

Metabolism of Tritiated Gibberellin A₂₀ in Maize¹

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ABSTRACT

After the application of 2.36 Curies per millimole [2,3-³H]gibberellin A₂₀ (GA₂₀) to 21-day-old maize (*Zea mays* L., hybrid CM7 × CM49) plants, etiolated maize seedlings, or maturing maize cobs, a number of ³H-metabolites were observed. The principal acidic (pH 3.0), ethyl acetate-soluble metabolite was identified as [³H]GA₁ on the basis of co-chromatography with standard [³H]GA₁ on SiO₂ partition, high resolution isocratic elution reverse phase C₁₈ high performance liquid chromatography and gas-liquid chromatography radiocounting. Two other acidic metabolites were identified similarly as [³H]GA₈ and C/D ring-rearranged [³H]GA₂₀, although gas-liquid chromatography radiocounting was not performed on these metabolites. Numerous acidic, butanol-soluble (e.g. ethyl acetate-insoluble) metabolites were observed with retention times on C₁₈ high performance liquid chromatography radiocounting similar to those of authentic glucosyl conjugates of GA₁ and GA₈, or with retention times where conjugates of GA₂₀ would be expected to elute. Conversion to [³H]GA₁ was greatest (23% of methanol extractable radioactivity) in 21-day-old maize plants. In etiolated maize seedlings, the C/D ring-rearranged [³H]GA₂₀-like metabolite was the major acidic product, while conversion to [³H]GA₁ was low.

regulation of levels of the biologically active GA(s), and hence should be considered a potential control point. In the present report, the metabolism of [³H]GA₂₀ at several stages of maize plant development is described.

MATERIALS AND METHODS

The metabolism of [³H]GA₂₀ was investigated in maturing maize cobs, husks, and kernels, in etiolated maize seedlings, and in 21-d-old maize plants. The [2,3-³H]GA₂₀ was prepared by hydrogenation of GA₅ methyl ester-16,17-epoxide with tritium gas (14). Immediately prior to applications, [³H]GA₂₀ underwent final purification on a gradient-eluted SiO₂ partition column (5).

Plant Material.

21-d-Old Maize Plants. Three kernels of the early maturing maize (*Zea mays* L.) hybrid CM7 × CM49 (17) were planted in each of 12 plastic pots (13 × 20 cm) filled with a mixture of peat moss and sand. Pots were placed in a growth room at 25/15°C (day/night), 14-h photoperiod (300 μE m⁻² s⁻¹). After emergence, plants were thinned to one per pot. Twenty-one days after planting (at which time the plants were still in the vegetative growth phase), 0.22 μCi [2,3-³H]GA₂₀ (2.36 Ci/mmol) in 0.4 ml 60% aqueous ethanol was pipetted into the leaf whorl. Twenty-four, 48, 96, and 144 h after the addition of [³H]GA₂₀, three plant shoots were excised at the soil surface and 4 cm above the surface, yielding shoot cylinders which contained the apical meristems. These were rinsed with H₂O and then homogenized separately at -40°C in MeOH:H₂O (80:20, v/v).

Maturing Maize Cobs and Husks. Plants were raised under field conditions as previously described (17). On August 28, 1981, at which time kernels contained milky endosperm, about 1 μl [³H]GA₂₀ in 0.3 ml 50:50 EtOH:H₂O (v/v) was injected 0.5 cm into the shank of each ear directly below the cob. After black-layer maturity was reached (4), cobs were harvested and air-dried. Husks and kernels were removed and the cobs or husks were frozen, lyophilized, and then homogenized as above (50 ml 80% MeOH/g dry weight tissue). Three cobs were bulked for analysis of metabolites.

Maturing Maize Kernels and Etiolated Maize Seedlings. Mature kernels of the hybrid DK 23 were placed pericarp-side-down in Petri dishes containing two filter paper discs and 5 ml H₂O. Dishes were wrapped in aluminum foil and stored at 22°C for 72 h. Under a green safelight, 1 μCi [³H]GA₂₀ in 30 μl 50% aqueous ethanol was pipetted onto the germinated embryo after the seedling had been turned over (pericarp up). After 16 h incubation in the dark, the seedling was excised from the kernal remnant and homogenized in 5 ml 80% aqueous MeOH at -40°C.

