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Metabolism of [^{3}H]Gibberellin A_5 by Immature Seeds of Apricot (*Prunus armeniaca* L.)[1]

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ABSTRACT

Immature seeds of apricot (*Prunus armeniaca* L.) were fed the native gibberellin A_5 (GA_5) as 1- and 1,2-[^{3}H]GA_5 (5.3 Curies per millimole to 16 milliCuries per millimole) at doses (42 nanograms to 10.6 micrograms per seed) 2 to 530 times the expected endogenous level. After 4 days of incubation, seeds were extracted and free [^{3}H]GA-like metabolites were separated from the highly H_2O-soluble [^{3}H]metabolites. For high specific activity feeds the retention times (Rts) of radioactive peaks were compared with Rts of authentic GAs on sequential gradient-eluted → isocratic eluted reversed-phase C_{18} high performance liquid chromatography (HPLC) -radiocounting (RC). From high substrate feeds (530 and 230 × expected endogenous levels) HPLC-RC peak groupings were subjected to capillary gas chromatography-selected ion monitoring (GC-SIM), usually six characteristic ions. The major free GA metabolites of [^{3}H]GA_5 were identified as GA_1, GA_3, and GA_6 by GC-SIM. The major highly water soluble metabolite of [^{3}H]GA_5 at all levels of substrate GA_5 had chromatographic characteristics similar to authentic GA_1-glucosyl ester. Expressed as a percentage of recovered radioactivity, low substrate [^{3}H]GA_5 feeds (2 × expected endogenous level) yielded a broad spectrum of metabolites eluting at the Rts where GA_1, GA_3, GA_5 methyl ester, GA_6, GA_{22}, GA_{29} (17, 14, 1.6, 7, 1.1, 0.5%, respectively) and GA glucosyl conjugates of GA_1, GA_3, GA_5, and GA_8 (33, 11, 1, 0.1%, respectively) elute. Metabolites were also present at Rts where GA glucosyl conjugates of GA_6 and GA_{29} would be expected to elute (8 and 0.1%, respectively). Only 5% of the radioactivity remained as GA_5. Increasing substrate GA_5 levels increased the proportion of metabolites with HPLC Rts similar to GA_1, GA_6, and especially GA_1 glucosyl ester, primarily at the expense of metabolites with HPLC Rts similar to GA_3, GA_3-glucosyl ester, and a postulated conjugate of GA_6. There was evidence that high doses of substrate GA_5 induced new metabolites which often, but not always, differed from GA_1, GA_3, and GA_6 in HPLC Rt. These same metabolites, when analyzed by GC-SIM yielded m/e ions the same as the M^+ and other characteristic m/e ions of the above GAs, albeit at differing GC Rt and relative intensities.

Immature seeds or fruits are rich in GAs,[4] and the initial characterizations of GAs in higher plants has been from these tissues (5, 10, 11, 17, 25, 26, and see references in Bearder [2]). Even though related species contain structurally similar GAs (2), the interconversion pathways and rapidity of metabolism may differ with the plant organ and its developmental stage (10–12). Gibberellin A_5 has been identified by GC-SIM as being native to immature seeds of apricot (*Prunus armeniaca*) (4). It is a potential precursor of GA_1 (14), GA_3 (9, 14), and GA_{29} (14), of which GA_1 and GA_{29} are known to be native to immature seeds of apricot (4). Gibberellin A_5 is also a possible, though unlikely, precursor of GA_{32}. In the present work we examine the metabolism of [^{3}H]GA_5 of varying substrate amounts in this tissue.

MATERIALS AND METHODS

Plant Material. Apricot (*Prunus armeniaca*) fruits, with seeds weighing 0.25 to 0.5 g f.w. (very little embryo development) were collected 3 to 4 weeks after anthesis in the Waite experimental orchard, Glen Osmond, South Australia.

