Phytochemistry, Vol. 24, No. 4, pp. 663–671, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

METABOLISM OF [^{3}H]GIBBERELLIN A_5 IN DEVELOPING *PHARBITIS NIL* SEEDS

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(*Revised received* 20 *July* 1984)

Key Word Index—*Pharbitis nil*; Convolvulaceae; metabolism; [^{3}H]gibberellin A_5; gibberellins; gibberellin glucosyl conjugates.

Abstract—The native gibberellin A_5 (GA_5), as [1-^{3}H]GA_5 (3.2 Ci/mmol) was fed to seed capsules (0.58 μCi/capsule) of *Pharbitis nil* cv Violet at the 2-week stage of development, and its metabolism in the seeds was investigated after 43 hr. Extractable radioactivity in free GA metabolites was 38%, with 56% in GA glucosyl conjugate-like substances. Only 2.5% of the extractable radioactivity remained as [^{3}H]GA_5. Tentative identifications, based on comparisons with authentic standards after sequential chromatography on silica gel partition column → gradient-eluted C_{18} HPLC → isocratic-eluted C_{18} HPLC–radiocounting (RC), showed that [^{3}H]GA_5 was converted to at least six free GAs, GA_1, GA_3, GA_6, GA_8, GA_{22}, GA_{29}, a GA_5 methyl ester-like metabolite, and at least twelve GA glucosyl conjugate-like substances, GA_5-glucoside (GA_5-G), GA_5-glucosyl ester (GA_5-GE), GA_1-O(3)-G, GA_1-O(13)-G, GA_1-GE, GA_3-O(3)-G, GA_3-O(13)-G, GA_3-GE, GA_6-G or GE, GA_8-O(2)-G, GA_{22}-G or GE and GA_{29}-O(2)-G. After lower specific activity feeds of [1,2-^{3}H]GA_5 (74 mCi/mmol; 0.1 μCi/capsule) at approximately the same stage of development, the presence of GA_1, GA_3, GA_5, GA_6, GA_8 and GA_{29} was further confirmed by sequential (after C_{18} HPLC–RC) capillary gas chromatography–selected ion monitoring (GC–SIM), using six characteristic ions. However, for GA_{22} only a trace of the parent ion was present at the appropriate retention time.

INTRODUCTION

The gibberellins (GAs) GA_1 (**2**), GA_3 (**10**), GA_5 (**15**), GA_8 (**8**), GA_{17} (**25**), GA_{19} (**26**), GA_{20} (**1**), GA_{26} (**22**), GA_{27} (**23**), GA_{29} (**6**), GA_{44} (**24**) and GA_{53} (**27**) have been characterized from immature seeds of *Pharbitis nil* [1–8] but GA metabolism in that tissue has not been studied except to a limited extent by Barendse *et al.* [9] who investigated [^{3}H]GA_1 metabolism and noted at least two unknown acidic metabolites, and the formation of [^{3}H]GA_1 conjugate-like substances. In the present report, the metabolism of native GA_5 in developing (2 week old) seeds of *P. nil* cv Violet was studied in order to attempt characterization of its metabolites under circumstances where metabolism might be expected to approximate the norm (e.g. high specific radioactivity feeds where the level of GA_5 entering the seed was near the expected endogenous level). We also wanted to determine whether GA_5 is converted to GA_6 (**20**), since GA_6 has been suggested to be an intermediate in the conversions of [^{3}H]GA_5 to [^{3}H]GA_3 [10], and [^{3}H]GA_5 to [^{3}H]GA_8 [11], and [^{3}H]GA_6 was shown to be converted to GA_8 glucoside (**9**), and to GA_1/GA_3 and GA_8-like substances [12]. [^{3}H]GA_5 of lower specific activity was also fed in an attempt to obtain levels of metabolites sufficient for characterization by the more definitive means of GC–SIM.

RESULTS AND DISCUSSION

Separation and identification of metabolites of [^{3}H]GA_5 (*Table* 1)

Each of the silica gel partition column fraction groupings I, II, III and IV of the free GA metabolites from the [1-^{3}H]GA_5 feed (Fig. 1) contained two to five significant peaks when run sequentially on gradient-eluted → isocratic-eluted C_{18} HPLC–RC. The retention times (R_ts) of radioactive HPLC peaks from fraction I coincided with authentic GA_5 and GA_5 methyl ester [GA_5-Me (**18**)]. The R_ts of radioactive HPLC peaks from fraction II coincided with those of authentic GA_5 and GA_6. The R_ts of radioactive HPLC peaks from fraction III coincided with those of GA_6, GA_1, GA_3 and GA_{29}. The R_ts of radioactive peaks from fraction IV coincided with authentic GA_8, GA_{29} and GA_{22} (**19**). From fraction IV two unknown polar radioactive compounds were obtained, one of which co-chromatographed with authentic GA_{32} (**14**) (R_t 6–7 min), the other of which eluted earlier than GA_{56} (**21**) (Table 1).

