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# METABOLISM OF [$^3$H]GIBBERELLIN $A_4$ IN SOMATIC SUSPENSION CELL CULTURES OF CARROT

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**Key Word Index**—*Daucus carota*; Umbelliferae; somatic cell cultures; metabolism; GAs; GA glucosyl conjugates.

**Abstract**—The native gibberellin $A_4$ ($GA_4$), in radioactive form ([1,2-$^3$H]$GA_4$, 1.06 Ci/mmol), was fed to carrot somatic cell cultures (suspension and immobilized cell systems) and its metabolism over a 48 hr period was investigated. It was found that the [$^3$H]$GA_4$ was metabolized to at least two GAs, [$^3$H]$GA_1$ and [$^3$H]$GA_8$, six GA glucosyl conjugates, [$^3$H]$GA_1$-0(3)-glucoside, [$^3$H]$GA_1$-0(13)-glucoside, [$^3$H]$GA_1$-glucosyl ester, [$^3$H]$GA_4$-glucoside, [$^3$H]$GA_4$-glucosyl ester, a [$^3$H]$GA_8$ glucosyl conjugate(s) and a previously unknown [$^3$H]$GA_1$ glucosyl conjugate ([$^3$H]$GA_1$-0(3,13)-diglucoside-like compound). The $GA_1$-diglucoside-like compound was found only in extracts of cells and was present in significant amounts (33% of total extractable radioactivity). All other metabolites were present in both cells and medium. For extracts of the medium, no differences between the suspension and immobilized cultures existed in types of [$^3$H]$GA_4$ metabolites although quantitative differences were apparent.

## INTRODUCTION

Gibberellins (GAs) $A_1$ (**2**), $A_4$ (**1**), $A_7$ (**17**) and $\Delta^{1(10)}GA_1$ counterpart (**13**) have been characterized from somatic suspension cultures of anise and carrot by GC/MS [1]. Conversion of [$^3$H]$GA_1$ to [$^3$H]$GA_8$ (**3**), [$^3$H]$GA_8$-0(2)-glucoside [$GA_8$-0(2)-G, (**10**)] and [$^3$H]$GA_1$-0(3)-G (**8**) has been reported in somatic embryos and proembryos of anise suspension cultures [2], and conversion of [$^3$H]$GA_4$ to [$^3$H]$GA_1$, [$^3$H]$GA_2$ (**14**), [$^3$H]$GA_8$ and [$^3$H]$GA_{34}$ (**4**) in several other plant species has been reported [1–9]. The conversion of a GA to GA glucosyl conjugates was discussed by Noma *et al.* [2] and Yamane *et al.* [9].

The present paper discusses the metabolism of [1,2-$^3$H]$GA_4$ to $GA_1$ and $GA_8$ and to glucosyl conjugate-like substances in somatic cell cultures and an immobilized cell system [10–12] of carrot.

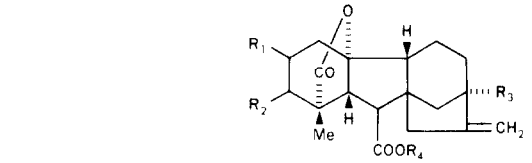
## RESULTS AND DISCUSSION

### *Distribution of radioactivity*

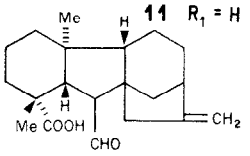
The distribution of applied radioactivity in the cultured cells and their medium is shown in Table 1. In the suspension cell culture system 16.2%, 63.7% and 20.1% of the radioactivity was found in the medium extract, cell extract and cell residue, respectively. The medium bathing the immobilized cells was not significantly different from medium bathing the cell suspension cultures with regard to types of [$^3$H]$GA_4$ metabolites, but the quantities in each category of metabolite differed somewhat (Table 1). For the immobilized cell systems, the extracts of MES medium [12] tended to have more residual precursor [$^3$H]$GA_4$, but they also had more radioactivity in peaks III and V–VII (Table 2). Uptake of precursor and re-uptake of metabolites may thus be less efficient for cells bathed in MES medium. Only suspension culture cells were used to examine cell metabolism. The distribution of radioactivity within the cell extract was 1.4% and 98.6% in the acidic ethyl acetate-soluble fraction and the highly water-soluble fraction, respectively. This suggests that [$^3$H]$GA_4$ and its acid metabolites are almost completely metabolized to highly water soluble compounds (e.g. mainly GA glucosyl conjugate-like substances) in the cells.

