Metabolism of [^3H] Gibberellin A₅ in Cell Suspension Cultures of *Pharbitis nil*

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Gibberellin A₅ (GA₅), a native GA of immature seeds of *Pharbitis nil*, was fed to *Pharbitis nil* cell suspension cultures as [C-1, ^3H] GA₅ (3.1 Ci/mmol), and its metabolism over a 48 hr period was investigated. Radioactivity in free GA metabolites was 13.1%, with 79.9% in GA glucosyl conjugate-like metabolites. Only 7.0% of the radioactivity remained as [^3H] GA₅. Tentative identifications were based on comparison with retention times of authentic free GAs and/or glucosyl conjugates after sequential chromatography on Si gel partition column → gradient-eluted C18 HPLC-radiocounting (RC) → isocratic-eluted C18 HPLC-RC, and showed that [^3H] GA₅ was converted to [^3H] GA₁ (2%), [^3H] GA₃ (4%), [^3H] GA₆ (2%), [^3H] GA₂₂ (1%) and their glucosyl conjugates, and also to [^3H] GA₈ glucoside, and [^3H] GA₅ glucosyl conjugates. The major conjugate-like substances were [^3H] GA₁ and [^3H] GA₃ glucosyl esters, at 15% and 34%, respectively, of the total extractable radioactivity.

Gibberellins: GA₁, GA₃, GA₅, GA₈, GA₁₇, GA₁₉, GA₂₀, GA₂₆, GA₂₇, GA₂₉, GA₄₄ and GA₅₃ have been found in immature seeds of *Pharbitis nil*.^{1~6)} To date study of the metabolism of native GAs in *Pharbitis* has been restricted to GA₁ (Barendse *et al.*⁷⁾) and GA₅ (Koshioka *et al.*⁸⁾).

The metabolites of [^3H] GA₁ were not definitively characterized.⁷⁾ However, the metabolites of [^3H] GA₅ in immature seeds of *Pharbitis nil* were identified by reasonably definitive means, and at least six free GAs, GA₁, GA₃, GA₆, GA₈, GA₂₂, GA₂₉, and their putative glucosyl conjugates were found.⁸⁾

In this paper, the metabolism of [C-1, ^3H] GA₅ to free GAs and putative glucosyl conjugates in cell suspension cultures of *Pharbitis nil* is studied to find out how it is metabolized and also to determine whether GA₅ is converted to GA₆ which was suggested to be an

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Abbreviations: GA-G, gibberellin glucoside; GA-GE, gibberellin glucosylester.

intermediate in the conversion of [^3H] GA₅ to [^3H] GA₃, and to [^3H] GA₈^{8~10)} in cell suspension cultures.

MATERIALS AND METHODS

Culture origin and maintenance. The culture of *Pharbitis nil* L. var. Violet was derived from hypocotyl tissues of a germinated seed. The suspension cultures were maintained by two weekly passages on sucrose-containing medium at 27°C in the dark on a shaker (60 ppm).¹¹⁾

Metabolism of [^3H] gibberellin A₅ by the cells. Cell suspension cultures of *Pharbitis nil* (10-day-old inoculum) were pre-incubated for 2 days in 20 ml of sucrose-free medium. This medium was replaced by a sucrose-containing medium before addition of sterile [C-1, ^3H] GA₅ (5 μCi , 3.1 Ci/mmol,¹²⁾ dissolved in 50 μl of 95% EtOH) to the medium. The incubation was continued for further 2 days. Cell suspension cultures were harvested and the cells were frozen and freeze-dried.

Extraction of cells. Freeze-dried cells (0.12 g) were extracted with 20 ml of aq. 80% MeOH and the extract was purified by the method of Koshioka *et al.*¹³⁾

Analytical methods. Gradient-eluted Si gel partition column chromatography¹⁴⁾ was followed by gradient-eluted C18 reverse phase HPLC-RC¹⁵⁾ for Si gel partition column fraction groupings, and for the highly water soluble fraction.¹³⁾ Isocratic- or very shallow gradient-eluted HPLC-RC was used to further separate discrete peak fractions or hydrolyzed peak fractions from the earlier gradient-eluted HPLC-RC as in our previous report.⁸⁾

Hydrolysis of GA glucosyl conjugate fractions. 1) The sample was dissolved in 0.2 M acetate buffer (pH 4.0, 0.4 ml), to which was added 0.2 ml 1% β -glucosidase solution. The mixture was then left at 37°C for 16 hr. 2) Sample was dissolved in 0.5 ml of 0.1 M HCl and heated at 100°C for 1 hr or dissolved in 0.5 ml of 0.5 M H₂SO₄ and heated at 100°C for 4 hr. 3) Sample was dissolved in 0.5 ml of 0.1 M NaOH and heated at 100°C for 1 hr.

RESULTS AND DISCUSSION

Separation and identification of metabolites of [^3H] GA₅

Because the [^3H] GA₅ had a high specific activity, tentative identifications were based on a series of sequential chromatography steps, with comparison of retention times (t_{R} s) of authentic standards at each step. However, the

FIG. 1. Elution Pattern from a Si Gel Partition Column of [^3H] GA₅ and Its Metabolites in an Extract from a Suspension of Cells of *Pharbitis nil*.