From the highly water-soluble fraction of the [1-^{3}H]GA_5-fed sample, six significant peaks were observed on gradient-eluted C_{18} HPLC–RC (Fig. 2). These gradient-eluted HPLC peak fraction groupings each contained from one to four peaks when run sequentially on isocratic-eluted C_{18} HPLC–RC. The R_t of fraction I (Fig. 2) coincided with that of GA_8-O(2)-G (**9**) and one of three hydrolysis products (by enzyme, base or acid) of the peak coincided with GA_8 on gradient-eluted C_{18} HPLC (Table 1). The R_ts of radioactive compounds in fraction II

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Table 1. Separation and identification of the metabolites of high specific activity [^{3}H]GA_5 (3.2 Ci/mmol, 0.58 μCi fed per capsule) in developing seed of *P. nil* by sequential silica gel partition → gradient reverse-phase C_{18} HPLC–RC → isocratic-eluted C_{18} HPLC–RC

Unknown compounds and authentic standards (free GAs)	R_t (min) of peak: Gradient-eluted (10 → 73% MeOH in aq. 1% HOAc) HPLC–RC*	R_t (min) of peak: Isocratic or very shallow gradient-eluted HPLC–RC systems (A–E) (see Experimental)	Identity†
Free GAs from silica gel partition column fractions*			
Fraction I (a)	32–33	9–10 (D) and 28–29 (C)	GA_5 (**15**)
(b)	35–36	19–21 (D)	GA_5-methyl ester (**18**)
Fraction II (a)	28–29	11–12 (C) and 21–22 (B)	GA_6 (**20**)
(b)	32–33	28–29 (C)	GA_5 (**15**)
Fraction III (a)	24–25		GA_{29} (**6**)
(b)	26–27	8–9 (C) and 24–26 (A) 35–37 (E)	GA_3 (**10**)
(c)	26–27	8–9 (C) and 28–30 (A) 40–42 (E)	GA_1 (**2**)
(d)	28–29	11–12 (C)	GA_6 (**20**)
Fraction IV (a)	6–7		unknown
(b)	10–11		unknown
(c)	14–15		GA_8 (**8**)
(d)	24–25		GA_{29} (**6**)
(e)	28–29	13–14 (C)	GA_{22} (**19**)
GA conjugates from HPLC–RC fractions†			
Fraction I (a)	13–14		GA_8-G (**9**)
Hydrolysate	14–15		GA_8 (**8**)
Fraction II (a)	19–24		GA_{29}-G (**7**)
Hydrolysate	24–25		GA_{29} (**6**)
(b)	19–24	8–13 (A) contained 2 peaks	GA_3-O(3)-G (**11**) and GA_3-O(13)-G (**12**)
Hydrolysate	26–27	24–26 (A)	GA_3 (**10**)
(c)	19–24	10–16 (A) contained 2 peaks	GA_1-O(3)-G (**3**) and GA_1-O(13)-G (**4**)
Hydrolysate	26–27	28–30 (A)	GA_1 (**2**)
Fraction III (a)	25–27	11–13 (A)	GA_3-O(3)-G (**11**)
Hydrolysate	26–27	24–26 (A)	GA_3 (**10**)
(b)	25–27	19–21 (A)	GA_3-GE (**13**)
Hydrolysate	26–27	24–26 (A)	GA_3 (**10**)
(c)	25–27	13–16 (A)	GA_1-O(3)-G (**3**)
Hydrolysate	26–27	28–30 (A)	GA_1 (**2**)
(d)	25–27	21–23 (A)	GA_1-GE (**5**)
Hydrolysate	26–27	28–30 (A)	GA_1 (**2**)
Fraction IV (a)	28–30	16–17 (B)	GA_6-G or -GE
Hydrolysate	29	11–12 (C) and 21–22 (B)	GA_6 (**20**)
(b)	28–30	24–25 (B)	GA_{22}-G or -GE
Hydrolysate	29	13–14 (C)	GA_{22} (**19**)
Fraction V (a)	31–33	16–18 (C)	GA_5-G (**16**)
Hydrolysate	32–33	27–29 (C)	GA_5 (**15**)
(b)	31–33	21–22 (C)	GA_5-GE (**17**)
Hydrolysate	32–33	27–29 (C)	GA_5 (**15**)
Standards			
GA_1 (**2**)	26–27	8–9 (C) and 28–30 (A) 40–42 (E)	
GA_1-GE (**5**)	25–26 [13, 15]		
GA_1-O(3)-G (**3**)	23–25 [13, 15]		
GA_1-O(13)-G (**4**)	21–24 [13, 15]		

Table 1. (*Continued*)

Unknown compounds and authentic standards (free GAs)	R_t (min) of peak: Gradient-eluted (10 → 73% MeOH in aq. 1% HOAc) HPLC–RC*	R_t (min) of peak: Isocratic or very shallow gradient-eluted HPLC–RC systems (A–E) (see Experimental)	Identity†
GA$_3$ (**10**)	26–27	8–9 (B) and 24–26 (A) 35–37 (E)	
GA$_3$-GE (**13**)	25–26 [13, 15]		
GA$_3$-O(3)-G (**11**)	23–25[13, 15]		
GA$_3$-O(13)-G (**12**)	21–24[13, 15]		
GA$_5$ (**15**)	32–33	9–10 (D) and 27–29 (C)	
GA$_5$-G (**16**)	31–32 [15]		
GA$_5$ methyl ester (**18**)	35–36	19–21 (D)	
GA$_6$ (**20**)	28–29	11–12 (C) and 21–22 (B)	
GA$_8$ (**8**)	14–15		
GA$_8$-O(2)-G (**9**)	13–14		
GA$_{22}$ (**19**)	28–29	13–14 (C)	
GA$_{29}$ (**6**)	24–25		
GA$_{32}$ (**14**)	6–7		
GA$_{56}$ (**21**)	13–14		

*As shown in Figs. 1 and 2.

