Metabolism of Gibberellin A$_{12}$-7-Aldehyde by Soybean Cotyledons and Its Use in Identifying Gibberellin A$_7$ as an Endogenous Gibberellin

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ABSTRACT

The level of gibberellin(GA)-like material in cotyledons of soybean (Glycine max L.) was highest at mid-pod fill—about 10 nanograms GA$_3$ equivalents per gram fresh weight of tissue, assayed in the immersion dwarf rice bioassay. This amount is about 1000-fold less than in Pisum and Phaseolus seed, other legume species whose spectrum of endogenous gibberellins (GAs) is well known. The metabolism of [14C]-GA$_{12}$-7-aldehyde (GA$_{12}$ald)—the universal GA precursor—by intact, mid-pod-fill, soybean cotyledons and their cell-free extracts was investigated. In 4 hours, extracts converted GA$_{12}$ald to two products—[14C]GA$_{12}$ (42% yield) and [14C]GA$_{15}$ (7%). Within 5 minutes, intact embryos converted GA$_{12}$ald to [14C]GA$_{12}$ and [14C]GA$_{15}$ in 15% yield; 4 hour incubations afforded at least 22 products (96% total yield). The putative [14C]GA$_{12}$ was identified as a product of [14C]GA$_{12}$ald metabolism on the basis of co-chromatography with authentic GA$_{12}$ on a series of reversed and normal phase high pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) systems, and by a dual feed of the putative [14C]GA$_{15}$ and authentic [14C]GA$_{12}$ to cotyledons of both peas and soybeans. The [14C]GA$_{15}$ was identified as a metabolite of [14C]GA$_{12}$ald by capillary gas chromatography (GC)-mass-spectrometry-selected ion monitoring, GC-radiocounting, HPLC, and TLC. By adding the [14C] metabolites of [14C]GA$_{12}$ald to a different and larger extract (about 0.2 kg fresh weight of soybean reproductive tissue) and purifying endogenous substances co-chromatographing with these metabolites, at least two GA-like substances were obtained and one identified as GA$_2$ by GC-mass spectrometry. Since [14C]GA$_{12}$ was not found as a [14C]metabolite of [14C]GA$_{12}$ald, soybean embryos might have a pathway for biosynthesis of active, C-19 gibberellins like that of the cucurbits; GA$_{12}$ald → GA$_{12}$ → GA$_{15}$ → GA$_{24}$ → GA$_{34}$ → GA$_2$ → GA$_5$.

Soybean (Glycine max) has been the subject of many studies on phytohormones, but few, if any, deal with endogenous GAs or their metabolism. Identifying endogenous soybean GAs and elucidating the pathways for their synthesis is a first step in understanding the roles that GAs play in the physiology of this major crop. Since seeds of many legumes are relatively rich sources of GAs (e.g. up to 125 μg/g fresh weight in Phaseolus coccineus [8]), this suggests soybean seeds should be the first tissue examined for this purpose.

The GAs in extracts of higher plants usually have been prepared for identification by solvent partitioning, followed by either silica gel partition chromatography or reverse phase HPLC (5). After one or two such LC steps, fractions demonstrating biological activity, or pools of fractions with LC Rts similar to known GAs can usually be analyzed by GC-MS (e.g. 1). However, this approach has not yielded detectable levels of any GAs in immature seed or seedlings of soybean (V Soensel, personal communication to P Birnberg and M Brenner). The failure of these traditional methods to identify any GAs in soybeans suggests either that soybean GA levels are lower than in most seeds, or that their presence is masked by compounds which are not removed by the minimal purification procedures used. Therefore, we decided to study soybean cotyledon metabolism of [14C]GA$_{12}$ald—the universal, committed precursor of GAs (11)—because: (a) developing cotyledons have proved to be the most abundant source of GAs in other legumes (6, 7); (b) the radioactive products may allow identification of the GA pathways in soybeans; and (c) the [14C] products could serve as markers for endogenous GAs during purification of large-scale extracts of soybean seeds.

In a test system, Maki et al. (19) found that [14C]GA$_{12}$ald was metabolized by intact pea cotyledons primarily by the early-13-hydroxylation pathway,

$$[14C]GA_{12}ald \rightarrow [14C]GA_{33} \rightarrow [14C]GA_{44} \rightarrow [14C]GA_{19,17} \rightarrow [14C]GA_{20}$$