### *Separation and identification of metabolites of* [$^3$H]$GA_4$

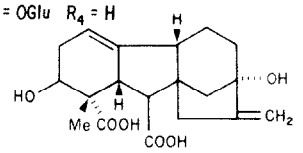
Si gel partition column fraction groupings 7–11, 13–16 and 18–21 (Fig. 1) of each sample (one cell extract and three different medium extracts) contained only one significant peak when run on gradient- and isocratic-eluted HPLC-RC and/or GLC-RC. These peaks coincided with known standards of [$^3$H]$GA_4$, [$^3$H]$GA_1$ and [$^3$H]$GA_8$ for fraction groupings 7–11, 13–16 and 18–21, respectively. In grouped fractions 24–26 a significant radioactive peak was observed ($R_t$ 27–28 min on gradient-eluted HPLC-RC) but the compound was not further identified. Seven GA glucosyl conjugate-like compounds were separated from the highly water-soluble fraction on gradient-eluted HPLC-RC (Fig. 2). The $R_t$ of peak I (Table 3) coincided on gradient-eluted HPLC with that of $GA_8$-0(2)-G. One of the three hydrolysis products (enzyme, base or acid) coincided on gradient- and isocratic-eluted HPLC with $GA_8$, and the other two products were probably epimerized $GA_8$ or C/D rearranged $GA_8$. The $R_t$s of peaks III–V (Table 3) coincided with those of $GA_1$-0(13)-G (**9**), $GA_1$-0(3)-G and $GA_1$ glucosyl ester [$GA_1$-GE(**7**)], respectively. Their hydrolysis products (enzyme, base or acid) coincided with $GA_1$, 3-epi $GA_1$ [13] or C/D rearranged $GA_1$ [14]. The $R_t$s of peaks VI and VII (Table 3) coincided with those of $GA_4$-G (**6**) and $GA_4$-GE (**5**), respectively. Their hydrolysis products (enzyme, base or acid) coincided with $GA_4$ or 3-epi $GA_4$. Peak II (Table 3) was found only in the cell extract,



| | | | | |
|---|---|---|---|---|
| **1** | $R_1$ = H | $R_2$ = OH | $R_3$ = H | $R_4$ = H |
| **2** | $R_1$ = H | $R_2$ = OH | $R_3$ = OH | $R_4$ = H |
| **3** | $R_1$ = OH | $R_2$ = OH | $R_3$ = OH | $R_4$ = H |
| **4** | $R_1$ = OH | $R_2$ = OH | $R_3$ = H | $R_4$ = H |
| **5** | $R_1$ = H | $R_2$ = OH | $R_3$ = H | $R_4$ = Glu |
| **6** | $R_1$ = H | $R_2$ = OGlu | $R_3$ = H | $R_4$ = H |
| **7** | $R_1$ = H | $R_2$ = OH | $R_3$ = OH | $R_4$ = Glu |
| **8** | $R_1$ = H | $R_2$ = OGlu | $R_3$ = OH | $R_4$ = H |
| **9** | $R_1$ = H | $R_2$ = OH | $R_3$ = OGlu | $R_4$ = H |
| **10** | $R_1$ = OGlu | $R_2$ = OH | $R_3$ = OH | $R_4$ = H |
| **11** | $R_1$ = H | $R_2$ = OGlu | $R_3$ = OGlu | $R_4$ = H |



**12**



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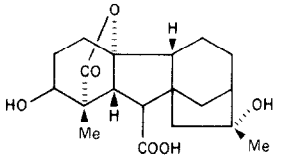

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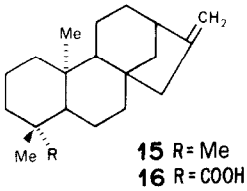
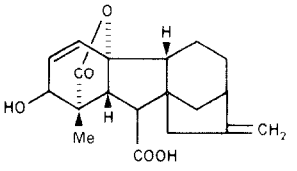


**15** R = Me
**16** R = COOH



**17**

Table 1. Recovery of radioactivity (percentage of applied radioactivity*) from cell cultures incubated with [1,2-$^3$H] $GA_4$ for 48 hr

| | Suspension cell culture | Immobilized cell cultures | |
|---|---|---|---|
| | | MES medium | 71V medium |
| Medium extract | | | |
| Total | 16.2 | 21.2 | 14.3 |
| Acidic, ethyl acetate phase | 7.6 | 12.7 | 6.9 |
| Si gel effluent | 4.9 | 8.4 | 4.3 |
| Si gel methanol wash | 2.7 | 4.3 | 2.6 |
| Acidic, butanol phase | 5.7 | 4.7 | 3.7 |
| Residual water phase | 2.9 | 3.8 | 3.7 |
| Cell extract | | | |
| Total | 63.7 | — | — |
| Si gel effluent | 0.9 | — | — |
| Si gel methanol wash | 62.8 | — | — |
| Cell residue | 20.1 | — | — |

*2.6 $\mu$Ci was applied to each cell culture.