†Identifications based on sequential silica gel partition → gradient-eluted HPLC → isocratic eluted HPLC R_ts, relative to R_ts of authentic GAs and GA-glucosides (-G) and glucosyl esters (-GE), and on the expected HPLC R_t (i.e. 1 to several min earlier than the free GA moiety) of GA glucosyl conjugates from refs. [13] and [15], and on the R_t of their free GA moiety on gradient-eluted and/or isocratic-eluted HPLC after enzymatic hydrolysis.

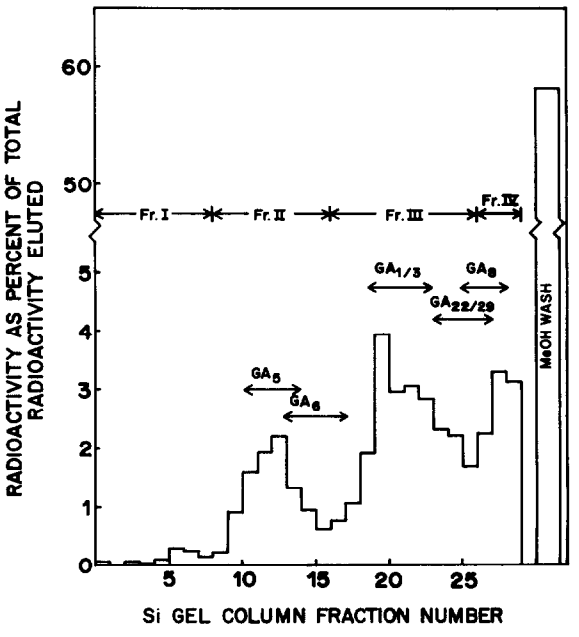
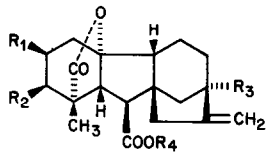
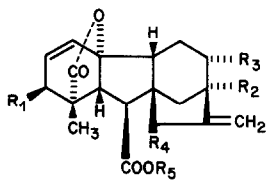


Fig. 1. Silica gel partition column chromatogram of the ethyl acetate–methanol-soluble portion of a methanolic extract of *P. nil* seeds incubated with [^{3}H]GA$_5$ for 43 hr.

(Fig. 2) coincided with the expected R_ts [13] on isocratic-eluted C$_{18}$ HPLC for GA$_{29}$-O(2)-G (**7**), GA$_1$-O(3)-G (**3**), GA$_1$-O(13)-G (**4**), GA$_3$-O(3)-G (**11**) or GA$_3$-O(13)-G (**12**), respectively (Table 1). Their hydrolysis products coincided on gradient and/or isocratic C$_{18}$ HPLC with GA$_{29}$, GA$_1$ or GA$_3$ (Table 1). The R_ts of radioactive compounds in fraction III (Fig. 2) coincided on isocratic-eluted C$_{18}$ HPLC with the expected R_ts [13] for GA$_1$-O(3)-G, GA$_1$-GE (**5**), GA$_3$-O(3)-G or GA$_3$-GE (**13**) and their hydrolysis products coincided on gradient- and/or isocratic-eluted C$_{18}$ HPLC with GA$_1$ and GA$_3$ (Table 1). Fraction IV (Fig. 2) may contain GA$_6$ glucosyl conjugate and GA$_{22}$ glucosyl conjugate, since the R_ts of the hydrolysis products of fraction IV (Fig. 2) coincided on gradient- and/or isocratic-eluted C$_{18}$ HPLC with authentic GA$_6$ and GA$_{22}$ (Table 1). The radioactive compounds in fraction V (Fig. 2), when chromatographed subsequently on isocratic-eluted C$_{18}$ HPLC, coincided with the expected R_ts of GA$_5$-G (**16**) or GA$_5$-GE (**17**), and their hydrolysis products coincided with GA$_5$ on gradient-eluted HPLC (Table 1).