the major GA pathway which had been suggested by enzymolog-

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3 Abbreviations: GA, gibberellin; BSTFA, bis-trimethylsilyl-trifluoroacetamide; n-BuOH, 1-butanol; DAF, days after flowering; DMAP, N,N-dimethyl-amino pyridine; EGME, ethiocholane-3o-ol-17 one glucuronide methyl ester; EtOAc, ethyl acetate; EtOH, 95% ethanol (aq); FID, flame ionization detector; GC-SIM, gas chromatography-selected ion monitoring; HOOAc, acetic acid; iPrOH, 2-propanol; KA, ent-kaurenio acid; KRI, Kovats Retention Index; LC, liquid chromatography; RC, radiocounting; Rt, retention time; Sys, system; amu, atomic mass units.
ical studies (13) and by identification of endogenous GAs (7, 9). These radioactive compounds were also used as markers to co-purify endogenous GAs from pea reproductive tissue, yielding, after three HPLC steps, >95% chemically pure GA$_{17/19}$, GA$_{20}$, GA$_{44}$, and GA$_{53}$, and providing the first identification of GA$_{15}$ as an endogenous component of pea tissue. A similar procedure has been followed with soybeans and the results are presented herein.

**MATERIALS AND METHODS**

**HPLC Systems.** System 1a employed a RoSi C$_{18}$ column 10 × 150 mm to and a solvent flow rate of 4 ml/min. All other columns were 4.6 × 150 mm with solvent flow rates of 1 ml/min. Systems 1b, 2a-2g, and 6a-6c employed a Nucleosil 5 μm C$_{18}$ column; system 3 a PRP-1 column; and systems 4 and 5a-5b a Chromosorb Silica column. All compounds were methylated with CH$_{3}N$_2 prior to separation on systems 4, 5, and 6. Systems 1a-1b, 3, and 4 employed the following linear solvent gradient programs—Systems 1a-1b with solvents (A) 0.1 M HOAc(aq), and (B) 0.1 M HOAc in CH$_3$CN; gradient from 100% to 80% A/20% B in 2 min, gradient to 35% B in 15 min, gradient to 75% B in 15 min, gradient to 100% B in 2 min, isotropic 100% B for 7 min; system 3 with solvents (C) 10 mM Na$_2$HPO$_4$ in 10% EtOH(aq) and (D) 1 mM Na$_2$HPO$_4$ in 75% EtOH(aq); gradient from 100% C to 100% D in 26 min, isotropic 100% D for 7 min; and system 4, isotropic 1% iPrOH/99% C$_2$H$_4$$_2$O$_2$ for 2 min, gradient to 25% iPrOH/75% C$_2$H$_4$$_2$O$_2$ in 24 min, isotropic 25% iPrOH/75% C$_2$H$_4$$_2$O$_2$ for 4 min. System 2a-2g, 5a-5b, and 6a-6c employed isocratic elution with 45% A/55% B (2a), 50% B (2b), 45% B (2c), 40% B (2d), 35% B (2e), 27% B (2f); 0.2% iPrOH/9.8% C$_2$H$_4$$_2$O$_2$ (5a), 1% iPrOH/99% C$_2$H$_4$$_2$O$_2$ (5b); 75% CH$_3$CN(aq) (6a), 58% CH$_3$CN(aq) (6b), and 55% CH$_3$CN(aq) (6c).

Injection volumes were about 500 μl. Radioactive compounds eluting from the columns were detected by continuous scintillation counting with a Packard Trace 7140; and organic material by UV absorbance at 205 nm (the detection limit for GA$_{17}$ was about 100 ng).

**TLC Systems.** Silica gel plates were developed with toluene: EtOH:conc. NH$_4$$_3$O$_4$ (aq) (155:83:1, v/v) for system I; CH$_2$Cl$_2$:EtOAc:HOAc (70:30:1, v/v) for system IIa, 50:50:1 for system IIb, and 40:60:1 for system IIc; or hexanee:iPrOH:HOAc (90:10:1) for system III.

**Gas Chromatography-Mass Spectroscopy.** GC-MS was performed with a Kratos MS-25 at 70 eV essentially as described (19). Prior to GC-MS, all GA had been methylated. They were trimethylsilylated by incubation with 10 μl pyridine + 10 μl BSTFA + 5 μl DMAP at 22°C for 20 min. Ten-μl samples were injected into the GC. KRI were estimated from parallel runs with 'Parafilm' dissolved in hexane (10). Recording of mass spectra was initiated 6 min after the GLC column reached 270°C. KRI of some standards were: Me-KA, 2457; Me-GA$_{3}$, 2500; MeTMS-GA$_{20}$, 2605; MeTMS-GA$_{22}$, 2614; MeTMS-GA$_{3}$, 2614; and MeTMS-GA$_{5}$, 2700. GC-SIM analysis of all samples except l$^{14}$CJA$_{15}$ was performed on this system.

**Gas Chromatography-Selected Ion Monitoring of l$^{14}$CJA$_{15}$.** GC-SIM was performed essentially as described in Bottini et al. (3) (with a final GLC temperature of 250°C).

