid occurs at about 9 ml with the over-all recovery now at 39%. [^{14}C]IAA-containing tubes were pooled, dried at 12 mm Hg, dissolved in about 50 μl methanol, and methylated with 100 μl diazomethane in ether (35). After dissolving in dry, peroxide-free tetrahydrofuran, the over-all recovery of methylated IAA, from samples containing 300 to 1,500 ng IAA, was 20%.

The methylated mixture of IAA and d_4-IAA was still contaminated by hydroxycinnamic (3) and dimethoxyhydroxycinnamic acids (Schultz, Champman, and Bandurski, unpublished) so that determination of IAA by UV and fluorescence assays was not possible. Thus, the methyl ester mixture was injected into a gas chromatographic 3.3-m × 2-mm column of 3% SP2250 coupled to a mass spectrometer and monitoring masses, 130.2, 134.2 (base peak for methyl IAA and d_4-methyl IAA, respectively) and 189.2, 193.2 (the molecular ions for methyl IAA and d_4-methyl IAA). Both the molecular ion and base peak ratios are monitored so that contaminant ions would be detected, since contaminants would not affect both ratios equally. As can be seen from Figure 2, the samples are almost free of contaminant ions except for a small 193.2 peak at a retention time of 7.4 min, substantially separated from IAA at 8.1 min.

Since no d_4-IAA was contributed by the plant, the following computation may be made (Fig. 2). There are 200,188 counts at mass (130.2 plus mass 134.2) with 143,814 counts at mass 134.2 = 71.8% at mass 134.2. There are 47,472 counts at mass (189.2 plus mass 193.2) with 33,900 at mass 193.2 = 71.4% at mass 193.2. The mean amount of d_4-IAA is 71.6%. The isotope dilution equation (33) is $Y = ([C_i/C_f] - 1)X$; where Y = amount of compound in the tissue; C_i = the initial percentage of d_4-IAA in the internal standard (100 in this case, since no 130.2 or 189.2 is found in the d_4-IAA used as an internal standard); C_f = the percentage of d_4 found (71.6%); and X = the amount of d_4-IAA added (3.18 μg as estimated at A_{282} (3), $\epsilon = 6.06 \times 10^3$, multiplied by the percent at 4 mass units above IAA (0.72) = 2.290 μg) and corrected for the 89% of IAA ions occurring at 4 mass units below

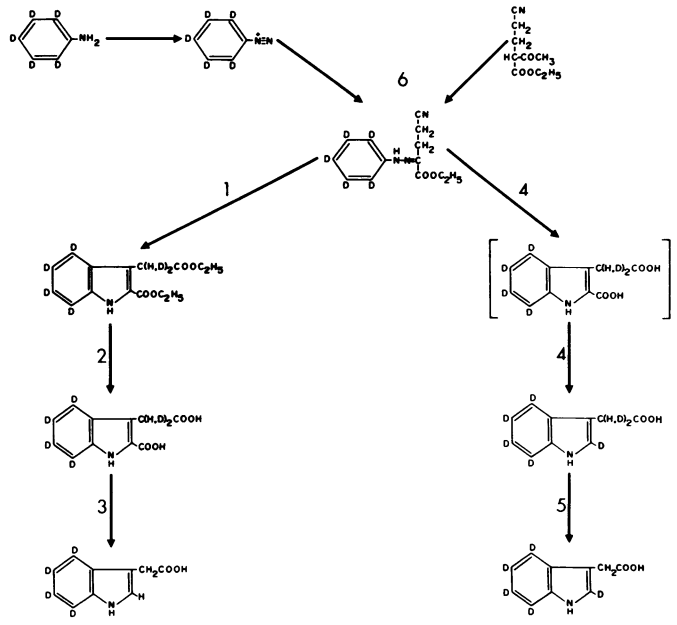

FIG. 1. Syntheses of IAA-4,5,6,7-d_4 and IAA-2,4,5,6,7-d_5. Conditions used for the indicated steps, were as follows: 1, DCl in C_2H_5OD/D_2O, at reflux; 2, KOH/C_2H_5OH, at room temperature; 3, 1 equivalent KOH, at 200 C; 4, DCl/D_2O-D_3PO_4-pyridine, at reflux; 5, 14% aqueous KOH, at 120 C; 6, NaOH-50% aqueous ethanol, at −15 C.