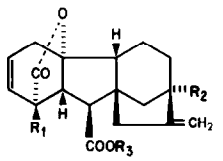
Low specific activity feeds of [1,2-^{3}H]GA$_5$ were also made to developing seed capsules for more definitive identification of metabolites. After 43 hr, 1.2×10^6 dpm was extracted from the 90 seeds (4.3 g) obtained from 57 capsules, which represents 9.5% of the radioactivity fed to the capsule. The elution profiles of the silica gel partition column and subsequent HPLC were similar to the high



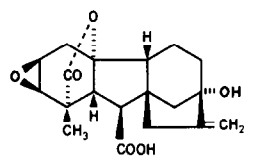


1 R_1 = H R_2 = H R_3 = OH R_4 = H
2 R_1 = H R_2 = OH R_3 = OH R_4 = H
3 R_1 = H R_2 = OGLU R_3 = OH R_4 = H
4 R_1 = H R_2 = OH R_3 = OGLU R_4 = H
5 R_1 = H R_2 = OH R_3 = OH R_4 = GLU
6 R_1 = OH R_2 = H R_3 = OH R_4 = H
7 R_1 = OGLU R_2 = H R_3 = OH R_4 = H
8 R_1 = OH R_2 = OH R_3 = OH R_4 = H
9 R_1 = OGLU R_2 = OH R_3 = OH R_4 = H

10 R_1 = OH R_2 = OH R_3 = H R_4 = H R_5 = H
11 R_1 = OGLU R_2 = OH R_3 = H R_4 = H R_5 = H
12 R_1 = OH R_2 = OGLU R_3 = H R_4 = H R_5 = H
13 R_1 = OH R_2 = OH R_3 = H R_4 = H R_5 = GLU
14 R_1 = OH R_2 = OH R_3 = OH R_4 = OH R_5 = H



15 R_1 = CH_3 R_2 = OH R_3 = H
16 R_1 = CH_3 R_2 = OGLU R_3 = H
17 R_1 = CH_3 R_2 = OH R_3 = GLU
18 R_1 = CH_3 R_2 = OH R_3 = CH_3
19 R_1 = CH_2OH R_2 = OH R_3 = H



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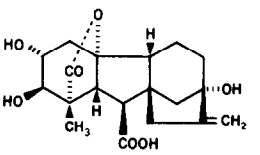

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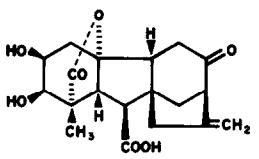

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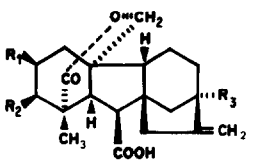
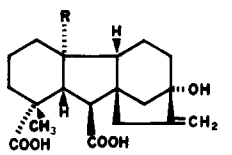


23 R_1 = OH R_2 = OH R_3 = H
24 R_1 = H R_2 = H R_3 = OH



25 R = COOH
26 R = CHO
27 R = CH_3

specific activity feeds with regard to the qualitative spectrum of metabolites, although there were quantitative differences (see Table 4). The results of sequential analysis by silica gel partition → C_{18} HPLC → GC-SIM are shown in Tables 1 and 3. Gibberellins A_1, A_3, A_5, A_6, A_8 and A_{29} were identified using GC–SIM for six characteristic ions (Table 3). The GA_{22}-like compound (silica gel fraction IV, gradient-eluted HPLC R_t 28–29 min) was monitored at m/z 504 $[M]^+$, 489, 472, 401, 387 and 370, which are characteristic of GA_{22}-Me, TMSi. However, only a trace of the parent ion at m/z 504 was found at R_t 13.6 min, which corresponds with the R_t of GA_{22}-Me, TMSi.

Metabolism of high specific activity [^{3}H]GA_5 (*Table* 2)

After 43 hr, 3.96×10^6 dpm was extracted from the 31 seeds (1.53 g) obtained from 28 capsules, which represents 11% of the radioactivity fed to the capsule. Of the extracted radioactivity, 38.4% existed as free GAs, with 56.4% as GA glucosyl conjugate-like substances. Based on the identifications noted above, the main metabolites of [^{3}H]GA_5 were [^{3}H]GA_1, [^{3}H]GA_3 and their purported glucosyl conjugates, i.e. [^{3}H]GA_1-O(3)-G, [^{3}H]GA_1-O(13)-G, [^{3}H]GA_1-GE, [^{3}H]GA_3-O(3)-G, [^{3}H]GA_3-O(13)-G and [^{3}H]GA_3-GE (Table 2). Thus, 22.7% of the extracted radioactivity was in the form of C-3 hydroxy-

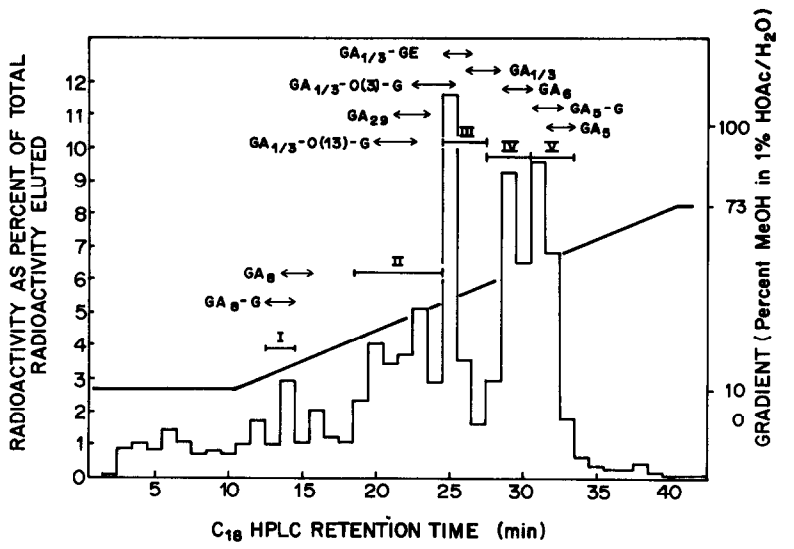


Fig. 2. Gradient-eluted reverse phase C_{18} HPLC profile of the combined highly water-soluble fraction [e.g. methanol wash from silica gel partition column chromatography (Fig. 1) plus the water-soluble fraction in the extraction procedure [14]].