Table 2. Levels of radioactivity (percentage of extracted radioactivity) in extracts of cells, or medium from cell cultures, incubated with [$^3$H]$GA_4$ for 48 hr

| GA fractions | Suspension cell culture | | Immobilized cell cultures | |
|---|---|---|---|---|
| | Cell* | Medium† | Medium‡ | Medium (71V)§ |
| GAs | | | | |
| $GA_4$ | 0.5 | 6.8 | 9.4 | 4.9 |
| $GA_1$ | 0.4 | 2.5 | 1.9 | 2.8 |
| $GA_8$ | 0.2 | 2.5 | 4.7 | 1.4 |
| Tailing or other peaks | 0.6 | 18.5 | 23.6 | 21.0 |
| GA conjugate-like substances | | | | |
| Peak I [$GA_8$-0(2)-G] | 1.8 | 2.5 | 1.9 | 2.8 |
| Peak II ($GA_1$-diglucoside-like conjugate) | 32.8 | — | — | — |
| Peak III [$GA_1$-0(13)-G] | 2.1 | 6.8 | 7.5 | 7.0 |
| Peak IV [$GA_1$-0(3)-G] | 2.9 | 3.7 | 1.4 | 2.8 |
| Peak V ($GA_1$-GE) | 31.4 | 7.4 | 5.2 | 6.3 |
| Peak VI ($GA_4$-G) | 14.9 | 6.8 | 8.0 | 4.8 |
| Peak VII ($GA_4$-GE) | 1.0 | 2.5 | 2.8 | 2.8 |
| Tailing or other peaks | 11.5 | 22.2 | 15.6 | 18.2 |

Values estimated from radioactivity eluted from Si gel partition column (GAs) and isocratic gradient HPLC (GA conjugate-like substances).

*1.656 $\mu$Ci extracted in total.

†0.421 $\mu$Ci extracted in total.

‡0.551 $\mu$Ci extracted in total.

§0.372 $\mu$Ci extracted in total.

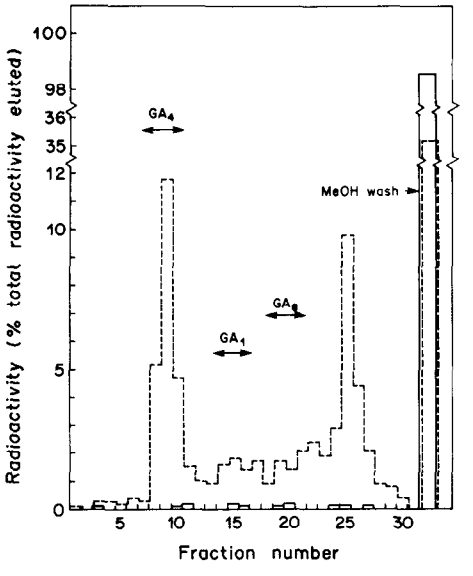



Fig. 1. Si gel partition column chromatograms of an extract of carrot somatic cells in liquid suspension culture and the medium therefrom. [$^3$H]$GA_4$ (2.6 $\mu$Ci) was incubated for 48 hr in the suspension cultures. (———) Cell extract; (– – – –) medium extract (acidic, ethyl acetate phase).

not in the medium extracts. The hydrolysis products (enzyme, base or acid) coincided with $GA_1$, 3-epi $GA_1$ [13] or C/D rearranged $GA_1$ [14]. The $R_f$s of the radioactive moiety of peak II after hydrolysis with 0.13 M acetic acid (at room temperature for 4 days) coincided with those of the original peak, $GA_1$-0(13)-G, $GA_1$-0(1)-G and $GA_1$ on isocratic HPLC-RC. This suggests that peak II might be $GA_1$-0(3,13)-diglucoside (**11**).