Extraction, purification, and chromatography of [³H]GA metabolites were performed as previously described (5, 18). Briefly, after 12 h shaking at 4°C, extracts were filtered, 0.25 M pH 8.0 phosphate buffer was added (2 ml for each 10 ml 80% MeOH), and the MeOH was removed *in vacuo* at 35°C. Partitioning against diethyl ether (18) was performed only on extracts from the 21-d-old seedlings and husks as these samples contained moderate amounts of Chl. The aqueous phase was acidified to pH 3.0 with

Hedden *et al.* (10) have recently characterized GA₅₃,⁵ GA₄₄, GA₁₇, GA₁₉, and GA₂₀ from immature maize tassels by GLC-MS. Further, data from selected ion current monitoring GLC-MS indicate that GA₁, GA₂₉, and GA₈ are probably also native in maize (10). These GAs are members of the probable early 13-hydroxylation pathway, a pathway which begins with GA₁₂-aldehyde. The 13-hydroxylation pathway also exists in *Phaseolus vulgaris* (9) where GA₂₀ is apparently a precursor of either GA₁ or GA₂₉ (19). In other plant systems, two principal metabolites have been tentatively identified following the application of [³H]GA₂₀: [³H]GA₁ and/or [³H]GA₂₉ (6-8, 13, 16). Since both GA₁ and GA₂₉ appear to be native in maize (10), a branch point in the metabolic pathway in maize may exist after GA₂₀. The biologically active GA₁ (1) may be produced through C-3 hydroxylation, or alternatively, C-2 hydroxylation may yield the biologically inactive GA₂₉. Given the different biological activities of these potential metabolites, a branch point following GA₂₀ may be a site of

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⁵ Abbreviations: GA, gibberellin; BuOH, butanol; C/D R GA₂₀, C/D ring-rearranged GA₂₀; EtOAc, ethyl acetate; GLC-RC, GLC-radiocounting; MeOH, methanol; MeTMSi, trimethylsilyl ether of the methyl ester; Rt, retention time.

1 N HCl and partitioned four times into EtOAc. The acidic, EtOAc phase was taken to dryness *in vacuo*, solubilized in 0.1 M pH 8.0 phosphate buffer, and purified on polyvinylpyrrolidone (Polyclar AT) (as per Ref. 18) and the eluant from this column was adjusted to pH 3.0 with 1 N HCl prior to partitioning four times with equal volumes of ethyl acetate. The acidic, EtOAc phase was dried and chromatography was accomplished on gradient-eluted Woelm SiO₂ for partition (5, 15, 19). Forty 10-ml fractions were collected rather than larger fractions previously used. Gradient elution reverse phase C₁₈ HPLC was carried out using a Waters Associates ALC/GPC R-401 liquid chromatograph with two model 6000 pumps, a model 660 solvent flow programmer, and a model U6K injector. A Berthold LB 503 radioactivity monitor was used on-line with a heterogeneous counting cell. The MeOH and H₂O for HPLC were purified by fractional distillation, followed by filtration through Millipore HATF or FHUP filters, respectively. Solvent A consisted of 89:10:1 (v:v:v) H₂O:MeOH:acetic acid and solvent B was 100% MeOH. Following sample injection, there was a 10-min flush of 0% B, then a 30-min gradient from 0 to 70% B, both at 1.8 ml min⁻¹. The isocratic elution C₁₈ reverse phase HPLC-RC was carried out using a Whatman M9 Partisil 10 ODS 2 column (9.4 mm × 50 cm). Eluant was 62.3:37:0.7 H₂O:MeOH:acetic acid (v:v:v) at 1.8 ml min⁻¹. After HPLC-RC, samples were converted to the MeTMSi derivatives prior to injection on GLC-RC on a Packard model 430 GLC coupled to a Packard model 884 gas proportional counter. Glass columns were packed with 2% SE30 (2 mm × 2 m) or 3% OV 101 (2 mm × 2 m). Column, detector, and injector temperatures were 230, 210, and 250°C, respectively, and 50 ml min⁻¹ carrier He was used.