lated GA metabolites, [^{3}H]GA_1, [^{3}H]GA_3 and their purported glucosyl conjugates. However, the percentage of [^{3}H]GA_3 and its conjugates may be underestimated if loss of ^{3}H at C-1 occurred during metabolism. Only 9.6% of the extracted radioactivity remained as GA_5 or its conjugates [[^{3}H]GA_5 (2.5%), [^{3}H]GA_5-Me-like substance (0.4%) and [^{3}H]GA_5 glucosyl conjugate-like substances (6.7%)]. The [^{3}H]GA_5-Me-like substance was classed as a [^{3}H]GA_5 metabolite, rather than as an artefact of our extraction and purification procedure because the methyl ester of GA_5 was not observed in another feeding where authentic [^{3}H]GA_5 was subjected to the same methods [14] in the presence of a plant extract (M. Koshioka, A. Jones and R. P. Pharis, unpublished work). As expected [11, 12], [^{3}H]GA_8 and a GA_8 glucoside-like substance were observed, but the conversion rate was lower than that reported for developing bean seeds [11]. [^{3}H]GA_6 and a [^{3}H]GA_6 glucosyl conjugate-like compound were observed as significant peaks, i.e. 4.5 and 1.1‰, respectively (Table 2), and this suggests that [^{3}H]GA_6 exists as an intermediate in metabolism from [^{3}H]GA_5 to [^{3}H]GA_1, although [^{3}H]GA_3 and/or [^{3}H]GA_{29} have also been proposed as intermediates [10–12]. It is noteworthy that a GA_{22}-like compound (i.e. silica gel gradient-eluted HPLC → isocratic-eluted HPLC → capillary GC R_ts the same as authentic GA_{22}, with GC–SIM for low specific activity feeds also yielding a trace of the parent ion at m/z 504) is present, as is a GA_{22} glucosyl conjugate-like compound whose isocratic-eluted HPLC R_t (Table 1) is consistent with that expected of a GA_{22} glucosyl conjugate [13, 15]. The GA_{22} glucosyl conjugate-like substance eluting from the isocratic HPLC was then subjected to enzyme hydrolysis, and the resulting hydrolysate yielded a radioactive compound which subsequently co-chromatographed with authentic GA_{22} on isocratic HPLC solvent C (Table 1). A tentative scheme for the metabolism of [^{3}H]GA_5 in *P. nil* seeds is shown in Fig. 3.

The effect of increasing the dosage of GA_5 by a factor of 7.45 × (i.e. from 59.6 ng applied per capsule in the high specific activity feeds to 444 ng applied per capsule in the low specific activity feeds) is noted in Table 4 for both free GA and GA conjugate-like metabolites. While there are no remarkable differences with regard to the proportion of total free GA metabolites relative to GA conjugate-like metabolites, within each group several trends are apparent. Firstly, within the free GA metabolites, increasing the uptake of GA_5 especially increased the proportion of GA_5 that was recovered from the seeds, and also increased proportions of GA_6, GA_{22}, GA_{29} and other/tailing radioactivity. These increases were accomplished primarily at the expense of GA_1, GA_3 and GA_8. Secondly, within the GA glucosyl conjugate-like fraction there was more than a three-fold increase in GA_5-G/GE-like substances, and also appreciable increases in GA_8- and GA_{29} conjugate-like metabolites, for the low specific activity feeds. Concomitantly, $GA_{1/3}$ and $GA_{6/22}$ conjugate-like metabolites decreased, as did radioactivity classed as others/tailing.

Thus, increasing the precursor dosage has differential effects on the metabolism. Expressed as a percentage, both GA_1 and GA_3 (which are highly biologically active GAs) and $GA_{1/3}$ conjugate-like metabolites are reduced (relative to high specific activity feeds), while concomitantly the amounts of GA_6 and GA_{22} increase (primarily GA_6 based on GC–SIM m/z intensity results, data not shown), as do the amounts of GA_5 and GA_5 conjugate-like metabolites. If GA_6 is an intermediate between GA_5 and GA_1, GA_3 and GA_{29} (see Fig. 3), then the build-up of GA_6 in the low specific activity feeds would indicate that conversion of the epoxide GA_6 to GA_1 and GA_3 is very much a rate-limiting step, whereas the conversion to GA_{29} is appreciably less affected by increased substrate. Although in the low specific activity feed the proportion of GA_8 is reduced relative to high specific activity feeds, conversion to a GA_8 conjugate-like substance almost