**Gas Chromatography-Radio Counting.** Methylated GAs were treated with BSTFA and then subjected to GLC on an OV-101 column (2 mm × 183 cm) with a Hewlett Packard 5880A instrument. The carrier gas was He, flowing at 2.7 ml/min. The column was held at the injection temperature of 255°C for 10 min, linearly raised to 275°C in 2 min, and held at 275°C for 10 min. Chromatography on OV-17 was accomplished similarly with a 2 mm × 122 cm column held at 6 ml/min with a temperature program of 1 min at 158°C, 10 min linear increase to 300°C, and hold for 5 min. Retention times were determined by FID for all compounds except l$^{14}$CJD.1 and l$^{14}$CJD.2, and by RC (Barber Coleman Co., Series 5000) for l$^{14}$CJD.1, l$^{14}$CJD.2, and l$^{14}$CJA$_{5}$. The delay between the FID and RC responses for the l$^{14}$CJA$_{5}$ was used to calibrate the system, and all Rts adjusted accordingly.

**Silica Partition Chromatography of Conjugates.** The procedure of Koshioka et al. (17) was followed except that free GAs were eluted with EtOAc/HOAc (99/1), and column dimensions were 25 × 7 mm i.d. A model apolar conjugate, EGE$_{5}$, eluted in the methanol fraction and GA$_{5}$ in the EtOAc/HOAc fraction.

**Plants.** Glycine max (var Evans) seeds were planted in the field in St. Paul, MN on July 1, 1983 and July 5, 1984 in 76-cm rows, 3.8 cm apart, and plants were thinned to 7.6 cm 3 weeks later. Newly formed, white flowers on the fifth to seventh nodes were tagged 35 d after planting for future sampling. The pea plants used for studying l$^{14}$CJA$_{15}$ metabolism were grown and seeds harvested 22 DAF as described (19).

**Isolation and Incubation of Reproductive Tissue with Radioactive Substrates.** Soybean pods harvested 7 to 20 DAF were immediately stored at −80°C until extraction of the whole pods. Otherwise, cotyledons were isolated and stored at −80°C or treated with l$^{14}$CJA$_{5}$, l$^{14}$CJA$_{5}$ald or l$^{14}$CJA$_{5}$, as follows. A single cotyledon was placed on its abaxial surface upon moist filter paper and 5 μl of EtOH containing the l$^{14}$CJA$_{5}$ (ald) (about 50,000 dpm, 35 ng) was placed on the adaxial surface. After incubation in the dark at 22°C, cotyledons were frozen on dry ice and stored at −80°C until extraction.

**Extraction and Isolation of GA.** Tissues were extracted twice in 80% MeOH, and in the case of bulk extracts, partitioned against EtOAc as described (19). After acidic EtOAc partitioning, the aqueous phase of bulk extracts was partitioned against four volumes of n-BuOH.

**Preparation of Radioactive Markers and Their Addition to Bulk Extracts.** The radioactive products from a 4-h incubation of 24-DAF (about 50% filled) cotyledons with l$^{14}$CJA$_{5}$ald consisted of a mixture of at least 20 [%]C, [%]C, [%]C, etc. These were added to bulk extracts, and the extracts then further purified. For purification of endogenous material co-eluting with [%]C and [%]C, 80-gm batches of 24-, 32-, and 52-DAF embryo tissue were pooled. For [%]C through [%]C, 30 gm of 7-to-20-DAF pods were pooled with 160 g of 24-DAF embryo tissue.

**Purification of Bulk Extracts.** The acidic EtOAc fractions of bulk extracts, mixed with the radioactive markers as described above, were concentrated in vacuo and subjected to HPLC system 1a with fractions collected every min. After aliquots were removed for bioassay, the 1-min fractions were pooled into peaks as indicated in Figure 1B. Each fraction was then separated on two or three more HPLC systems as follows: Fraction B—system 2b, then system 5a, then system 6a; D—2e, 5b, 6b; E—2a, 6c; F—2d, 3, 4; G—2e, 3, 4; H—2e, 3, 4; J—2f, 3, 4; and K—2f, 4, 3. After each step, fractions co-eluting with the major [%]C peaks were concentrated with an air stream and then lyophilized, except that after the last step, they were air-dried into 400-μl conical vials with iPrOH added to aqueous samples to hasten evaporation.

**Bioassay of GA.** The immersion, dwarf-rice (cv Tan-ginbozu) bioassay (23) was used. Quantifications were made by a standard curve which afforded a linear relationship between log (ng of GA$_{5}$) and log (length of second leaf sheath minus length of control [no GA]) over the range 1 to 100 ng; 1 ng GA$_{5}$ was barely detectable. Samples were assayed with one vial of five seedlings at each dilution.