### *Metabolism of* [$^3$H]$GA_4$

In carrot somatic cell cultures most of the absorbed [$^3$H]$GA_4$ was converted to [$^3$H]GA-G/GE (98% of the absorbed radioactivity) (Table 2). The main metabolites of [$^3$H]$GA_4$ were the [$^3$H]$GA_1$-0(3,13)-diglucoside-like compound (32.8%) and [$^3$H]$GA_1$-GE (31.4%). Thus, 69.6% of the absorbed radioactivity was converted to [$^3$H]$GA_1$ and its glucosyl conjugate-like metabolites and only 16.4% remained as [$^3$H]$GA_4$ and [$^3$H]$GA_4$ glucosyl conjugate-like metabolites [e.g. [$^3$H]$GA_4$-G (14.9%), [$^3$H]$GA_4$-GE (1.0%) and precursor [$^3$H]$GA_4$ (0.5%)]. This pattern of metabolism was very different from that found for anise cell cultures [15] where 60% of the absorbed radioactivity still remained as [$^3$H]$GA_4$ and its glucosyl conjugate-like metabolites (e.g. [$^3$H]$GA_4$-G and [$^3$H]$GA_4$-GE) after 348 hr of incubation. No [$^3$H]$GA_1$-0(3,13)-diglucoside-like compound was observed in the anise cultures. In comparison with the levels of [$^3$H]$GA_1$ glucosyl conjugate-like metabolites, the levels of free [$^3$H]$GA_1$, free [$^3$H]$GA_8$ and [$^3$H]$GA_8$-0(2)-G were very low (0.4%, 0.2% and 1.8%, respectively). This suggests that the rate of conversion from [$^3$H]$GA_1$ to its conjugates was much greater than the rate of conversion from [$^3$H]$GA_4$ to [$^3$H]$GA_1$, from [$^3$H]$GA_1$ to [$^3$H]$GA_8$ and from [$^3$H]$GA_8$ to [$^3$H]$GA_8$ glucosyl conjugate. The very high level of [$^3$H]$GA_1$-0(3,13) diglucoside-like compound (32.8% of the absorbed radioactivity) suggests that its formation from [$^3$H]$GA_1$-0(3)-G (2.9% of the radioactivity) and/or [$^3$H]$GA_1$-0(13)-G (2.1% of the radioactivity) is very rapid. In the culture medium (acidic, ethyl

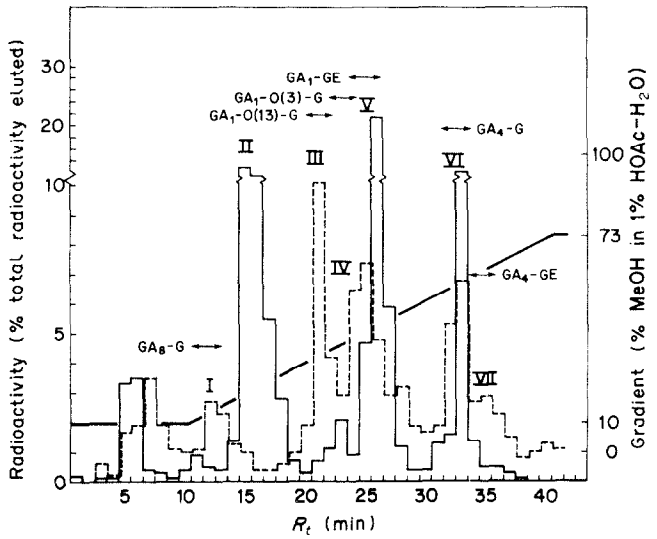



Fig. 2. Gradient-eluted reverse phase $C_{18}$ HPLC profile of the highly water-soluble fraction (e.g. methanol wash, which will contain GA glucosyl conjugates) from Si gel partition CC of the cell extract, and of the extract of the medium from carrot somatic cell suspension cultures in which 2.6 $\mu$Ci [$^3$H]GA$_4$ had been incubated for 48 hr. (———) Cell extract; (– – – –) medium extract.