The acidic, aqueous phase after partitioning with EtOAc was extracted three times with BuOH, and then the aqueous phase was adjusted to pH 7.0 with 1 N KOH, and again extracted three times with BuOH. A MeOH wash of the SiO₂ partition column after the hexane:EtOAc gradient was completed was used to remove very polar radioactivity (GA glucoside and GA glucosyl ester, which are sparingly soluble in EtOAc will elute in the MeOH wash). This MeOH wash was added to the acidic, and neutral, BuOH-soluble fractions to produce a final 'BuOH-soluble' fraction which was then subjected to gradient elution reverse phase C₁₈ HPLC. This final BuOH-soluble fraction will contain GA glucosyl conjugates and contained virtually all of the highly H₂O-soluble radioactivity.

RESULTS AND DISCUSSION

Following the [³H]GA₂₀ feed to the 21-d-old maize plants, three regions of acidic, EtOAc-soluble radioactivity were eluted from SiO₂ partition columns (Fig. 1). In extracts of the 24-h feed, most of the radioactivity co-chromatographed with authentic [³H]GA₂₀, while a second peak eluted in the more polar region coincidental with [³H]GA₁ (Fig. 1). A less polar shoulder on the [³H]GA₂₀ peak (where C/D R GA₂₀ elutes) was not observed in these samples.

While there was a rapid initial metabolism of [³H]GA₂₀ (Fig. 2), it leveled off by 48 h. Due to the anatomical organization of the leaf whorl surrounding the apical meristem, it probably was not possible to rinse off all of the applied [³H] GA₂₀ that had not been absorbed by the plant. Thus, at least a part of the ³HGA₂₀ peak at all harvests may not have been absorbed by the tissue.

Our SiO₂ partition column does not separate GA₁ from GA₂₉, the two likely principal products from GA₂₀ feeds. Since both GAs are probably native in maize, and GA₂₀ is native, it was of particular interest to characterize further the polar peak which co-chromatographed on SiO₂ with [³H]GA₁.

Jones *et al.* (11) reported that GA₁ and GA₂₉ are readily separated on gradient-eluted reverse phase C₁₈ HPLC and Davies *et al.* (3) also noted their separation. Thus, the ³H peaks from SiO₂ partition chromatography were further analyzed on gradient-

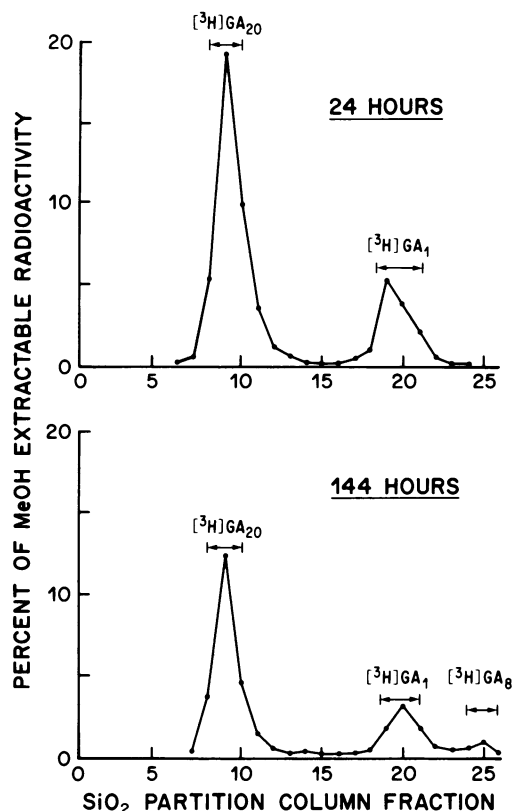


FIG. 1. Profile of ³H elution from gradient-eluted SiO₂ partition columns loaded with extracts from maize plants 24 or 144 h after administering [³H]GA₂₀ to the 21-d-old plants.