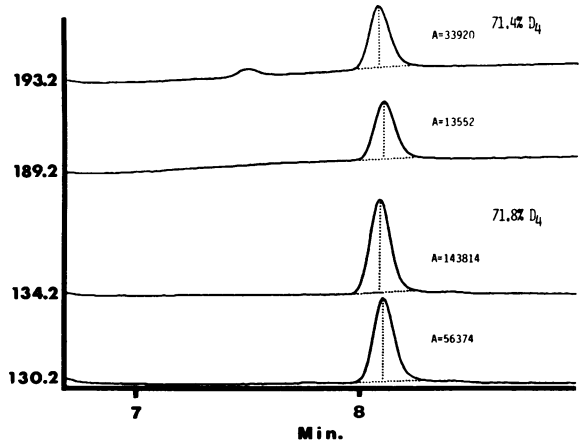

FIG. 2. A graph of ion current at the indicated masses *versus* gas chromatographic retention times. IAA of mass 193.2 and the fragmentation at 134.2 is owing to d_4-IAA added, whereas IAA at mass 189.2 and the fragment ion at 130.2 is owing to IAA from the plant. Details are in the text.

the d_4-IAA. Thus, for Figure 2:

$$0.89Y = \frac{([100/71.6] - 1)2.29\ \mu g}{0.01\ kg} = 91\ \mu g/kg, \text{ and}$$

$$Y = 102\ \mu g/kg$$

and this agrees with values for this lot of seeds obtained by [^{14}C]-IAA isotope dilution applied to seedlings grown under similar conditions (*cf.* 3, 5).

Routinely, it is best to measure X by adding d_4-IAA to a known amount of IAA, determine C_f on the methylated sample, and then use the isotope dilution equation $X = Y([C_i/C_f] - 1)^{-1}$ to find X. Determining X empirically obviates the need for corrections due to normal heavy isotope abundances and to differences in fragmentation caused by deuterium.

The precision of determination of percentage of d_4-IAA, measuring both peak area and peak height, as determined by 20 experiments, each replicated twice, was 0.96 ± 0.83% of the mean. Thus, computer and human variance in determining peak size or peak height is less than 1%. The over-all accuracy of the entire procedure for determining IAA, beginning with plant material, is better than ±5%. For example, a series of 11 experiments using two replicates/experiment, showed 61 ± 4 and 60 ± 3% of the free IAA to be on the lower side of a geotropically stimulated corn shoot as determined by peak area and peak height, respectively (*cf.* 37). Thus, errors involved in cutting plants in half and extracting, derivitizing, and chromatographing IAA adds only an additional 2 to 3% variance. For geostimulation in these experiments, 4-day-old seedlings were pinned through the endosperm and placed in a horizontal position on sheets of styrofoam. Geostimulation was for 2 h at 25 C, 90% humidity, and in darkness, and the harvested tissue included the portion from the tip down to 1 cm below the coleoptile node.

Addition of an amount of d_4-IAA approximately equal to the IAA expected in the sample provides greatest accuracy. Further, it is important that the GC column be "cleaned" after each sample, particularly if the percentage of d_4 in successive samples is widely different. To accomplish this, the column is vented to the air and injected with 1 μl of *N,O-bis*-(trimethylsilyl)-trifluoracetamide, followed by two 1-μl injections of tetrahydrofuran. A blank injection of tetrahydrofuran now gives no ion current at the masses and time of interest. Further, repeated injections of 25% d_4-IAA-75% IAA followed by repeated injections of 75% d_4-IAA-25% IAA do not show a memory effect.