All fruits without pedicels were rejected. Approximately 0.67 μCi of 1-[^{3}H]GA_5 ([18]; 5.3 Ci mM^{-1}), 0.67 μCi of 1-[^{3}H]GA_5 (48 mCi mM^{-1}), and 0.5 μCi of 1,2-[^{3}H]GA_5 ([9]; 16 mCi mM^{-1}) were diluted in 5 μl of aqueous 8 mM $KHCO_3$ solution, and injected into each seed (Fig. 1). Three lots of 50 seeds were treated. Thus, 1-[^{3}H]GA_5 was fed at approximately 2 and 230 times, and 1,2-[^{3}H]GA_5 at 530 times, the endogenous estimated levels of GA_5 of the tissue. We have assumed that endogenous GA_5 in the apricot seeds was about 20 ng/g f.w., this being the level obtained by physical methods of analysis by Yamaguchi *et al.* (25) for peach seeds. This value for peach roughly agrees with the amount of GA_5 quantified in these apricot seeds by the dwarf rice bioassay (4) (0.5 ng/g f.w.) multiplied by the potency of GA_5 relative to GA_3 in this assay (6).

The injection of [^{3}H]GA_5 was made through the pericarp at a point 5 mm from the pedicel along the suture, as described in the Figure 1 legend. Thus, a minimum of vascular tissue was disrupted. After 4 d each fruit was opened, the intact seed removed, weighed, frozen in liquid N_2, and freeze dried.

Extraction Procedure. Fifty seeds were homogenized and extracted as detailed in Bottini *et al.* (4).

Paper Chromatography. Acidic EtOAc and acidic BuOH frac-

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[4] Abbreviations: GAs, gibberellins; BuOH, 1-butanol; EtOAc, ethyl acetate; f.w., fresh weight; GA-GE, gibberellin glucosyl ester; GA-G, gibberellin glucoside; GA-Me, gibberellin methyl ester; GC-SIM, gas chromatography-selected ion monitoring; HPLC-RC, high pressure/performance liquid chromatography-radioactivity counting; MeOH, methanol; MeTMSi, methyl ester trimethylsilyl ether; Rt, retention time.

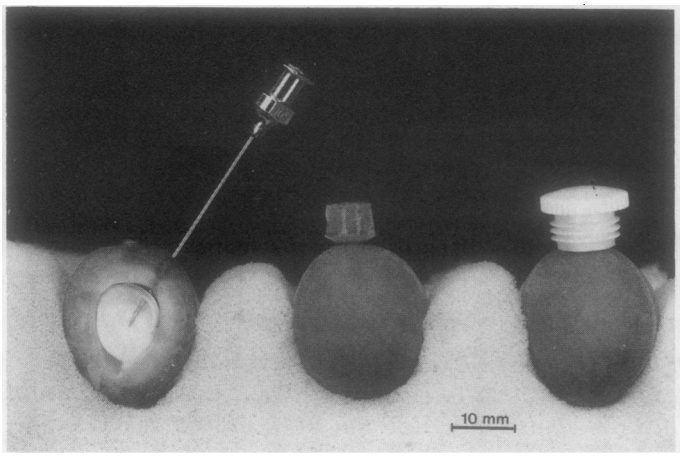

FIG. 1. Technique used to inject [^{3}H]GA_5. [^{3}H]Gibberellin A_5 was dissolved in aqueous 8 mM $KHCO_3$ solution and 5 μl injected into each seed. Fruit were collected 3 to 4 weeks after anthesis when seeds were between 0.25 and 0.5 g f.w. Pedicels were covered with agar (1%) and sucrose (2%) and the fruits were held upright at 100% RH, 20°C, 16 h daylength, for 4 d.

tions were chromatographed as in Bottini *et al.* (4). For chromatograms of the EtOAc-soluble fractions radioactive eluates (80% MeOH) were grouped as follows: R_F 0.2 to 0.4, 0.4 to 0.55, and 0.55 to 0.8. For chromatograms of the BuOH fractions radioactive eluates (50% MeOH) were grouped as follows: R_F 0.2 to 0.35 and 0.35 to 0.55.

SiO_2 Partition Column Chromatography. For the BuOH fractions each of the two R_F zones was subjected to a short SiO_2 partition column (15), eluted with EtOAc:*n*-hexane (95:5) first and then with 100% MeOH, in order to separate any "free, EtOAc-soluble GAs" (originating from either hydrolysis with time, or imperfect partitioning [14]) from GA glucosyl conjugates and from most of the other highly H_2O-soluble free GAs (such as GA_{32}).