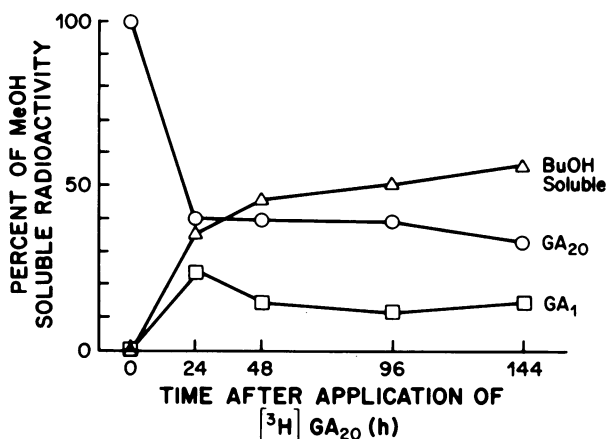


FIG. 2. Time course of changes in ³H-compounds following a [³H]GA₂₀ feed to 21-d-old maize plants. The BuOH-soluble fraction includes the MeOH wash from SiO₂ columns and would include any GA glucosyl conjugates if present.

eluted C₁₈ reverse phase HPLC. The Rt of [³H]GA₂₀ and standards of logical metabolites and some degradation products were determined by reverse phase HPLC-RC for comparison with actual metabolites (Fig. 3).

The [³H]GA₁-like peak from the SiO₂ partition columns for 21-d-old plants (Fig. 1) eluted from the gradient-eluted C₁₈ HPLC column as a single peak coincident with the Rt of [³H]GA₁. This peak was collected and subsequently developed isocratically on reverse phase C₁₈ HPLC-RC using a 50-cm Whatman Magnum 9 column capable of resolving authentic standards of [³H]GA₁ from [¹⁴C]GA₃ by almost 10 min (Fig. 4, upper half). When the [³H]

Table I. Distribution of ^3H following $[^3\text{H}]\text{GA}_{20}$ Feeds to Maize

Qualitative analysis is based on SiO_2 partition chromatography followed by gradient-eluted HPLC-RC, and in the case of GA_1 , by isocratic HPLC and GLC-RC.

Sample $[^3\text{H}]\text{GA}_{20}$ Feeds	Acidic, EtOAc-Soluble Fraction				BuOH-Soluble (Conjugate-like) Fraction				BuOH-Insoluble (very highly H_2O -soluble)	Total EtOAc-Soluble (excluding MeOH wash from SiO_2 partition columns)	Total EtOAc-Insoluble (including MeOH* wash from SiO_2 partition columns)
	C/D R GA_{20}	GA_{20}	GA_1	GA_8	C/D R GA_{20}	GA_{20}	GA_1	GA_8			
% total extractable dpm											
21-Day-old plants harvest at 24 h		41	23							64	36
Harvest at 144 h		32	14	2						48	52
Etiolated seedlings	7	54	4	1						66	34
Mature cobs		3	2		24	27	2		42	5	95
Husks		4	13	5		22	15	3	12	22	78
Kernels	4	18	3		52	12	1			25	75

* Represents the sum of the BuOH-soluble fraction, the BuOH-insoluble fraction (e.g. very highly H_2O -soluble), and the MeOH wash from the SiO_2 partition column after hexane:EtOAc gradient elution.