RESULTS AND DISCUSSION

Isotopic Purity by Mass Spectrometry. We determined the relative abundance of nondeuterated IAA and species containing 1 to 7 deuterium. These abundances, as determined by the molecular ion, are shown in Table I. The data show that the most abundant ions were, in fact, at the expected mass numbers with intensities approximately 15% less than theory. Every preparation of IAA-d_2 contained detectable quantities of the unlabeled acid, thus further decreasing its value as an internal standard. The ion intensities at 178 to 180 amu were larger than expected, indicating that the sample contains more than two equivalents of deuterium and these must be in the indole moiety. Base-catalyzed ring deuteration of IAA, although not unexpected (38), has not been previously reported. Attempts to minimize this reaction, with maximal side chain labeling, by varying the ratios and concentrations of IAA and potassium deuteroxide, during the exchange procedure, were not successful.

The predominant molecular ion of deutero-IAA obtained by cyclization of the deuterated hydrazone in DCl/D_2O-D_3PO_4-pyridine, appeared at 181 and 182 amu. Table I shows a deuterium content of 6 to 7 equivalents, so some deuterium must be in the side chain. Since mass spectroscopic purity was not adequate for use as an internal standard, the side chain label was removed by base-catalyzed exchange in H_2O. The resultant product was mainly IAA-d_5, m^+ = 180 amu. Comparison of the lower mass molecular ions, before and after alkali treatment, showed that a small amount of deuterium from the indole ring was lost in this step.

Isotopic Purity by NMR. In the 180 MHz ^{1}H-NMR spectrum, four of the aromatic protons of the unlabeled hydrazone appeared as an asymmetric doublet at δ = 7.3 ppm, with the fifth proton as a multiplet, possibly a sextet, at 6.9 ppm. The individual peaks of these two signals were 4 Hz apart, and deuterated samples showed small singlets at the same chemical shifts. The ratio of peak areas was about 4:1, and their area corresponded to a protium content of 0.5 to 0.8%. This accorded with the manufacturer's reported isotopic purity of the aniline-d_5 as greater than 99% D.

The indole ring proton signals were assigned according to (see below) published data using ^{13}C and ^{1}H-NMR spectra of tryptophan in trifluoroacetic acid and D_2O solutions (8, 25, 28). For our work, acetone-d_6 was the best solvent. Since solvent and concentration effects on the indole ^{1}H-NMR spectrum are possible (19, 32), the chemical shifts reported for tryptophan in trifluoroacetic acid-d_1 and D_2O, and the values observed for IAA in alkaline D_2O and acetone-d_6, are compared in Table II. The two downfield signals appear at closely corresponding δ values and can be assigned to H-4, lower field, and H-7, higher field. The remaining signals in the IAA spectrum were identified by homonuclear double-resonance experiments. For 2-carboxy-IAA diethyl ester, there were no differences with regard to H-4 and H-7 resonances, whereas the H-5 and H-6 signals were shifted to lower fields and did not overlap.

The ^{1}H-NMR spectra of three samples of 2-carboxy-IAA diethyl ester showed an average protium content in the benzene portion of the indole ring of 1.3 to 2.4%, with the largest amount being in position 5. The side chain of the ester contained 0.7 to 0.8 equivalents of deuterium derived, by acid-catalyzed exchange, from the C_2H_5OH/DCl used in its synthesis. MS showed that hydrolysis of the ester caused some loss of label from the side chain. Quantitative NMR analysis was not possible because the free deuterated 2-carboxy-IAA did not contain a completely protonated position usable as a reference. Decarboxylation of this compound to IAA-d_4 was accompanied by complete dedeuteration of the side chain, and the protium accumulated in the indole moiety could be monitored by NMR. No deuterium was present in position 2. The protium content in positions 4 to 7 had increased to an average level of 2.8%, its distribution being roughly the same as in deuterated 2-carboxy-IAA diethyl ester.