HPLC-RC. The eluates from the grouped R_F zones were subjected to reversed-phase C_{18} HPLC (13), as described in Bottini *et al.* (4), except that the following solvent programs were used: (a) 19% MeOH in 1% AcOH for 10 min, 19% to 50% MeOH in 1% AcOH from 10 to 40 min, then 100% MeOH from 40 to 50 min; (b) 10 to 13% MeOH in 1% AcOH for 70 min, then 100% MeOH from 70 to 80 min; (c) 23.5% MeOH in 1% AcOH for 40 min, then 100% MeOH from 40 to 50 min.

The radioactivity of the HPLC fractions was monitored by liquid spectrometry of aliquots, and the elution profiles compared with those of authentic GAs. All significant peaks of radioactivity from HPLC were subjected to GC-SIM analysis.

GC-SIM. Samples were converted to the MeTMSi derivatives as noted in Bottini *et al.* (4) prior to injection on a Hewlett-Packard 5790A GC and a 5970A Series Mass Selective Detector; details are given in Bottini *et al.* (4).

RESULTS

Figures 2 to 4 show a qualitative picture of most, but not all of the different [^{3}H] metabolites in extracts of immature apricot seeds fed with [^{3}H]GA_5 of low specific radioactivity (4.6 or 10.6 μg GA_5 per seed; 230 or 530 × expected endogenous levels). The use of high specific radioactivity [^{3}H]GA_5 (42 ng GA_5 per seed; 2 × expected endogenous level) showed some qualitative and quantitative changes (relative to feeds with high substrate levels) in the profiles of [^{3}H] metabolites; these are summarized in Table II.

For seeds fed with 10.6 μg 1,2-[^{3}H]GA_5, (530 × expected endogenous levels) analysis of the paper R_F 0.2 to 0.4 zone (EtOAc soluble) yielded one peak at the Rts of GA_3 and GA_1 (Fig. 2A). Using another gradient which separates GA_3 and GA_1, the main [^{3}H] metabolite eluted at Rt 57 to 61 min (Fig. 2B), between GA_3 and GA_1. GC-SIM analysis of the Rt 57 to 61 min peak indicated that neither GA_1 nor GA_3 were present, although two unknown substances with strong m/e 504 ions (the M+ ion of GA_3) were noted by GC-SIM. Other m/e ions characteristic of GA_1 and/or GA_3 were also present, but at relative intensities differing from GA_1 or GA_3. The [^{3}H] metabolite at Rt 47 to 50 min (Fig. 2B) yielded no 504, 506, or 594 ion peaks by GC-SIM.

For seeds fed 4.6 μg of 1-[^{3}H]GA_5, (230 × expected endogenous

Table I. *GC-SIM Data[a] for Putative GA_1, GA_3, and GA_6 Found in Fraction Groupings from HPLC-RC Containing Major Amounts of Radioactivity*

Based on calculations of specific activity and radioactivity injected, the compounds noted below could represent metabolites of [^{3}H]GA_5. GA_1 and GA_5 were detected from extracts of seeds which had not been fed GA_5 (4), but the amounts of GA_1 and GA_5 found in fractions noted below were in excess of the amounts found from similar seed weights (4), based on portion of each extract injected, and upon intensity of the M^+ ion. Gibberellins A_3 and A_6 were not detected in seeds which had not been fed GA_5 (4).

Compound	HPLC-RC[b] Rt on Gradient			Rt on Each of Two Capillary GC Columns	Constituent Ions						Tentative Identity
	A	B	C								
	min			*min*	*% relative intensity*						
GA_3	19–22	46–50	13–14	13.5	504 (24)	489 (3)	473 (2)	445 (3)	414 (2)	370 (4)	
GA_3	19–22	46–50	13–14	20.4	504 (201)	489 (18)	473 (6)	445 (10)	414 (4)	370 (30)	
1	19–22	46–50	12–15	13.5	504 (30)	489 (5)	473 (0)	445 (3)	414 (6)	370 (3)	GA_3
2			12–15	20.4	504 (201)	489 (32)		445 (8)	414 (33)	370 (8)	GA_3
GA_1	22–23	58–62	18–19	19.5	506 (21)	491 (2)	447 (3)	416 (1)	377 (5)	313 (3)	
4	20–23			19.5	506 (21)	491 (2)	447 (2)	416 (tr)	377 (8)		GA_1
GA_6	28–29		21–23	18.4	432 (127)	417 (14)		373 (25)	303 (25)	235 (178)	
5			21–23	18.4	432 (127)	417 (22)		373 (26)	303 (172)	235 (143)	GA_6
GA_5	36–37			16.4	416 (1362)	401 (243)	385 (413)	357 (267)	343 (243)	299 (632)	
6	36–37			16.5	416 (1362)	401 (238)		357 (227)	343 (246)	299 (770)	GA_5