Table 2. Radioactivity (as a percentage of extracted radioactivity) found in [^{3}H]GA_5 and its metabolites from developing seeds of *P. nil* after 43 hr of incubation with high specific activity [1-^{3}H]GA_5 (0.58 μCi of 3.2 Ci/mmol [^{3}H]GA_5 fed to capsule at week 2 of development)*

Purported gibberellins and GA conjugates [glucosides (-G) or glucosyl esters (-GE)]	Radioactivity (%)†
Free GA-like substances	
GA_1 (2)	2.8
GA_3 (10)	4.9
GA_5 (15)	2.5
GA_5-methyl ester (18)	0.4
GA_6 (20)	4.5
GA_8 (8)	1.0
GA_{22} (19)	1.3
GA_{29} (6)	1.1
Others and/or tailing	19.9
Sub-total	38.4
GA conjugate-like substances	
GA_1-GE (5)	1.2
GA_1-O(3)-G (3) and -O(13)-G (4)	2.6
GA_3-GE (13)	3.4
GA_3-O(3)-G (11) and -O(13)-G (12)	7.8
GA_5-GE (17)	4.0
GA_5-G (16)	2.7
GA_6-G or -GE	1.1
GA_8-G (9)	2.2
GA_{22}-G- or -GE	5.6
GA_{29}-G (7)	1.6
Others	24.2
Sub-total	56.4
Work-up losses	5.2
Total	100

*Identifications are based on sequential silica gel partition column → gradient-eluted C_{18} HPLC → isocratic C_{18} HPLC elution R_t relative to authentic GAs/GA-glucosyl conjugates (Table 1 and [13, 15]).

†Values were estimated from the silica gel partition column (GA_5) and a combination of gradient-eluted and isocratic-eluted HPLC–RC.

doubles, indicating that this conjugation step, like those of GA_{29} to GA_{29}-G/GE-like substances and GA_5 to GA_5-G/GE-like substances, is considerably less rate-limiting in the presence of increased levels of precursor.

We attempted to obtain an estimate of the endogenous GA_5 present in seeds fed [^{3}H]GA_5 of high specific activity by using GC–SIM, and injecting approximately 1/20 of the radioactivity originally present at the GA_5 R_t at the silica gel stage of analysis [i.e. < 200 pg of [^{3}H]GA_5 was injected; this takes into account work-up and usage (HPLC–RC) losses]. We were unable to detect the presence of a *m/z* 416 ion, even though estimates from Yamaguchi *et al.* [8] imply that 13.5 ng of GA_5 might be expected in such an injection aliquot (e.g. 9 ng of GA_5 was extracted per seed in their study, assuming a 50% water content and an average seed dry weight of 45 mg). However, the values of Yamaguchi *et al.* [8] are based on collections from a wide range of seed ages, and variation in GA_5 amounts of 100-fold with varying seed age is to be expected (N. Murofushi, personal communication). Since our GC–SIM procedure was capable of detecting *ca* 1–2 ng of authentic GA_5, we thus conclude that the level of GA_5 present in our seeds was less than *ca* 0.7–1.4 ng per seed. Estimates of levels of other endogenous GAs from the high specific activity [^{3}H]GA_5-fed seeds were not attempted since the ^{3}H-labelled fractions were fully utilized in the various HPLC–RC analyses.

If we thus assume that our 2-week-old immature seeds had levels of endogenous GA_5 that were less than 1 ng per seed, then our data (expressed as pg per seed, Table 4) indicates that uptake from the high specific activity feed was at least 4–8 times the endogenous GA_5 level (assuming that all of the radioactivity entered the seed as GA_5), and the low specific activity feed was made at a level of at least 18–37 times the endogenous level of GA_5. We thus consider that the high specific activity feed was made at a level which should not unduly perturb 'normal' GA metabolism in the seed.

EXPERIMENTAL

Plant material and application of [^{3}H]GA_5. [1-^{3}H]GA_5 (3.2 Ci/mmol [16]: 0.58 μCi/2 μl of 95% EtOH/capsule of *ca* 4 seeds) and [1,2-^{3}H]GA_5 (74 mCi/mmol [10]: 0.1 μCi/2 μl of 95% EtOH/capsule of up to 4 seeds) were injected into seed capsules of *P. nil* cv Violet *in situ* at the 2-week stage of development, respectively. After 43 hr capsules were harvested and lyophilized.

Extraction of tissue. For high sp. act. feeds 31 lyophilized seeds (1.53 g) from 28 capsules were extracted and extracts purified according to the method of Koshioka *et al.* [14]. For low sp. act. feeds 90 lyophilized seeds (4.3 g) from 57 capsules were extracted and processed as noted above.