**Cell-Free Reactions.** Cell-free extracts were prepared at 4°C by homogenizing cotyledons with 40 mM K-Hepes (pH 7.0), 0.6 M mannitol, 2.5 mM MgCl$_2$, 1 mM DTT, and 1 g/L BSA (1.6 ml buffer/g tissue). After centrifugation (12,000g × 30 min), three
volumes of the supernatant were incubated for 4 h at 22°C with one volume of reaction mix prepared to give the following final concentrations: NADPH, 1 mM; MgSO₄, 5 mM; FeSO₄, 0.5 mM; ascorbic acid, 5 mM; 2-oxoglutarate, 5 mM; [¹⁴C]GA₃, about 0.2 mM; and pH 7.0. Reaction products were analyzed by HPLC system 1b.

Enzymic Hydrolysis of Conjugates. Presumptive conjugates were incubated for 0, 4, or 24 h at 22°C in 10 mM KP (pH 5.0) containing A. niger cellulase (3 mg/ml), T. viride cellulase (3 mg/ml), amon β-glucosidase (3 mg/ml), and A. niger pectinase (1 mg/ml). This mixture is similar to Boots Pectinolysin (26).

Chemical Reactions. (a) Acetylation was accomplished by incubation overnight in pyridine/Ac₂O (2/1), and was monitored by TLC system III for GA₄/7 and by HPLC system 1b for [¹³C]-D. (b) Saponification with NaOH was performed as described (13) and was monitored by HPLC system 1b. Methylation was performed with CH₃N₂ (19).

Materials. Co-factors and enzymes were purchased from Sigma. HPLC matrices were obtained from Alltech (RoSil C₁₈); Hamilton (PRP-1); Macherey-Nagel (Nucleosil C₁₈); or Merck (Spherisorb). EGME was synthesized by methylation of the corresponding acid (purchased from Sigma).

Authentic GA and KA were obtained or synthesized as follows: KA (24); GA₃, GA₄, and GA₅ (>95%) from Abbott Laboratories; [⁴C]GA₃ from Amersham; GA₁₅ for GC-SIM from J. MacMillan; a mixture of GA₃ and GA₄ for GLC-RC by isolation from *Thlaspi arvense* L. (20); [³H]GA₃ and [³H]GA₅₀ (21); [³H]GA₃ from A. Crozier (30). [2,3,5,6,9,11,13,14-'⁴C]GA₁₂ (about 200 Ci/mol) and [2,3,5,6,9,11,13,14-'⁴C]GA₁₂ (about 200 Ci/mol) were biosynthesized (2), as were [¹⁴C]GA₄₄ and [¹⁴C]GA₅₃ (19).

RESULTS

GA-Like Activity. To estimate the amount of GA-like activity in soybean cotyledons, extracts were made at five stages of development (Table I). The free GA-like substances were partitioned into acidic EtOAc and highly polar substances (most of the conjugated GAs) into acidic n-BuOH. The free GA were separated by HPLC system 1a into 30 fractions. Each HPLC fraction was assayed on Tan-ginbozu at two dilutions, 0.4 or 4 g fresh weight of tissue equivalents per vial. The n-BuOH fractions were each assayed without prior HPLC at 0.04, 0.4, and 4 g equivalents. No significant GA-like activity was detected in the n-BuOH fractions. The most active free GA preparation—from 24-DAF cotyledons—had many subfractions with GA-like activity (Fig. 1). Qualitatively similar patterns were obtained with the other preparations except for the 52-DAF extract, which had negligible bioactivity. GA-like bioactivity from soybean seeds was present in many fractions, but little was associated with fractions where GA₇ or GA₁₀ (the major bioactive 'early 13-hydroxy' GAs [7]) would have eluted (Fig. 1).

Total GA-like activity in all fractions (Table I) was highest at

<p>| Table 1. Levels of Total GA-Like Substances in Soybean Reproductive Tissue at Five Stages of Development |
|--------------------------------------------------------|--------------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Days after Flowering</th>
<th>Percent of Maximum Seed Fresh Wt</th>
<th>Amt in GA Equiv. (ng/g fresh wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7–20</td>
<td>0–40</td>
<td>3e</td>
</tr>
<tr>
<td>24</td>
<td>50</td>
<td>3b 10f</td>
</tr>
<tr>
<td>32</td>
<td>75</td>
<td>3b</td>
</tr>
<tr>
<td>42</td>
<td>100</td>
<td>2b</td>
</tr>
<tr>
<td>52</td>
<td>75a</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Soybean cotyledons (24–52 DAF) or whole pods (7–20 DAF) were isolated from field-grown plants, extracted, and bioassayed on dwarf-rice as in Figure 1.  
  a 1983 harvest.  
  b 1984 harvest.  
  c Dehydration phase.