[a] The M^+ ion of GA_1, GA_3, and GA_6 has been normalized in some, but not all, cases to the M^+ ion of apricot putative GAs, and the other diagnostic ions of the authentic GA adjusted accordingly. [b] Figures 2 to 3 show the C_{18} HPLC elution profiles of certain of compounds 1 to 6, relative to authentic GAs.

Table II. *Distribution of Radioactivity (in Percent) of the Different Metabolites Obtained from Extracts of Immature Seeds of Apricot Fed with 1-[^{3}H]GA_5 and 1,2-[^{3}H]GA_5 at Several Specific Radioactivities*

Free GAs or GA-like Substances	1-[^{3}H]GA_5 42 ng/seed (high specific radioactivity)	1-[^{3}H]GA_5 4.6 μg/seed	1,2-[^{3}H]GA_5 10.6 μg/seed
GA_5[a]		(3.7)	(3.7)
GA_5-like[b]	5.3	3.7	3.7
GA_6[a]		(10.0)	(6.0)
GA_6-like[c] (m/e 432)		(5.1)	(5.4)
GA_6-like[b]	6.6	16.1	11.4
GA_1[a]		(17.8)	(0.3)
GA_1-like[c] (m/e 506)		(0.7)	(2.7)
GA_1-like[c,d] (m/e 504)		(1.4)	(21.1)
GA_1-like[b]	17.4	19.9	24.1
GA_3[a]		(9.5)	(5.4)
GA_3[c] (m/e 504)			(0.9)
GA_3-like[b]	14	9.5	6.3
GA_{22}-like[b]		0.5	
GA_{29}-like[b]	0.5		
GA_{32}-like[b]	0.1		
Others & Tailing	1.7		
Total free [^{3}H] metabolites of [^{3}H] GA_5	45.5%	48.7%	45.5%
Putative [^{3}H] conjugates[e]			
GA_5-G/GE[b]	2.0		Not analyzed
GA_6-G/GE[b]	8.0	3.9	
GA_1-GE[b]	33.4	46.1	
GA_3-GE[b]	10.6		
GA_8-G[b]	0.1		
GA_{22}-G/GE[b]	0.1		
GA_{29}-G[b]	0.1		
Others & Tailing	0.2		
Total putative [^{3}H] conjugates	54.5%	51.3%	54.5%

[a] Identified on the basis of HPLC Rt, capillary GC Rt, and the relative intensities of five or six characteristic ions (see Table I). [b] Identified only on the basis of HPLC-Rt of the radioactive peak at or near the Rt of the authentic substance noted, or for putative conjugates of GA_5 and GA_{22}, where the conjugate should elute (13, 22). In the case of the high specific activity feed (42 ng/seed) sufficient substrate (by calculation) was not present for GC-SIM analysis. [c] Identified on the basis of HPLC Rt, and from a subsequent GC-SIM where the presence of a m/e which was the same as the M^+ of the GA in question occurred at an appropriate intensity based on specific radioactivity and amount of radioactive substance injected on the GC-SIM. However, based on differing GC-Rt and relative ion intensities, the unknown substance is not the GA in question. GC-MS was performed in some instances, and although the full spectrum was indicative of a gibberellin, it was not any of the 72 known GAs or their catabolites. [d] Two substances present with m/e 504 ions. [e] Separated from putative [^{3}H] free GA-like substances by partitioning and use of a SiO_2 partition column step (15).

level), analysis of paper R_F 0.2 to 0.35 (EtOAc soluble) on HPLC-RC yielded the same elution profile as in Figure 2A. An aliquot of the single peak (20–23 min) was subjected to HPLC-RC using the gradient that separates GA_1 from GA_3, and almost all of the radioactivity eluted at the Rt of authentic [^{3}H]GA_1 (data not shown). Further, this fraction grouping (Rt 20–23 min; Fig. 2A) yielded GC-SIM data indicating the presence of GA_1 (compound 4, Table I).