Analytical methods. Gradient eluted silica gel partition CC [17] was followed by gradient-eluted (see Fig. 2) C_{18} reverse-phase HPLC [15] for silica gel partition column fraction groupings, and for the H_2O-soluble fraction [14]. Isocratic or very shallow gradient-eluted HPLC was used to separate discrete peak groupings further from the earlier gradient-eluted HPLC. The solvent systems shown in Table 1 were: (A) 15% MeOH in aq. 1% HOAc, (B) 23.5% MeOH in aq. 1% HOAc, (C) 30% MeOH in aq. 1% HOAc, (D) 40% MeOH in aq. 1% HOAc, (E) 10% MeOH to 13% MeOH in aq. 1% HOAc over 60 min; flow rate 2 ml/min. GC–SIM conditions: A Hewlett–Packard 5972A gas chromatograph and a 5970A Series mass selective detector fitted with a direct capillary interface for on-column injection were used. Column: cross-linked 5% phenylmethyl silicone, length 12.5 m, film thickness 0.3 mm, i.d. 0.2 mm; head pressure 6 psi, E.M. Volts 1400, initial temp. 60°, final temp. *250°, **260°, ***270° initial time 1 min, programme rate 25°/min, interface temp. 280°. The HPLC fractions from the low sp. act. feeds were analysed by using GC–SIM after derivatization to the methyl ester, TMSi ether, using ethereal CH_2N_2 and TRI-SIL (Pierce Chemical Co.).

Hydrolysis of GA glucosyl conjugate fractions. Procedures were as described in ref. [13].

Acknowledgements—We gratefully acknowledge with thanks samples of authentic GAs and/or GA glucosyl conjugates from N. Takahashi (University of Tokyo, Japan), G. Sembdner (Institut für Biochemie der Pflanzen, Halle, G.D.R.) and J. MacMillan (University of Bristol, U.K.). This work was supported by Natural Sciences and Engineering Research Council Canada Grant A-2585 to R.P.P.

Table 3. Sequential analysis (silica gel R_f → gradient-eluted C_{18} HPLC R_t → capillary GC R_t → GC–SIM) from extracts of 90 seeds, where low specific activity (74 mCi/mmol) [1,2-^{3}H]GA$_5$ (0.1 μCi per capsule) was fed to capsules of *Pharbitis*

Gibberellin or compound	Fraction No. on silica gel partition column	R_t (min) on HPLC–RC	R_t (min) on GC–SIM	SIM spectrum* with relative abundance in parentheses (m/z value)						Identity
Compound†	Fraction II	32–33	12.3	416 (380)	401 (66)	385 (12)	357 (98)	343 (106)	299 (217)	GA$_5$
GA$_5$†	11–13	32–33	12.3	416 (380)	401 (61)	385 (10)	357 (50)	343 (47)	299 (98)	
Compound‡	Fraction II	28–29	12.5	432 (456)	417 (44)	403 (20)	373 (55)	303 (465)	235 (193)	GA$_6$
GA$_6$‡	13–16	28–29	12.5	432 (456)	417 (59)	403 (23)	373 (69)	303 (414)	235 (257)	
Compound‡	Fraction III	26–27	13.2	506 (407)	491 (41)	447 (30)	416 (9)	377 (48)	313 (45)	GA$_1$
GA$_1$‡	19–22	26–27	13.2	506 (407)	491 (40)	447 (34)	416 (10)	377 (54)	313 (33)	
Compound‡	Fraction III	26–27	13.5	504 (658)	489 (54)	473 (15)	445 (46)	414 (4)	370 (72)	GA$_3$
GA$_3$‡	19–22	26–27	13.5	504 (658)	489 (64)	473 (15)	445 (44)	414 (6)	370 (73)	
Compound‡	Fraction III	24–25	13.2	506 (519)	491 (54)	477 (21)	447 (31)	375 (39)	303 (157)	GA$_{29}$
GA$_{29}$‡	23–26	24–25	13.2	506 (519)	491 (48)	477 (13)	447 (27)	375 (64)	303 (88)	
Compound§	Fraction IV	14–15	13.6	594 (1019)	579 (67)	535 (68)	504 (25)	448 (202)	379 (108)	GA$_8$
GA$_8$§	26–28	14–15	13.5	594 (1019)	579 (48)	535 (62)	504 (22)	448 (230)	479 (182)	

*Based on estimates of amounts of endogenous GAs that might be expected in these immature seeds (see ref. [8]), and on amounts (by calculation of specific activity) that would be derived from the [^{3}H]GA$_5$ feed (Table 4). GA$_3$, GA$_5$, GA$_8$ and GA$_{29}$ SIM data may represent both endogenous GAs and metabolites of [^{3}H]GA$_5$; whereas for GA$_1$, GA$_5$ and GA$_6$, SIM data probably represent only [^{3}H]GA$_5$ or metabolites of [^{3}H]GA$_5$. For each of the parent ion m/z scans, there was only one peak (at the GC R_t noted) of significant intensity. This indicates that the radioactive peak eluted from the HPLC did not contain significant amounts of other metabolites with the same parent ion.

†GC column temperature 250°.

‡GC column temperature 260°.

§GC column temperature 270°.