The NMR spectrum of IAA-d_5, prepared by side chain dedeuteration of IAA-$d_{6,7}$ with KOH, showed the presence of a small amount of protium in position 2 of the indole ring. This position, again, contained the majority of deuterium which entered the indole ring during side chain labeling of IAA with potassium deuteroxide. Thus, the slow, base-catalyzed deuterium-protium exchange in the indole ring, shown by the mass spectrometric data (Table I) mainly, involves position 2.

Correlation of isotopic purity of IAA-d_4 as determined by ^{1}H-NMR and by MS was attempted since the IAA-d_4 mass spectrum is relatively undistorted by deuterium isotope effects. The presence of contaminant protium in any of the four positions distinguishable by NMR contributed to only one IAA-d_3 ion in the mass spectrum. Its predicted abundance calculated from the NMR data given in the experimental section is, therefore, 0.77 + 3.52 + 1.43 + 5.57 = 11.29%, which is in excellent agreement with the 12% ion abundance at 178 amu in the mass spectrum of this sample (Table I).

Stability of the Label in Deuterated IAA during Use as an Internal Standard. The susceptibility of deuterated IAA to hydro-

Table I. *Ion Abundances of Deuterated IAA in % of Cumulative Ionization in the Molecular Ion Region*

Sample	m^+ =									
	175	176	177	178	179	180	181	182	183	184
IAA-methylene-d_2										
Theoretical			89	10	1					
Observed	1	6	75	15	3	1				
IAA-4,5,6,7-d_4										
Theoretical					89	10	1			
Observed			2	12	72	12	2			
IAA-2,4,5,6,7-d_5										
Theoretical						89	10	1		
Deutero-IAA obtained by cyclization of the phenylhydrazone with DCl/D_2O-D_3PO_4-pyridine			1	1	1	6	26	56	8	1
IAA-2,4,5,6,7-d_5 obtained by treatment of the above sample with aqueous KOH			2	2	9	74	10	2		

Table II. *Deduction of the Chemical Shifts of the Ring Protons of IAA and 2-Carboxy-IAA Diethyl Ester from Corresponding Values for Tryptophan*

Compound	Conditions	Chemical Shifts (δ) Relative to Tetramethylsilane for the Protons				
		H-4	H-7	H-2	H-6	H-5
Tryptophan after Norton and Bradbury (28)	TFA-d 220 MHz	7.58	7.43	7.25	7.30	7.17
Tryptophan after McDonald and Leigh (25)	D_2O, pD = 6.5 c^2 = 25 mM 220 MHz	7.7	7.5	ND[a]	ND	ND
IAA	D_2O + 2 eq Na_2CO_3 c^2 = 100 mM 180 MHz	7.62	7.49	7.22	7.18[b]	
IAA	acetone-d_6 c = 100 mM 180 MHz	7.61	7.40	7.30	7.01[c]	6.93[d]
IAA	acetone-d_6 c = 15 mM 180 MHz	7.64	7.42	7.32	7.09[b]	
2-Carboxy-IAA diethyl ester	acetone-d_6 c = 65 mM 180 MHz	7.61	7.40		7.29	7.07

[a] ND, Not determined; [b] not resolved; [c] irradiated at H^5; [d] irradiated at H^6.

gen exchange reactions was checked under conditions encountered during the isolation and purification of plant IAA and the hydrolysis of its bound forms. The data show that deuterium in the side chain is more labile and, thus, the stability of ring-labeled IAA was tested under conditions that led to hydrogen exchange in the side chain deuterated acid.

IAA decomposes in strong acid, and thus the only "acidic" conditions tested were: chromatography on Dowex 50-X2 in the sodium form (12), with elution with aqueous 2-propanol, and binding to DEAE (A-25) and QAE-Sephadex in their acetate forms, and eluting with 2 or 5% acetic acid in 50% aqueous 2-propanol. These experiments were performed with IAA-d_2, and no loss of deuterium was observed. Thus, moderate acidity, such as used in chromatographic procedures, can be tolerated with IAA-d_2.