For seeds fed with 10.6 μg 1,2-[^{3}H]GA_5 analysis of paper R_F 0.4 to 0.55 (EtOAc soluble) on HPLC-RC yielded two radioactive peaks (Fig. 3A), one at 26 to 28 min (Rt of authentic GA_6), and the other at 19 to 22 min, slightly before and at the Rt of GA_3. Sequential shallow gradient HPLC-RC—GC-SIM yielded ambiguous results (data not shown except for compound 1, Table I). In order to get a better resolution of paper chromatogram R_F 0.4 to 0.55, a second portion of the eluate was analyzed on an isocratic solvent (Fig. 3B). The presence of GA_3 (compound 2, Table I), GA_6 (compound 5, Table I), and two unknown substances with m/e 504 and 506 ion peaks, respectively (data not shown), were indicated from GC-SIM. The two unknown substances also had other m/e ions characteristic of GA_1 and/or GA_3, but at GC Rts and/or relative intensities differing from GA_1 and GA_3.

For seeds fed with 10.6 μg of 1,2-[^{3}H]GA_5, HPLC analysis of paper R_F 0.55 to 0.80 zone (EtOAc soluble) yielded a main peak (data not shown), identified as GA_5 by GC-SIM (compound 6, Table I), with minor radioactive peaks eluting at the Rts of GA_1/GA_3, GA_6, and GA_5-Me (data not shown).

For the highly water-soluble [^{3}H] metabolites (*i.e.* present initially in the acidic BuOH fraction, and subsequently eluting only in the MeOH wash from the short SiO_2 column [15]) two HPLC gradients were used sequentially. In Figure 4A, a single peak eluting at the Rt of [^{3}H]$GA_{1/3}$ is shown. Subsequent HPLC (Fig. 4B) placed most of the radioactivity at 56 to 61 min, at or slightly before GA_1. Based on the known chromatographic behavior of GA glucosyl conjugates (13, 15, 22), this highly water soluble [^{3}H] metabolite has chromatographic characteristics similar to GA_1-GE. A smaller peak eluted just before [3H] GA_3, where GA_3-GE would be expected to elute (13, 15, 22).

The free GA fraction (*i.e.* eluting from the short SiO_2 partition column in *n*-hexane:EtOAc) present initially in the BuOH fraction (paper R_F 0.35–0.55 from seeds fed 4.6 μg of 1-[^{3}H]GAs), yielded a radioactive peak on HPLC at Rt 28 to 29 min (data not shown), and GC-SIM data indicative of the presence of GA_6