Table 4. A comparison of metabolism between high (3.2 Ci/mmol, 0.58 μCi fed/capsule) and low (74 mCi/mmol, 0.1 μCi fed/capsule) specific activity feeds of [3H]GA_5 to developing (week 2) seeds of *P. nil**

Purported gibberellins and GA conjugates [glucosides (-G) or glucosyl esters (-GE)]	% radioactivity† and pg [^{3}H]GA‡ per seed present 43 hr after feeding [^{3}H]GA_5§			
	High sp. act		Low sp. act	
	%	pg	%	pg
Free GA-like substances				
GA_8	1.0	59	0.3	80
GA_{29}	1.1	65	2.9	771
$GA_{1/3}$	7.7	455	4.8	1277
$GA_{6/22}$	5.8	343	15.5	4123
GA_5	2.4	142	10.5	2793
GA_5-Me	0.4	24	—	—
Others/tailing	19.9	1178	9.9	2633
Sub-total	38.4	2273	43.9	11677
GA conjugate-like substances				
GA_8-G/GE	2.2	130	4.2	1117
GA_{29}-G/GE	1.6	95	2.4	638
$GA_{1/3}$-G/GE	15.0	888	11.4	3032
$GA_{6/22}$-G/G	6.7	397	2.6	692
GA_5-G/GE	6.7	397	23.0	6118
Others/tailing	24.2	1433	11.1	2953
Sub-total	56.4	3339	54.7	14550
Work-up losses	5.2	308	1.4	372
Total	100.0	5920	100.0	26600

*The high (HSA) and low (LSA) specific activity feeds of [1-^{3}H]GA_5 and [1,2-^{3}H]GA_5, respectively, were made at week 2 of seed development, and the seed was harvested 43 hr after injection of the GA_5 into the seed capsule.

†Values were estimated from the silica gel partition column (GA5) and the gradient-eluted HPLC-RC.

‡Calculated from specific activity; values may be underestimated if loss of ^{3}H took place during metabolism.

§The dosage of GA_5 injected into the capsules in the LSA feed was 7.45 times that of the HSA feed, but uptake of GAs (calculated from specific activity) into the seed was only 4.5-fold higher for the LSA feed. Identifications for HSA metabolites are as noted in Table 1, and for LSA free GA metabolites are as noted in Table 3. Identifications for LSA conjugate-like metabolites are based on sequential silica gel partition column → gradient-eluted C_{18} HPLC–RC elution R_t (see Table 1 for R_t of authentic GA glucosyl conjugates). Certain radioactive fractions are grouped in Table 4 (e.g. $GA_{1/3}$, $GA_{6/22}$) since for LSA feeds the entire peak groupings from gradient-eluted C_{18} HPLC were subjected to GC–SIM without prior separation of the component GAs by isocratic C_{18} HPLC.

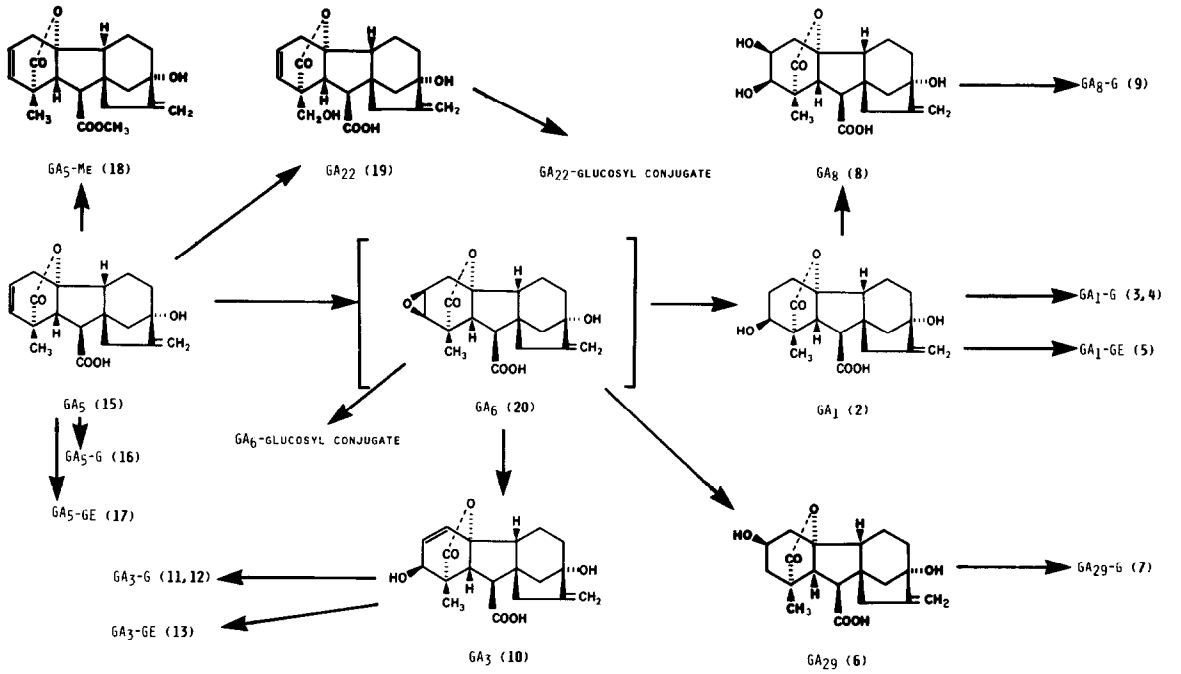


Fig. 3. Pathways of [^{3}H]GA$_5$ metabolism in *P. nil* seeds. The square brackets around GA$_6$ indicate that its role as an intermediate is only postulated.

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