Fig. 1. A to C, Reversed-phase HPLC separation of the products of GA₇ ald metabolism by half-filled (24-DAF) soybean cotyledons. Each cotyledon was incubated with [¹⁴C]GA₇ ald (about 40 ng, 200 Ci/mol) in the dark for 0.5, 4, or 24 h, extracted, and the products separated on HPLC system 1a. Each peak was assigned a letter from A to P. There was a slight malfunction of the HPLC pumps during run B (causing the [¹⁴C]GA₇ ald to elute slightly early). Significant (P < 0.05) biological activity of an extract from equivalent soybean cotyledons in the immersion, dwarf-rice assay. The extract’s acidic EtOAc fraction was separated as in (A–C), and 1 min fractions collected. Aliquots representing 4 g of tissue were assayed in duplicate. Fractions were also assayed in the presence of 10 ng GA₇; only for five fractions was the resulting activity less than expected. The apparent inhibition of the GA₇ exerted by these five fractions (indicated by roman numerals) were: (i) 50%; (ii) and (iii) 100% (total inhibition); (iv) 10%; and (v) 30%.

about the mid-pod-fill stage as in peas and runner beans, but was about 1000-fold lower (8, 9). The apparently low bioactivity does not reflect significant loss during sample purification since recovery of both polar and nonpolar [¹³C]GAs added as internal standards was about 70% through this procedure; and since analyzing mid-pod-fill peas in a similar manner gave the expected results (26). Endogenous inhibitor(s) of the immersion assay, probably ABA based on the Rt, may have obscured some GA bioactivity eluting with Rt 14-15 (Fig. 1D).

GA₇ Aldehyde Metabolism. Preliminary experiments indicated that the metabolism of [¹⁴C]GA₇ ald was similar in 20, 24, 32, and 42 DAF cotyledons (40, 50, 75, and 100% filled, respectively). In a subsequent experiment with half-filled cotyledons, 0.5 h of metabolism afforded at least 12 [¹³C]metabolites, and 4 h at least 20 (Fig. 1, A and B). After 24 h, the [¹³C] products, most of which eluted prior to GA₃ on C₁₈ HPLC, were so numerous that the radioactivity response was well above baseline during the first half of the HPLC run (Fig. 1C).

When this experiment was repeated with metabolism terminated after shorter time intervals (5, 15, and 30 min), peak [¹³C]J was the first product formed and peak [¹³C]D₂ the second. Since, [¹³C]J was postulated to be [¹³C]GA₁₀ (see below), the experiment was repeated with [¹³C]GA₁₀. Most of the same [¹³C] peaks were obtained, with [¹³C]D₂ (later shown to be [¹³C]GA₁₃) as the earliest major product.

The large number of [¹³C] peaks in Figure 1, B and C suggested that some were GA conjugates. This hypothesis was tested in
two ways. First, aliquots of each peak [\(^{14}C\)CB to \(^{14}C\)J] were individually separated on small, silica-partition columns into fractions which should contain free or conjugated GA (17). All the radioactive activity associated with peaks N to O eluted in the putative conjugate fraction, as did about half of that from I, and about 20% of that from E. Second, each peak, [\(^{14}C\)CB to \(^{14}C\)J], was treated with a mixture of hydrolytic enzymes similar to Boots Pectinolysin (26) and the products analyzed on TLC system IIa, IIb, or IIc. Most of the radioactive substances in peaks E and I were converted to less polar products after 24 h (as determined by TLC), a result consistent with these peaks containing conjugated GA. The polarity of the radioactive compounds in the other peaks was unchanged by the enzymic treatment. Therefore, based on these two tests, even after 24 h of metabolism, only about one-quarter of the radioactive metabolites appear to be conjugated.

Cell-Free Metabolism. Since it has been reported (11) that cell-free preparations produce metabolites representative of endogenous GAs, the metabolism of [\(^{14}C\)GA13] was investigated with crude enzyme extracts of cotyledons. Only three products were formed, but these products had the same HPLC system 1 Rts as [\(^{14}C\)CB] and [\(^{14}C\)J], and in one case, [\(^{14}C\)CB] (chromatograms not shown). With extracts from 24 DAF cotyledons, a 4 h incubation afforded a 42% yield of [\(^{14}C\)B], 7% of [\(^{14}C\)D], and 1% of [\(^{14}C\)I]. Extracts of older cotyledons (32, 42, and 52 DAF) afforded less [\(^{14}C\)B], and no [\(^{14}C\)D] or [\(^{14}C\)I]. With [\(^{14}C\)GA13] as substrate, only [\(^{14}C\)B] was obtained—in 25% yield with extracts of 24 DAF extracts, and less with extracts of older tissues. Thus, soybean cotyledons contain enzymes capable of converting GA13 to B (GA12) and then to D (GA13), with the younger tissues, which had more GA-like bioactivity than the older ones (Table 1), having a greater capacity for converting GA12 to GA13.