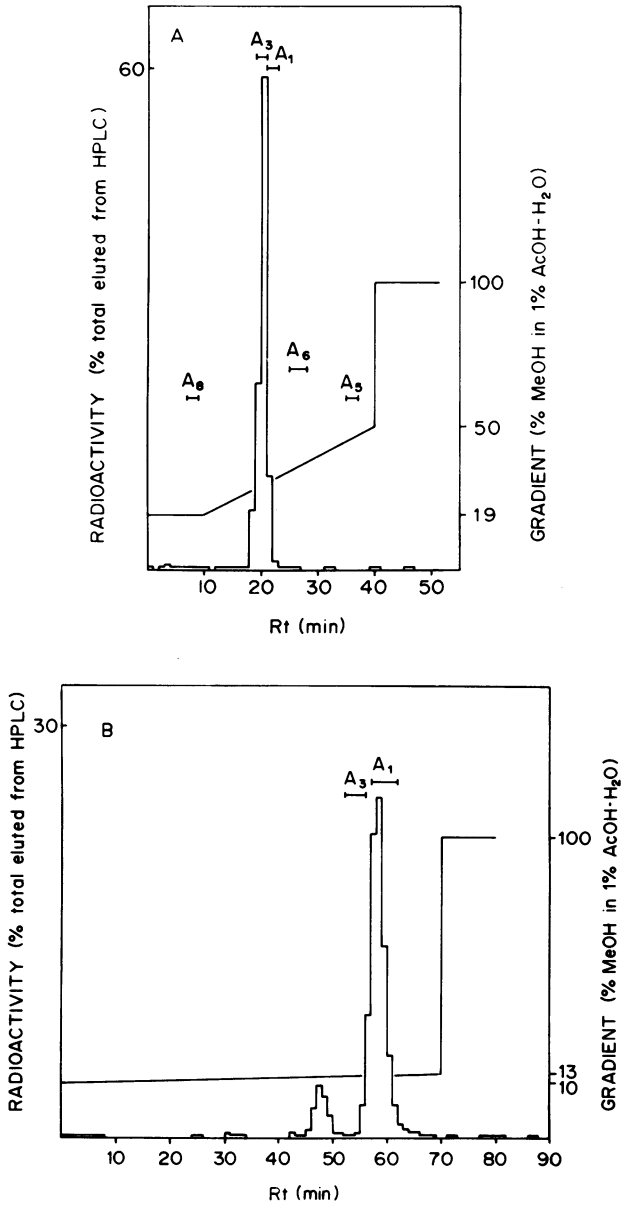

FIG. 2. A, Radioactive profile of free [^{3}H]GA_5 metabolites from C_{18} HPLC (19–50% MeOH in 1% acetic acid) of paper chromatogram R_F 0.2 to 0.4 (acidic, EtOAc-soluble substances). 1,2-[^{3}H]GA_5 was fed at 10.6 μg GA_5 per seed; B, radioactive profile from C_{18} HPLC (10–13% MeOH in 1% acetic acid) of discrete peak eluting at min 19 to 22 from (A).

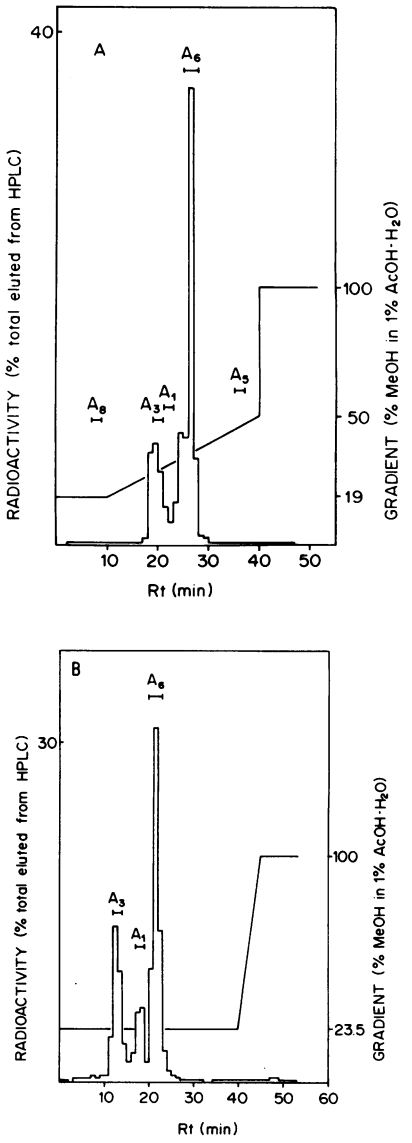

FIG. 3. A, Radioactive profile of free [^{3}H]GA_5 metabolites from C_{18} HPLC (19–50% MeOH in 1% acetic acid) of paper chromatogram R_F 0.4 to 0.55 (acidic, EtOAc-soluble substances). 1,2-[^{3}H]GA_5 was fed at 10.6 μg/seed; B, Radioactive profile of free [^{3}H]GA_5 metabolites from C_{18} HPLC (23.5% MeOH in 1% acetic acid, isocratic elution) of paper chromatogram R_F 0.4 to 0.55 (acidic, EtOAc soluble substances). 1,2[^{3}H] GA_5 was fed at 10.6 μg per seed. The peak at Rt 10 to 12 min yielded GA_3 (compound 2, Table I) and the peak at Rt 21 to 23 min yielded GA_6 (compound 5, Table I) by GC-SIM.