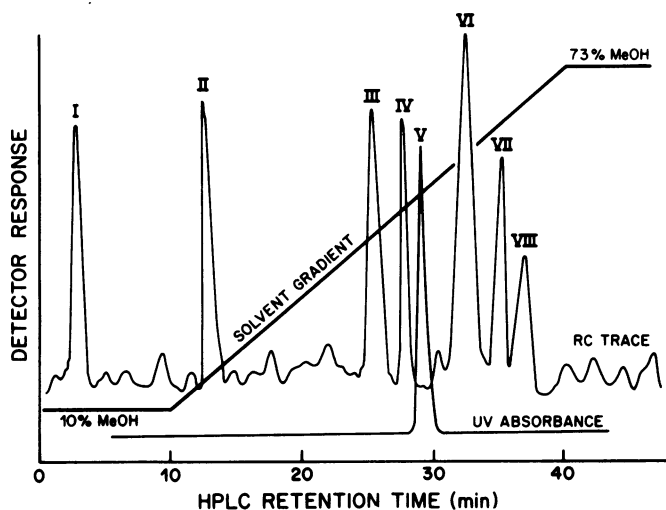


FIG. 3. Gradient elution C_{18} reverse phase HPLC-RC (on-line RC with flow-through cell) of $[^3\text{H}]\text{GA}_{20}$, logical metabolites ($[^3\text{H}]\text{GA}_8$ and $[^3\text{H}]\text{GA}_1$), a reference standard (ABA) which was detected via UV A at 265 nm, and some degradation products. The chromatogram is a composite trace made by superimposing traces from HPLC runs with pairs of $[^3\text{H}]\text{GA}_{20}$ and one other ^3H -compound in each run. I, Unidentified ^3H -degradation product of $[^3\text{H}]\text{GA}_{20}$ which elutes with injection solvent; II, $[^3\text{H}]\text{GA}_8$ (12.5 min); III, $[^3\text{H}]\text{GA}_1$ (25.0 min); IV, unknown H-degradation product of acid-treated $[^3\text{H}]\text{GA}_{20}$ (27.5 min); V, ABA (28.5 min); VI, $[^3\text{H}]\text{GA}_{20}$ (32.0 min); VII, $[^3\text{H}]\text{GA}_{20}\text{-Me}$ (35.0); VIII, C/D R $[^3\text{H}]\text{GA}_{20}$ (36.6 min).

GA_1 -like peak from gradient-eluted HPLC was coinjected with a small spike of $[^{14}\text{C}]\text{GA}_3$, it eluted at the same Rt as authentic $[^3\text{H}]\text{GA}_1$ (Fig. 4, lower half). Thus, the $[^3\text{H}]\text{GA}_1$ -like peak in Figure 1 apparently consisted of only a single metabolite which was not $[^3\text{H}]\text{GA}_{20}$ and probably was $[^3\text{H}]\text{GA}_1$.

While attempts at subsequent GLC-RC of the $[^3\text{H}]\text{GA}_1$ -like peak from the $[^3\text{H}]\text{GA}_{20}$ feed to 21-d-old maize plants failed, a feed of $[^3\text{H}]\text{GA}_{20}$ to maturing maize cobs gave a chromatographically identical metabolite. After initial purification of SiO_2 partition chromatography, about 1.5×10^5 dpm of the $[^3\text{H}]\text{GA}_1$ -like metabolite was developed on gradient-eluted C_{18} HPLC-RC; it

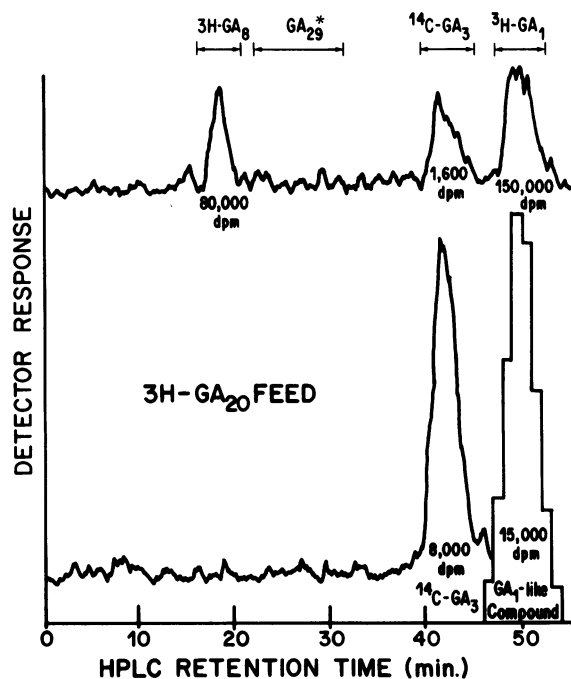


FIG. 4. Isocratic elution C_{18} reverse phase HPLC-RC with elution of $[^3\text{H}]\text{GA}_8$, $[^{14}\text{C}]\text{GA}_3$, $[^3\text{H}]\text{GA}_1$ (upper trace), and of $[^{14}\text{C}]\text{GA}_3$ coinjected with the acidic, polar ^3H -metabolite of $[^3\text{H}]\text{GA}_{20}$ from maize which had co-chromatographed previously with $[^3\text{H}]\text{GA}_1$ on a gradient-eluted SiO_2 partition column (lower trace).