Alkaline conditions are used to hydrolyze bound forms of IAA. The inositol and glucose esters are hydrolyzed by 1 N KOH, at room temperature for 1 h (3), and amide linked IAA is hydrolyzed with 7 N KOH for 3 h at 100 C (4). Partial, side chain dedeuteration of 2-carboxy-IAA diethyl ester (see above) occurs during hydrolysis in ethanolic KOH at room temperature, probably enhanced by the greater basicity of the ethoxide anion. We chose to study deuterium incorporation into the unlabeled acid as a means of measuring exchangeability of the IAA side chain hydrogen in weakly alkaline medium at elevated temperatures. The changes in abundance of the molecular ion caused by heating IAA in buffered D_2O are shown in Table III. Deuterium incorporation occurred even at pH 8. Since deuterium-protium exchange is reversible, except for small isotope effects, a comparable amount of deuterium would be lost from IAA-d_2 and its esters when heated in H_2O buffered at the same pH values.

The influence of hydrolysis with 7 N KOH at 100 C (4) on the

Table III. *Incorporation of Deuterium into the Side Chain of IAA*

Treatment[a]	Ion Abundances[b] in % of Cumulative Ionization in Molecular Ion Region m^+ =		
	175	176	177
None, theoretical abundances	88	11	1
None, experimental values	86	11	3
Na_3PO_4/D_2O (pH 8.0)	78	19	3
$Na_2B_4O_7/K_2CO_3/D_2O$ (pH 9.5)	77	19	4
$NaHCO_3/K_2CO_3/D_2O$ (pH 10.1)	74	22	4
K_2CO_3/D_2O (pH 10.7)	75	22	4

[a] 100 mg of IAA was dissolved in the following solutions: 2 ml of 1 M K_2CO_3, 2 ml of 0.5 M $NaHCO_3$ + 0.7 ml of 1 M K_2CO_3, 2 ml of saturated aqueous $Na_2B_4O_7$ + 0.4 ml of 0.5 M K_2CO_3, 2 ml of 1.5 M Na_3PO_4. Further procedures were as described for the preparation of IAA-methylene-d_2. The pH values were determined after the exchange reaction and are uncorrected values read on a pH meter equipped with a glass electrode.

[b] Arithmetic mean from two to three determinations.

Table IV. *Loss of Deuterium from Side Chain and Ring Deuterated IAA in 7N KOH for 3 h at 100 C*

Compound	Ion Abundances in % of the Cumulative Ionization in the Molecular Ion Region m^+ =								
	175	176	177	178	179	180	181	182	183
IAA-methylene-d_2[a] before alkali treatment		4	65	24	5	1			
IAA-methylene-d_2 after alkali treatment	79	15	6						
Unlabeled IAA theoretical abundances	89	10	1						
IAA-2,4,5,6,7-d_5 before alkali treatment	2	1	2	2	9	70	10	3	2
IAA-2,4,5,6,7-d_5 after alkali treatment	3	2	5	4	12	59	10	3	3

[a] Not the same sample used in Table I.

abundance of the molecular ion of IAA-d_2 is shown in Table IV and, as can be seen, the sample was almost completely dedeuterated. These conditions also influenced the mass spectrum of ring-labeled IAA-d_5, since the abundance of the nominal molecular ion decreased by about 10%. Based on results shown above, it is certain that this loss of deuterium was from the indole position 2. IAA-d_4, which does not contain deuterium in the pyrrole ring, should therefore be the best internal standard.

Acknowledgments—We acknowledge Dr. R. Chapman for his help in developing GC-column chromatographic and MS-SIM-monitoring techniques and for the use of MSU-DOE (EY-76-C-02-1338) and National Institutes of Health (RR00480) mass spectrometry equipment. Mr. Frank J. Bennis provided help in obtaining and interpreting NMR spectra. Ms. Prudence Hall and Dr. J. Cohen aided in the assays while Dr. W. Reusch made valuable suggestions concerning the synthesis of d_4-IAA and Dr. A. Ehmann originally suggested the value of polydeutero ring-labeled IAA. Drs. H. L. Gibble and D. Keglević facilitated personnel exchange, and Ms. P. Pengelly and M. La Haine helped in manuscript preparation.