TABLE I. RADIOACTIVITY* FOUND IN EFFLUENT FROM SI GEL PARTITION COLUMN CHROMATOGRAPHY

Fraction No.	Si gel fractions	Percentage of radioactivity	Tentative Identification**
Free GAs			
I	1~8	0.4%	Trace of GA ₅
II	9~14	9.5%	GA ₅ and GA ₆
III	15~22	3.4%	GA ₁ , GA ₃ and GA ₆
IV	23~28	6.8%	GA ₁ , GA ₃ and GA ₂₂
Methanol wash		79.9%	GA-Glucosyl conjugate-like substances

* 5 μCi of [^3H] GA₅ fed: Extractable radioactivity from the suspension cells of *Pharbitis nil* was 3.8 μCi .

** Based on C18 HPLC (gradient-eluted $\rightarrow$ isocratic-eluted) t_{R} s.⁸⁾

reader is referred to Koshioka *et al.*⁸⁾ where capillary gas chromatography-selected ion monitoring (GC-SIM) was used, in addition to the sequential chromatography steps, to identify metabolites of low specific activity [^3H] GA₅ in immature *Pharbitis* seeds.

Each of the Si gel partition column fraction groupings, I, II, III, and IV, of the free GA metabolites (Fig. 1) contained one to three

FIG. 2. Elution Pattern from Gradient-eluted Reverse Phase C18 HPLC Column of [^3H] Glucosyl Conjugate-like Substances in the Methanol Wash of the Si Gel Partition Column (Fig. 1 and Table I) from the Extract of a Suspension of Cells of *Pharbitis nil*.

significant peaks when run sequentially on gradient-eluted $\rightarrow$ isocratic, or shallow gradient-eluted C18 HPLC with radiocounting (-RC) (Table I). From fraction (Fr.) grouping I, only a trace of [^3H] GA_5 was observed. The t_R s of radioactive HPLC peaks from Fr. II coincided with those of authentic [^3H] GA_5 and GA_6 ⁸⁾. The t_R s of radioactive HPLC peaks from Fr. III coincided with those of authentic GA_6 , [^3H] GA_1 and [^3H] GA_3 ⁸⁾. The t_R s of radioactive HPLC peaks from Fr. IV coincided with those of authentic [^3H] GA_1 , [^3H] GA_3 and GA_{22} ⁸⁾.

From the highly water-soluble fraction several significant peaks were observed on gradient-eluted C18 HPLC-RC (Fig. 2). The gradient-eluted HPLC peak fraction groupings I to IV each contained from one to four peaks when run sequentially on isocratic- or shallow gradient-eluted C18 HPLC-RC. The com-

pound in Fr. I and its hydrolysis products did not coincide with GA_{32} on gradient- and/or isocratic-eluted HPLC-RC. It might have four or more hydroxyls or be a glucosyl conjugate of a four or more hydroxylated GA since it eluted earlier than $\text{GA}_8\text{-O(2)-G}$ ($\text{GA}_8\text{-O(2)-glucoside}$) on HPLC-RC. The t_R s of Fr. II coincided with that of $\text{GA}_8\text{-O(2)-G}$ and one of the hydrolysis products (by enzyme, acid, or base) of the peak coincided with GA_8 on gradient-eluted C18 HPLC-RC. The t_R s of Fr. III coincided with the expected t_R s¹⁶⁾ on isocratic-eluted C18 HPLC-RC for $\text{GA}_1\text{-O(3)-G}$, $\text{GA}_1\text{-O(13)-G}$, $\text{GA}_3\text{-O(3)-G}$ and $\text{GA}_3\text{-O(13)-G}$, respectively. Their hydrolysis products coincided on gradient- and isocratic-eluted C18 HPLC-RC with GA_1 or GA_3 . The t_R s of radioactive compounds in Fr. IV coincided on isocratic-eluted C18 HPLC-RC with the expected t_R s¹⁶⁾ for $\text{GA}_1\text{-O(3)-G}$, $\text{GA}_1\text{-GE}$,

GA₃-O(3)-G or GA₃-GE and their hydrolysis products coincided on gradient-eluted and/or isocratic-eluted C18 HPLC-RC with GA₁ and GA₃. Fr. V may contain a GA₆ glucosyl conjugate and a GA₂₂ glucosyl conjugate, since the t_R s of hydrolysis products of Fr. V coincided on gradient- and/or isocratic-eluted C18 HPLC-RC with authentic GA₆ and GA₂₂. The radioactive compounds in Fr. VI, when chromatographed subsequently on isocratic-eluted C18 HPLC-RC coincided with the expected t_R ⁸⁽¹⁶⁾ of GA₅-G or GA₅-GE, and their hydrolysis products coincided with GA₅ on gradient-eluted HPLC-RC.