Identification of [\(^{14}C\)J] as [\(^{14}C\)GA12]. Purification of [\(^{14}C\)J] on HPLC system 2b yielded a radiochemically pure substance (based on two TLC and three HPLC systems) with the Rts of [\(^{14}C\)GA13] (system 2b, 10.6 min; system 5a, 9.0 min; and system 6a, 11.6 min). [\(^{14}C\)J] was immobile on TLC system 1 indicating that it was a dicarboxylic acid (28). Furthermore, on TLC systems IIa and IIb, the Rf of [\(^{14}C\)J] was slightly lower than that of GA8, as would be expected for GA13 (14).

Only a single step is required to oxidize GA13 to the dicarboxylic acid GA12, suggesting that [\(^{14}C\)J] was [\(^{14}C\)GA13]. This hypothesis was confirmed by the metabolism of [\(^{14}C\)J] to the metabolism of [\(^{14}C\)GA12] in pairs of cotyledons isolated from the same embryo of both soybean and pea. In each case, the chromatographic patterns of the products of [\(^{14}C\)CB] and [\(^{14}C\)J] were nearly identical (Fig. 2).

Identification of [\(^{14}C\)D.2] as [\(^{14}C\)GA15]. Peak D was separated into two radioactive compounds by HPLC system 2c—a minor, component 'D.1' (Rt = 10.6 min), and 'D.2' (Rt = 12.0 min). We had an inadequate amount of authentic GA15 for use as an LC standard, but [\(^{14}C\)D.2 co-chromatographed with GA15 in LC system 1 (Rf = 0.2). IIa (Rf = 0.8), and IIb (Rf = 0.95); and on HPLC system 2c and 6b (Rt = 11.4 min). GA15 and GA9 have similar LC properties (16). The formation of GA15 from GA13 would require only one oxidation at C-20 followed by chemical esterification of GA15-open lactone to GA15. The formation of GA15 from GA12 requires three steps [11]. [\(^{14}C\)D.2] was unchanged by acetylation or saponification, consistent with the fact that GA15 has no free hydroxyl or ester groups (the lactone reforms on removal of the base [13]); model compounds GA9 and GA13 did react quantitatively with Ac2O, and the model compound [\(^{14}C\)G]A14 was unchanged by saponification. We concluded that [\(^{14}C\)D.2] was likely to be [\(^{14}C\)GA15].

The methyl esters of δ-lactone GAs have distinctively long RT on GLC (5). [\(^{14}C\)Me-D.2 and Me-GA13] co-eluted on two packed column, GLC-RC systems (10.6 min on OV-101, 1.8 min after GA4 and only 0.1 min before GA5; 13.4 min on the more polar OV-17, 3.2 min after Me-GA9). Such Rts are very unusual for GAs as apolar as [\(^{14}C\)Me-D.2 except for δ-lactones. On neither of these columns was any radioactivity associated with Me-GA9 or any other FID peak except Me-GA15. Based on its long Rt of 10.3 min on OV-101, [\(^{14}C\)D.1 probably has a δ-lactone, and could be an artifact produced in isolating GA15.

Additional evidence that [\(^{14}C\)D.2 is [\(^{14}C\)GA15 was provided by capillary-column GC-SIM. Authentic Me-GA15 eluted at 11.74 min (sd = 0.05 min) as indicated by the co-elution of its characteristic ions—434 m/e (M+), 312, and 284. [\(^{14}C\)Me-D.2 afforded co-eluting ions of 360, 328, and 300 amu at 11.68 min as would be expected for [2,3,5,6,9,11,13,14-\(^{14}C\)]MeGA15, whose gisbene ring is 16 (8 x 2) amu heavier than that of [\(^{14}C\)]MeGA15. Peak D.2 also afforded the characteristic ions of [\(^{14}C\)]GA15 (m/e 344, 312, and 284), but it cannot be determined whether these are derived from endogenous GA15 or unlabeled GA15, produced from GA13. Too little [\(^{14}C\)Me-D.2 was available for the ratio of the characteristic ions to be meaningful, but in combination with the experiments described above, these GC-SIM data confirm that [\(^{14}C\)Me-D.2 is [\(^{14}C\)Me-GA15].

Purification of Bulk Extracts and Identification of GA15. The procedure (19) used to identify endogenous pea GAs was applied to soybean reproductive tissue. Markers of [\(^{14}C\)J] through [\(^{14}C\)]-K from a 4-h incubation of [\(^{14}C\)GA12] (Fig. 1) were added to the acidic EtOAc fractions of bulk extracts of soybean reproductive tissue and the extracts purified through four HPLC systems (cf. "Materials and Methods"). During gradient C8 HPLC, ma-
material co-eluting with each peak ([14]C)J to [14]C)K was collected separately; during each subsequent HPLC step, material co-eluting with each of the major, distinct radioactive peaks was saved and subjected to further purification.