LITERATURE CITED

1. ALLEN JRF, AM GREENWAY, DA BAKER 1979 Determinations of indole-3-acetic acid in xylem sap of *Ricinus communis* L. using mass fragmentography. Planta 144: 299–303
2. ATKINSON JG, JJ GSAKVARY, AT MORSE, RS STUART 1968 Base catalyzed deuterium exchange reactions. A simple synthesis of α-deutero carboxylic acids. *In* J Sirchis, ed, Proceedings of the 2nd International Conference on Methods of Preparing and Storing Labelled Compounds, Brussels (Belgium), 1966. Euratom, Brussels, pp 675–681
3. BANDURSKI RS, A SCHULZE 1974 Concentration of indole-3-acetic acid and its esters in *Avena* and *Zea*. Plant Physiol 54: 257–262
4. BANDURSKI RS, A SCHULZE 1977 Concentrations of indole-3-acetic acid and its derivatives in plants. Plant Physiol 60: 211–213
5. BANDURSKI RS, A SCHULZE, JD COHEN 1977 Photo-regulation of the ratio of ester to free indole-3-acetic acid. Biochem Biophys Res Commun 79: 1219–1223
6. BERTILSSON L, L PALMER 1972 Indole-3-acetic acids in human cerebrospinal fluid: Identification and quantification by mass fragmentography. Science 177: 74–76
7. BOWMAN RE, PJ ISLIP 1971 Decarboxylation of indole-2-carboxylic acids. Chem Ind (Lond) 154
8. BRADBURY JH, RS NORTON 1973 Carbon-13 NMR spectra of tryptophan peptides and of native and denatured proteins. Biochim Biophys Acta 328: 10–19
9. BROWN HC, C GROOT 1942 A convenient procedure for the preparation of deuterium chloride. J Am Chem Soc 64: 2223–2224
10. CARUSO JL, RG SMITH, LM SMITH, T-Y CHENG, GD DAVES 1978 Analysis of indole-3-acetic acid in Douglas fir using a deuterated analog and combined gas chromatography-mass spectrometry. Plant Physiol 62: 841–845
11. EHMANN A 1977 The van Urk-Salkowski reagent-A sensitive and specific chromogenic reagent for silica gel thin layer chromatographic detection and identification of indole derivatives. J Chromatogr 132: 267–276
12. EHMANN A, RS BANDURSKI 1972 Purification of indole-3-acetic acid myo-inositol esters on polystyrene-divinylbenzene resins. J Chromatogr 72: 61–70
13. FEOFILAKTOV VV, NK SEMENOVA 1953 Action of aromatic diazo compounds on compounds of the alkyl acetoacetic ester type as a method for the preparation of arylhydrazones of α-keto acids, α-amino acids, and nitrogen heterocycles. XIV. Synthesis of 2-carboxyindolyl-3-acetic acids substituted in the benzene ring and the corresponding indolyl-3-acetic acids. J Gen Chem USSR 23: 669–679
14. FINDLAY SP, G DOUGHERTY 1948 The synthesis of certain substituted indole-acetic acids. J Org Chem 13: 560–569
15. FRIVOLD OE, O HASSEL, S RUSTAD 1937 Brechungsexponent des schweren Ammoniaks und des schweren Chlorwasserstoffs im sichtbaren Gebiet. Physik Z 38: 191–195
16. GAFFNEY TE, CG HAMMAR, B HOLMSTEDT, RE MCMAHON 1971 Ion specific detection of internal standards labelled with stable isotopes. Anal Chem 43: 307–310
17. HENECKA H, H TIMMLER, R LORENZ, W GEIGER 1957 Zur Kenntnis der Japp-Klingemann-Reaktion. Chem Ber 90: 1060–1069
18. HOSKINS JA, RJ POLLITT 1975 Quantitative aspects of urinary indole-3-acetic acid and 5-hydroxyindole-3-acetic acid excretion. J Chromatogr 109: 436–438