eluted coincidentally with the Rt of authentic $[^3\text{H}]\text{GA}_1$ (24.7 min). This metabolite was then analyzed by GLC-RC. The MeTMSi derivative of the $[^3\text{H}]\text{GA}_1$ -like metabolite eluted coincidentally with authentic standards of $[^3\text{H}]\text{GA}_1$ and GA_1 (Rt 22.5 min) from a 2% SE 30 column. This GLC column adequately separates GA_1 from 3-epi- GA_1 , another possible metabolite of $[^3\text{H}]\text{GA}_{20}$ (6, 12). Thus, the principal acidic, EtOAc-soluble metabolite of $[^3\text{H}]\text{GA}_{20}$ in maize was identified as $[^3\text{H}]\text{GA}_1$.

No detectable $[^3\text{H}]\text{GA}_{29}$ was produced following any of the $[^3\text{H}]\text{GA}_{20}$ feeds to maize. As GA_{20} is a precursor of GA_{29} (8, 16) and as both GA_{20} and GA_{29} are native in maize, the absence of $[^3\text{H}]\text{GA}_{29}$

GA₂₉ was surprising.

It must be noted that since the [³H]GA₂₀ was labeled principally at the C-2 and C-3 positions, some ³H loss would occur during C-2 or C-3 hydroxylation (6). Thus, the conversion of [³H]GA₂₀ to [³H]GA₁ was probably greater than that noted in Table I and detection of [³H]GA₂₉ would also have been hindered. However, tritium loss by such conversions should have been equal in all experiments. Hence, comparisons of metabolic rates under different conditions or at different stages should still be valid.

While [³H]GA₁ was the principal acidic, EtOAc-soluble metabolite in most experiments, other acidic, EtOAc-soluble metabolites were also observed. In the feed to etiolated maize seedlings, the principal acidic, EtOAc-soluble compound (other than [³H]GA₂₀) co-chromatographed on a SiO₂ partition column and on C₁₈ HPLC-RC with C/D R [³H]GA₂₀ (Fig. 5). The origin of C/D R [³H]GA₂₀ is uncertain. However, it is known that C/D R [³H]GA₂₀ can be produced simply by treating [³H]GA₂₀ with acid (6). Extracts were repeatedly exposed to HCl, HCOOH, and CH₃COOH during experimental workup. Thus, it is possible that C/D R [³H]GA₂₀ in the extracts may be an artifact.

In most samples of small ³H-labeled peak eluted at the Rt of [³H]GA₈ on SiO₂ partition columns and on HPLC-RC. Davies and Rappaport (2) have previously shown that [³H]GA₁ is probably converted to [³H]GA₈ in maize, and GA₈ is probably native in maize (10). Thus, this very polar EtOAc-soluble metabolite (Figs. 1 and 5) is probably [³H]GA₈. As noted above, the loss of at least some ³H at the C-2 and C-3 positions during hydroxylation would cause an underestimation of the GA₈ produced from GA₂₀.

Another minor metabolite was also observed in the [³H]GA₂₀ feed to etiolated seedlings (Fig. 5). With a Rt on HPLC-RC of 16.4 min (Fig. 3), this ³H peak did not correspond to any known metabolite or catabolite for which we have HPLC Rt data.

Following the [³H]GA₂₀ feed to maturing maize cobs in field trials, the senesced husks contained more [³H]GA₁ than [³H]GA₂₀ (Fig. 6). However, the relative abundance of acidic, BuOH-soluble metabolites (which are grouped into [³H]GA₁-like or [³H]GA₂₀-like conjugates on the basis of their Rt) tended to be just the opposite, with a far greater abundance of [³H]GA₂₀-like conjugates than [³H]GA₁-like conjugates (Fig. 7). It must be noted here, however, that identification of these BuOH-soluble metabolites as GA conjugates is tentative, being based on known partitioning characteristics, Rt (relative to standard GA glucosyl conjugates) on gradient-eluted C₁₈ HPLC-RC, and the knowledge that [³H]