(*i.e.* trace of m/e 432, and a relatively strong m/e 235 at the Rt of GA_6 (data not shown). Other characteristic ions of GA_6 were undetectable, however. The highly water soluble fraction (*i.e.* eluting from the short SiO_2 partition column in the subsequent MeOH wash) present in the above-mentioned paper R_F fraction also yielded a radioactive peak at 28 to 29 min (Rt of GA_6), and may represent [^{3}H]GA_6-GE (GA-GEs often elute from C_{18} HPLC very close to the free GA moiety, whereas GA-Gs usually elute much earlier [13, 22]).

The highly water-soluble [^{3}H] metabolite with chromatographic characteristics similar to GA_1-GE was the most abundant metabolite (33–46%) (Table II). [^{3}H] Metabolites eluting at Rts of GA_1, GA_3, GA_6 and where their glucosyl conjugates would be expected to elute, were present at all levels of substrate GA_5 fed. Based on HPLC Rts, other minor metabolites were GA_8-, GA_{22}-, GA_{29}-like, their glucosyl conjugate-like forms, and a GA_5Me-like substance (Table II). Only 3 to 5% of the radioactivity corresponded to the precursor ([^{3}H]GA_5), even with very high amounts of substrate GA_5 fed.

As the amount of substrate GA_5 increased, the amount of [^{3}H] GA_1-like substance (and its putative conjugate) increased, mainly at the expense of all other metabolites (except one eluting at the Rt of GA_6, which also increased) (Table II).

DISCUSSION

Immature seeds of apricot fed with [^{3}H]GA_5 yielded GA_3, GA_1, and GA_6 as well as three highly water soluble metabolites eluting at the expected Rts of glucosyl conjugates of these GAs. A similar spectrum of metabolites of [^{3}H]GA_5 has been found in