In contrast to the results with peas (19), there were no detectable UV-absorbing (205 nm) peaks with the same Rf as the radioactive markers. Even so, each fraction was analyzed for nonlabeled GAs by GC-MS, and by GC-SIM for characteristic ions of appropriate 'early 3-OH' or 'early 3,13-OH' GAs (5). In only two cases were mass spectra resembling those of known GAs obtained (Table II)—E.2 (which represented 34% of peak E after 4 h of metabolism) and F.2.2 (14% of peak F).

The spectrum afforded by E.2 (hereafter called 'GA7'); HPLC Rts 5.4 min on system 2a and 8.8 min on system 6c) is not that of any known GA (5, 27) but has some fragmentation ions characteristic of Me-GA9 (5). However, its GC Rt, almost as long as that of Me-TMS-GA9, is several minutes more than either Me-GA9 or 15,16-endorearranged Me-GA9 (data not shown). Since its Rt on C18 HPLC was just less than that of GA9, it may be a glucose conjugate of a GA similar to GA9. However, no fragmentation ions characteristic of a TMS derivative of a sugar were detected (12).

The two GAs in fraction F.2.2 (HPLC Rts 21.2 on system 3 and 14.0 min on system 4) had mass spectra and KRI that matched GA7 and iso-GA7 (Table II). Iso-GA7 was consistently obtained, albeit in varying amounts, during derivatization and GC-MS of GA7 or GA9. The possibility that the GA7 may have been a contaminant was ruled out since the only form of GA7 present in the laboratory during these experiments was GA9. However, no characteristic ions of GA7 (418, 386, 284) were found in scans at or near KRI 2614, even though these two compounds will coelute through the purification scheme used.

Based on the height of the GC-GLC trace and accounting for recovery during purification, the bulk extract of soybean cotyledons contained about 5 ng/g fresh weight of GA7. Based on bioassy, peak F (HPLC fractions 22-24 in Fig. 1), contained about 0.5 ng/g fresh weight of GA7-like bioactivity, equivalent to 1.5 ng/g fresh weight GA7-like material in the immersion, dwarf-rice assay.

**DISCUSSION**

GA-like bioactivity in soybean cotyledons was much lower than in other legumes (cf. Table I) and more nearly approximated that found in seeds of rice (15). Unlike rice (15), GA-like bioactivity was also low in young soybean reproductive structures (Table I). The GA-like bioactivity in soybeans and the metabolites of [14]C)GA9ald were present in many different HPLC fractions. Although she did not attempt to identify the metabolites, Nash (22) observed a similar large number of metabolites of [14]H)GA9 following its injection into young runner bean seedlings.

Soybean cotyledons have an active system for metabolizing GA12ald. Within 5 min, intact cotyledons converted [14]C)GA12ald to [14]C)GA12 and [14]C)GA15 in 15% yield (data not shown), and within 4 h, 90% of [14]C)GA12ald was converted to at least 20 compounds (cf. Fig. 1). Although the half-filled soybean cotyledons had about 1000-fold lower levels of free GA-like substances than comparable pea tissue, the soybeans metabolized [14]C)GA12ald at about one-eighth the efficiency of peas (compare Figs. 1 and 19 and herein). Similarly, cell-free preparations of soybean metabolized [14]C)GA12 or [14]C)GA12ald at about one-fourth the efficiency of pea preparations—e.g. 25 and 40% oxidations of 0.2 µM GA12 and GA12ald in 4 h for soybean; 87% conversion of 17.4 µM GA12 to GA13; and 99% conversion of 2.4 µM GA12 to GA15 for pea under similar conditions (14). Since these substrate concentrations are near the reported Kms values of GA precursors with microsomal oxidases (0.2-11 µM [11]), these conversion percentages probably are approximately proportional to enzyme titers. The relatively rapid metabolizing potential (at least for GA12ald and GA13) of soybean cotyledons and their cell-free extracts, coupled with the very low levels of endogenous free GA, may indicate a high rate of GA turnover in the reproductive tissues to inactive compounds.

Since both intact cotyledons and their cell-free extracts converted [14]C)GA12ald to [14]C)GA12 and then to [14]C)GA15, this