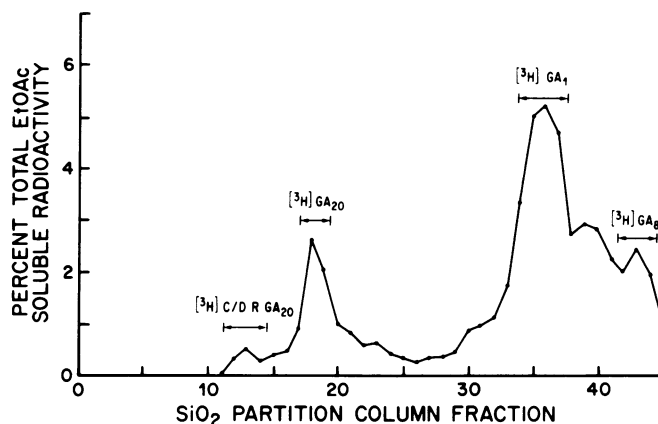


FIG. 6. Profile of ³H elution from a gradient-eluted SiO₂ partition column loaded with the acidic, EtOAc-soluble fraction from maize husks following a [³H]GA₂₀ feed to the cob.

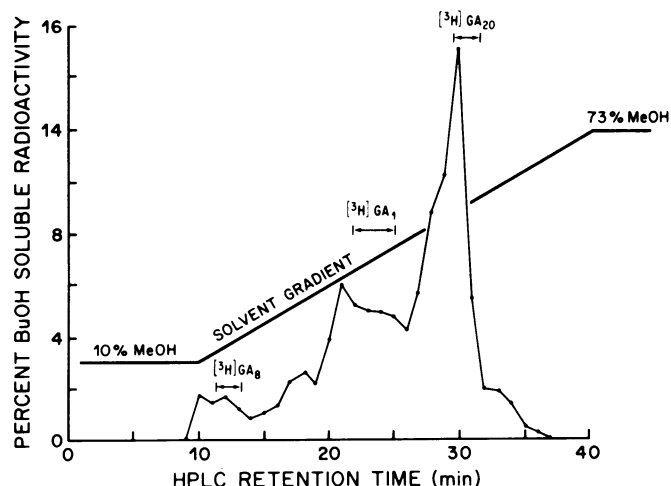


FIG. 7. Profile of ³H elution from C₁₈ reverse phase HPLC of the acidic, EtOAc-insoluble, BuOH-soluble fraction from maize husks following a [³H]GA₂₀ feed to the cob.

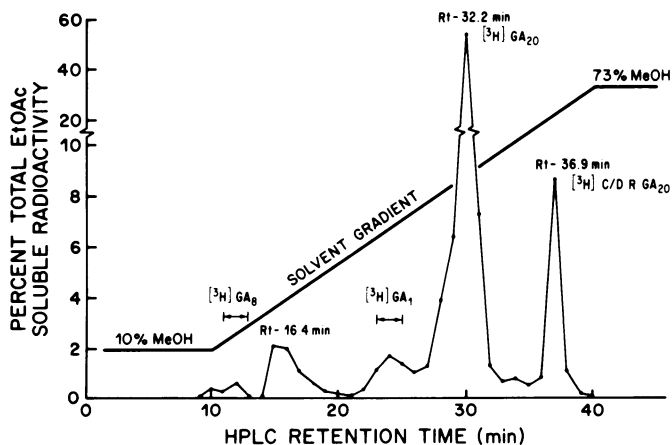


FIG. 5. Profile of ³H elution from gradient elution C₁₈ reverse phase HPLC of the acidic, EtOAc-soluble fraction from an extract of etiolated maize seedlings fed [³H]GA₂₀. Retention times included were determined by the integrator of the on-line HPLC-RC while plotted data points were calculated by liquid scintillation spectrometry of collected aliquots. In a subsequent HPLC analysis, authentic [³H]C/D R GA₂₀ eluted 4.6 min after authentic [³H]GA₂₀.