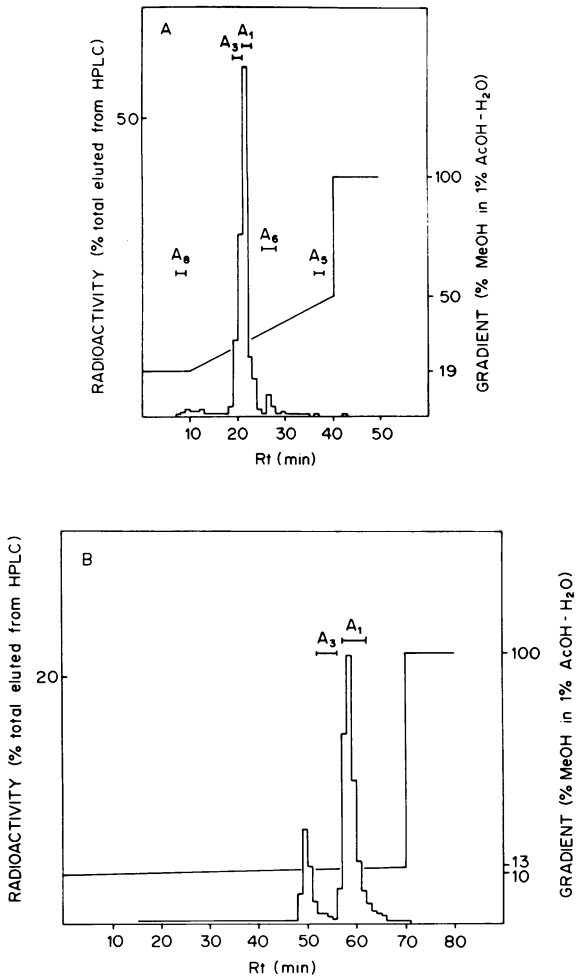

FIG. 4. A, Radioactive profile of highly water-soluble [^{3}H]GA_5 metabolites from C_{18} HPLC (19–50% MeOH in 1% acetic acid) of paper chromatogram R_F 0.2 to 0.35 (acidic, BuOH-soluble substances). 1-[^{3}H] GA_5 was fed at 4.6 μg per seed. This paper chromatogram eluate was chromatographed on a short SiO_2 partition column and the radioactivity (51.3%) eluting in the MeOH wash of the column (where GA glucosyl conjugates will elute) probably represents GA glucosyl conjugate-like substances (15). The major peak coincided with $GA_{1/3}$. An analogous peak was subsequently chromatographed on a shallower gradient (see [B]); B, radioactive profile from C_{18} HPLC (10–13% MeOH in 1% acetic acid) of a radioactive peak analogous to the peak at Rt 20 to 23 min of Figure 4a. The peaks may represent GA glucosyl ester-like conjugates of GA_3 (49–50 min) and GA_1 (56–60 min) (13, 14, 22).

limbs of peach (*Prunus persica* L.) (3, 8) and developing seeds of *Pharbitis nil* (14). The presence of several unknown [^{3}H] metabolites (Figs. 2, 3, and noted above in "Results") may be due to substrate overloading (see Refs. 7, 10, 11, 19, 24).

When substrate GA_5 dosage was increased from 2 times the estimated endogenous level to 230 and 530 times the estimated endogenous levels, there were substantial increases in the formation of [^{3}H] metabolites eluting at the Rts of GA_1 and GA_1-GE (Table I). Coincidentally, the trace amounts of [^{3}H] metabolites eluting at the Rts of GA_5Me, GA_{22}, GA_{29}, GA_{32}, and GA_8-G from the low substrate GA_5 feed (Table II) were, in general, reduced to almost undetectable levels.

Sembdner *et al.* (23) first proposed that GA_6 could be a logical intermediate between GA_5 and several of the more polar GAs that have subsequently been found as metabolites of [^{3}H] GA_5 in immature apricot seeds (Figures and Tables herein), in peach limbs (3, 8), and in immature *Pharbitis* seeds (14). Feeds with radioactive or stable isotope-labeled GA_6 have yet to be accomplished, and thus this question is not resolved by the present work.

Large amounts of GA_{32} were present in these and other immature apricot seeds (4, 5), and large amounts of GA_{32} were present in immature seeds of peach at varying stages of development (25, 26). However, there was no significant conversion of [^{3}H]GA_5 to the [^{3}H] metabolite eluting from the C_{18} HPLC at the Rt of authentic GA_{32} (Table II). Indeed, this very polar radioactive metabolite of GA_5 could be any one of several polyhydroxylated GA-like substances.

Past work indicates the presence of modest amounts of GA_5 (4, 25, 26) in immature seeds of two species of *Prunus*. In the present study [^{3}H]GA_5 was converted mainly to GA_1 and another unknown [^{3}H] metabolite eluting at or near the Rt of GA_1, depending upon whether gradient- or isocratic-eluted HPLC was used. At high substrate doses, especially, very large amounts of a highly water-soluble [^{3}H] metabolite eluting at the Rt of GA_1-GE were formed. It is thus possible that both GA_1 and GA_{32} function as biologically active GAs in developing apricot seeds, the former, however, being rapidly metabolized to a conjugate. Future studies on the kinetics of endogenous GA concentrations in developing seeds should examine not only changes in free GAs, but, insofar as possible, changes in GA glucosyl conjugates. Gibberellin glucosyl conjugates may well be a reflection of past events (*i.e.* high levels of GA_1-G/GE may imply effective amounts of GA_1 present in the recent past). Or, they may be a storage form of free GAs for subsequent use in a later stage of development or germination (1, 16, 20, 21).

High doses of substrate GA_5 promoted the formation of new metabolites which often (but not always) differed from GA_1, GA_3, and GA_6 in HPLC Rt, but retained the respective M^+ and other characteristic m/e ions, albeit at GC-Rt and relative intensities different from authentic GA_1, GA_3, or GA_6, respectively. Thus, physiologically meaningful studies may require the use of [^{3}H]GAs of high specific radioactivity, with confirmation of identity of metabolites coming from additional feeds of higher substrate level, and ideally stable isotope labeled substrate. Even here, every effort must be made to perform extensive and sequential analytical chromatographic separations (*e.g.* HPLC-RC and/or GC-RC) prior to GC-MS or GC-SIM.

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