**Table II. Mass Spectra of Gibberellin-Like Substances Isolated from Soybean Cotyledons**

<table>
<thead>
<tr>
<th>Putative Gibberellin</th>
<th>Associated Metabolite of [14]C)GA12ald</th>
<th>KRI</th>
<th>Mass Spectrum* m/e (% of base peak)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me-TMS-GA7</td>
<td>Me-TMS-F.2.2</td>
<td>2616</td>
<td>416 (M*, 17) 384 (42) 356 (50) 333 (24) 312 (28) 311 (24) 298 (24) 270 (33) 269 (26) 244 (30) 243 (21) 223 (59) 222 (100) 221 (40)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>416 (M*, 20) 384 (36) 356 (51) 312 (25) 311 (25) 298 (41) 281 (44) 270 (17) 269 (19) 244 (19) 242 (25) 223 (68) 222 (100) 221 (39)</td>
</tr>
<tr>
<td>Authentic Me-TMS-GA7</td>
<td></td>
<td>2614</td>
<td>416 (M*, 21) 384 (6) 356 (7) 326 (13) 313 (14) 299 (11) 298 (10) 282 (36) 281 (9) 267 (17) 244 (14) 238 (19) 223 (50) 222 (100)</td>
</tr>
<tr>
<td>Me-TMS-iso-GA7</td>
<td>Me-TMS-F.2.2</td>
<td>2633</td>
<td>416 (M*, 16) 384 (17) 356 (20) 326 (9) 313 (10) 312 (12) 299 (6) 298 (24) 282 (30) 281 (21) 267 (12) 223 (55) 222 (100) 221 (23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>344 (44) 330 (76) 317 (32) 316 (55) 303 (47) 302 (72) 298 (36) 288 (44) 281 (20) 272 (40) 271 (60) 270 (100) 257 (27) 256 (24) 243 (99) 242 (39) 229 (43) 228 (42) 214 (39) 211 (32)</td>
</tr>
<tr>
<td>'GA7'</td>
<td>Me-TMS-E.2</td>
<td>2655</td>
<td></td>
</tr>
</tbody>
</table>
probably represents an endogenous pathway. This sequence has been reported to begin two tips of pathways leading to active (non-28-hydroxylated) C-19 GAs:

\[
\text{GA}_3 + \text{GA}_1 + \text{GA}_3\text{(open lactone)} \rightarrow \text{GA}_2 + \text{GA}_3
\]

The steps in the upper pathway have been demonstrated enzymatically or by identification of endogenous GA in several other species (11, 13, 25). All of the enzymes of the lower pathway except the last have been isolated from *Cucurbita maxima* (11), and work with *Sechium edule* indicates that *Sechium* possesses the entire lower pathway (1, 4, 18). *Cyathura australis* may have both pathways except for the dehydrogenation of GA₂ to GA₃; this fern probably further oxidized GA₃ and GA₅ to 11-OH and 12-OH GAs (29). Soybeans may use the pathway to GA₅ just described since: (a) In 4 h, cotyledons did not convert [¹⁴C]-GA₁₅ to any detectable [¹⁴C]GA₉ (cf. GLC-RC results). (b) GA₅ was observed as an endogenous component of the reproductive tissue.

The procedure outlined by Maki et al. (19) allowed us to isolate GA₃ and the GA₅ component with sufficient purity to obtain clean mass spectra by GC-MS. Purification using bioassay alone probably would have been insufficient to yield fractions pure enough to identify by GC-MS. In fact, the bioactivity associated with GA₃ (Fig. 1), though significant, represented only 5% of the total bioactivity and thus might have been thought unimportant.

Two sets of observations require some discussion. First, most of the [¹⁴C]GA₁₅ metabolites did not serve as markers for detectable levels of endogenous GAs. Since each soybean cotyledon contained only about 1 ng of GA₁₅-like material, it is likely that the 40 ng of [¹⁴C]GA₁₅ fed to each cotyledon was well above the endogenous level of GA₁₅ and that some of the products were artifacts of substrate overloading. Although others may have been "natural" metabolites, equivalent nonlabeled endogenous GAs were presumably present at levels too low to detect.

Second, two prominent GA-like bioactive peaks (Fig. 1) which were theoretically present in sufficient quantities for GC-MS identification remain unidentified. The less polar one (RT = 29 min, Fig. 1) co-eluted with GA₁₂ on HPLC system 1. Since subsequent purification of material co-chromatographing with [¹⁴C]GA₁₂ afforded no known GAs, this bioactive peak contained no detectable endogenous GA₁₂.

The more polar bioactive peak (RT = 20–21 min, Fig. 1) is not a major product of exogenous GA₁₅ since the four major [¹⁴C]GA₁₅ metabolites which eluted with approximately this RT (Fig. 1) did not serve as markers for detectable endogenous GA. The bioactive substance with RT = 20 to 21 min may lie on a biosynthetic pathway occurring at a site which exogenously applied GA₁₅ does not reach. Alternatively, it could represent a trace amount of GA₃₆ which is about as active as GA₁₅ in the dwarf-riase assay and which elutes just before GA₁₅ on C₁₈ HPLC (16). Since a negligible amount of [¹⁴C]GA₃ was detected, probably very little of its logical precursors, [¹⁴C]GA₃₅ and [¹⁴C]GA₄₃, were present, and [¹⁴C]GA₃₆ could have been a minor, and therefore ignored, component of peaks [¹⁴C]GA₁₅ or [¹⁴C]CH₂.

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