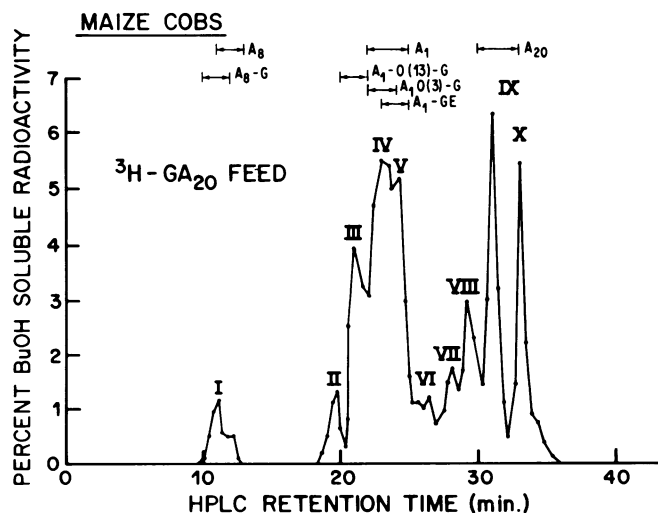


FIG. 8. Profile of ³H elution from C₁₈ reverse phase HPLC of the acidic, EtOAc-insoluble, BuOH-soluble fraction from maize cobs following a [³H]GA₂₀ feed to the cob.

GA glucosyl conjugate-like metabolites from other radioactive feeds (^3H GA₄ feed to anise and carrot somatic cell cultures; M. Koshioka, T. Douglas, D. Ernst, J. Huber, R. Pharis, and A. Jones, unpublished research), which had similar C₁₈ HPLC Rt to the peaks shown in Figures 6 and 7, yielded acidic, ^3H GA moieties upon hydrolysis.

There is apparent peak spreading in the HPLC chromatogram from the BuOH-soluble fraction of maize husks (Fig. 7), and this may have resulted from the large number of compounds which were not adequately resolved when large fractions were collected. However, for an acidic, BuOH-soluble fraction of an extract of maize cobs, a higher resolution reverse phase HPLC-RC analysis of the cobs was obtained by collecting 20-s fractions (Fig. 8) for liquid scintillation spectrometry. The cobs (which had been shelled and hence contained no kernels) contained at least 10 acidic, BuOH-soluble ^3H peaks (Fig. 8). Four of the first five peaks co-chromatographed on reverse phase gradient-eluted C₁₈-HPLC with authentic conjugates of GA₈ and GA₁ (Fig. 8). We had no authentic conjugates of GA₂₀; hence, no comparisons of metabolite peaks eluting in the less polar regions (Rt about 30 min) could be made. However, glucosyl conjugates elute prior to or almost coincident with the free acid GA in this HPLC system (M. Koshioka, S. Rood, R. Pharis, unpublished research) (Fig. 8). Thus, peaks VII, VIII, and IX may represent conjugate-like metabolites of ^3H GA₂₀. However, peak X was probably not a conjugate of ^3H GA₂₀ (Fig. 8). Since C/D R ^3H GA₂₀ elutes 9.1 min after ^3H GA₂₀ and also after peak X, it is possible that peak X represents a conjugate of C/D R ^3H GA₂₀ (Fig. 8).

It is noteworthy that the fate of ^3H GA₂₀ was dependent not only on the ontogenetic stage at GA application (Table I) and on incubation time (Figs. 1 and 2), but also appears to depend on the target tissue. For example, following ^3H GA₂₀ feeds to maturing maize cobs, very little of the radioactivity in the cobs partitioned into EtOAc at pH 3, while in the husks and kernels increasingly more radioactivity was EtOAc-soluble (Table I). The relative abundance of various acidic, BuOH-soluble metabolites also differed in the different tissues (Table I and Fig. 8). A more complete analysis of ^3H GA₂₀ at varying stages of ontogeny is thus warranted.

In summary, following ^3H GA₂₀ feeds to maize at a number of developmental stages, the principal acidic, EtOAc-soluble metabolite was usually a compound which, when analyzed sequentially on a number of different systems, co-chromatographed with ^3H GA₁, ^3H GA₈- and C/D R ^3H GA₂₀-like metabolites were also

produced, although C/D R GA₂₀ may be an artifact. An appreciable portion of radioactivity was EtOAc-insoluble (e.g. BuOH-soluble) and appears to represent GA glucosyl conjugates.

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