Metabolism of [³H] GA₅

In *Pharbitis* cell suspension cultures most (79.9%) of the extracted radioactivity consisted of putative [³H] GA-Gs and [³H] GA-GEs (Table II). The main metabolites of [³H] GA₅ were thus putative [³H] GA₃-GE (33.7%) and putative [³H] GA₁-GE (15.0%). Thus, 59.3% of the eluted radioactivity was in the form of the C-3 hydroxylated GA metabolites, [³H] GA₁, [³H] GA₃ and their putative conjugates. Only 11.9% of the eluted radioactivity remained as [³H] GA₅ and its putative glucosyl conjugates (e.g. [³H] GA₅ (7.0%), [³H] GA₅-G (4.7%) and [³H] GA₅-GE (0.2%)). As expected^{8,10,17} putative GA₈ glucosyl conjugate was observed in relatively large amounts (4.9%), but free GA₈ was not observed, unlike immature seeds of *Pharbitis*, which yielded both.⁸⁾ [³H] GA₂₂ and its putative glucosyl conjugate was also observed as a metabolite of [³H] GA₅. Previously,⁸⁾ GA₆ was identified by GC-SIM as a metabolite of low specific activity [³H] GA₅ fed to immature seeds of *Pharbitis nil*. In this study [³H] GA₆ and its glucosyl conjugate were also observed, but the conversion rate (0.9%) was lower than that reported for immature seeds (e.g. where GA₆ was 4.5% and putative GA₆ glucosyl conjugate was 1.1%⁸⁾). The presence of [³H] GA₆ as a metabolite of [³H] GA₅ in *Pharbitis* cell suspension cultures, *Pharbitis* immature seeds and also in *Prunus* tissues^{18,19)} suggested that [³H] GA₆ is an intermediate in metabolism from [³H] GA₅ to

TABLE II. RADIOACTIVITY* (AS A PERCENTAGE OF TOTAL RADIOACTIVITY ELUTED) FOUND IN [³H] GA₅ AND ITS METABOLITES FROM SUSPENSION CELLS OF *Pharbitis nil*

Putative gibberellins and GA conjugates	Percentage of radioactivity
Free gibberellins	
GA ₁	2.0
GA ₃	3.9
GA ₅	7.0
GA ₆	2.3
GA ₂₂	1.4
Others	3.5
Sub-total	20.1
GA glucosyl conjugate-like substances	
GA ₁ -O(3)-G plus GA ₁ -O(13)-G	6.5
GA ₁ -GE	15.0
GA ₃ -O(3)-G plus GA ₃ -O(13)-G	4.1
GA ₃ -GE	33.7
GA ₅ -G	4.7
GA ₅ -GE	0.2
GA ₆ -G or GA ₆ -GE	0.9
GA ₈ -G	4.9
GA ₂₂ -G or GA ₂₂ -GE	2.2
Others	7.7
Sub-total	79.9
Total	100.0

* 5 μ Ci of [³H] GA₅ was fed to the suspension cultures of *Pharbitis nil*. The extractable radioactivity was 3.8 μ Ci.

[³H] GA₁ and/or [³H] GA₃.^{8~10)} The low radioactivity present in [³H] GA₆ (relative to GA₁, GA₃ and their putative glucosyl conjugates) suggests that the [³H] GA₅ to [³H] GA₆ conversion may be a rate-limiting step, relative to the conversion of [³H] GA₆ to [³H] GA₁ and/or [³H] GA₃.

There was no evidence for a putative GA₅ methyl ester, GA₂₉, and putative GA₂₉-G in the [³H] GA₅ fed cell suspension cultures, although immature seeds of *Pharbitis* did yield all three as metabolites (0.4, 1.1, and 1.6%, respectively).

The production of putative GA glucosyl conjugates, and specifically GA₁ and GA₃ glucosyl conjugates, was appreciably enhanced in the cell suspension cultures (Table II) relative to immature seeds (Table II in ref. 8). Similarly high production of putative GA glucosyl conjugates from [³H] GA₄ was noted for anise

somatic cell cultures,¹⁶⁾ and this enhanced conjugation may be caused by the high levels of sugar used in the culture medium.

Gibberellins A₁ and A₃ and their putative glucosyl conjugates make up 65% of the radioactivity in *Pharbitis* cell suspension cultures (Table II), whereas in immature seeds they accounted for only 23% of the total radioactivity.⁸⁾ This increase (relative to immature seeds) for these C-3 hydroxylated metabolites of [^3H] GA₅ occurred primarily at the expense of unknown metabolites [present at 44% in immature seeds, compared to only 11% in cell suspension cultures (Table II)]. However, relative decreases in GA₆, GA₈, GA₂₉ and putative glucosyl conjugates of GA₅, GA₆, GA₈, GA₂₂ and GA₂₉ for cell suspension cultures, also accounted for a significant part of the difference between the two systems in their metabolism of [^3H] GA₅.

The pattern of GA₅ metabolism in cell suspension cultures of *Pharbitis* is thus similar to that found in immature seeds, although minor qualitative differences do exist (such as the absence of free GA₈ and GA₂₉ and putative GA₅ methyl ester and GA₂₉-G), and metabolism to certain metabolites (mainly GA₁, GA₃ and their putative glucosyl esters) is enhanced. Cell suspension cultures of *Pharbitis* may be a useful initial tool in examining the metabolism of other native *Pharbitis* GAs.

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