19. JARDINE RV, RK BROWN 1963 Determination of α or β substitution of the indole nucleus by nuclear magnetic resonance. Can J Chem 41: 2067–2073
20. KEIMATSU S, S SUGASAWA 1928 Studien zur Synthese der Indolderivate. II. Synthese einiger Indolcarbonsauren. J Pharm Soc Jap 48: 101–105
21. KING FE, PL ECUYER 1901 Synthesis of indoleacetic acids. J Chem Soc 1934: 1901–1905
22. LITTLE CHA, JK HEALD, G BROWNING 1978 Identification and measurement of indoleacetic and abscissic acids in the cambial region of *Picea sitchensis* (Bong.) Carr. by combined gas chromatography-mass spectrometry. Planta 139: 133–138
23. LOVENBERG W, K ENGELMAN 1971 Assay of serotonin related metabolites, and enzymes. *In* D Glick, ed, Methods of Biochemical Analysis—Analysis of Biogenic Amines and Their Related Enzymes. John Wiley and Sons, New York, pp 1–34
24. MANN JD, EG JAWORSKI 1970 Minimizing loss of indoleacetic acid during purification of plant extracts. Planta 92: 285–291
25. MCDONALD GG, JS LEIGH JR 1973 A new method for measuring longitudinal relaxation times. J Magnetic Resonance 9: 358–362
26. 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
27. MIKHAILOVA VN, VV PIGULEVSKII 1957 The synthesis of some esters of β-chloropropionic acid. J Appl Chem USSR 30: 1911–1915
28. NORTON RS, JH BRADBURY 1976 Kinetics of hydrogen-deuterium exchange of tryptophan and tryptophan peptides in deutero-trifluoroacetic acid using proton magnetic resonance spectroscopy. Mol Cell Biochem 12: 103–111
29. OAKWOOD TS, CA WEISSGERBER 1955 Benzoyl cyanide. Org Synth Coll Vol 3: 112–118
30. PHILLIPS RR 1959 The Japp-Klingemann reaction. *In* R Adams, ed, Organic Reactions, Vol 10, John Wiley and Sons, Inc., New York, pp 143–178
31. PÜTTER R 1965 Methoden zur Herstellung und Unwandlung aromatischer Diazoniumsalze. *In* E Müller, ed, Methoden der Organischen Chemie Band X/3, G. T. Verlag, Stuttgart, pp 1–212
32. REINECKE MG, HW JOHNSON JR, JF SEBASTIAN 1969 The solvent and concentration dependence of the nuclear magnetic resonance spectra of indoles. J Am Chem Soc 91: 3817–3822
33. RITTENBERG D, DL FOSTER 1940 A new procedure for quantitative analysis by isotope dilution with application to the determination of amino acids and fatty acids. J Biol Chem 133: 737–744
34. ROBINSON JR 1957 The synthesis of 3-indoleacetic acid-^{14}C labelled in the benzene ring. Can J Chem 35: 1570–1571

35. SCHLENK H, JL GELLERMAN 1960 Esterification of fatty acids with diazomethane on a small scale. Anal Chem 32: 1412–1414
36. STEWART R, RH CLARK 1947 Convenient synthesis of β-chloropropionitrile. J Am Chem Soc 69: 713–714
37. THIMANN KV 1977 Hormone Action in the Whole Life of Plants. University of Massachusetts Press, Amherst, pp 95–104
38. ZATSEPINA NN, YL KAMINSKY, IF TUPITSYN 1969 Hydrogen isotopic exchange in five-membered aromatic heterocycles. III. Furan, thiophene, pyrrole, N-methylpyrrole and their benzologues. Reaktsionnaia sposobnost (i mekhanizmy reaktsii) organicheskikh soedinenii 6: 766–777