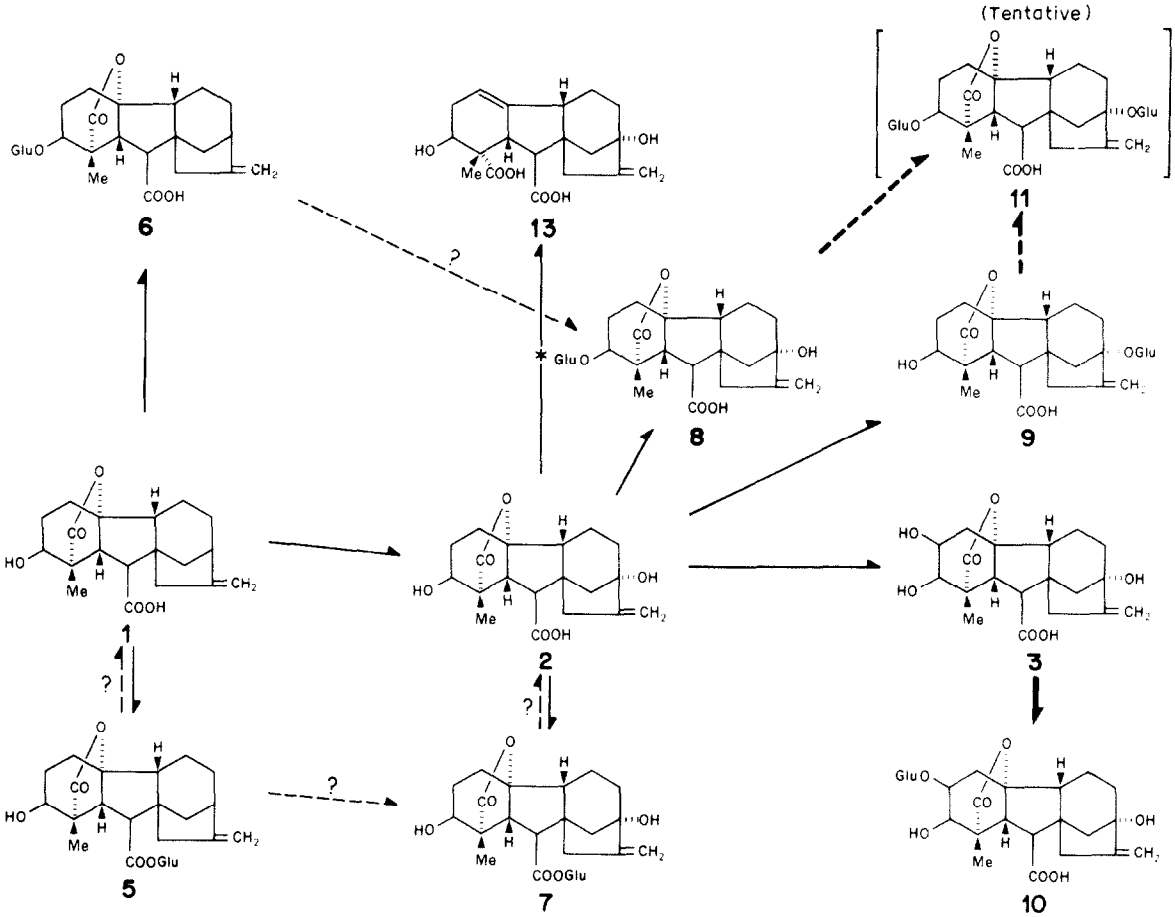



Fig. 3. Possible pathways of [$^3$H]GA$_4$ metabolism (2 → 13) in carrot somatic cell suspension cultures; based on Noma *et al.* [1, 2].

Table 3. Separation and identification of [$^3$H]$GA_4$ and its metabolites by gradient and isocratic HPLC-RC after 48 hr incubation of [$^3$H]$GA_4$ with suspension cell cultures of carrot

| Unknown compounds and authentic standards | $R_t$ (min) Gradient-eluted HPLC-RC | Isocratic HPLC-RC (for conditions see ref. [15]) | Identity* |
|---|---|---|---|
| From Si gel partition CC fractions | | | |
| Peak A (fr. 7–11)† | 37–38 | 35–37(III) | $GA_4$(**1**) |
| Peak B (fr. 13–16) | 25–26 | 26–27(II) | $GA_1$(**2**) |
| Peak C (fr. 18–21) | 12–13 | 22–23(I) | $GA_8$(**3**) |
| From HPLC fractions | | | |
| Peak I | 11–12 | 17–18(I) | $GA_8$-0(2)-G(?)(**10**) |
| Hydrolysate of I‡ | 12–13 | 22–23(I) | $GA_8$(**3**) |
| Peak II | 15–17 | 9–10(II) | $GA_1$-conjugate (**11**?) |
| Hydrolysate of II‡ | 25–26 | 26–27(II) | $GA_1$(**2**) |
| Peak III | 21–22 | 17–18(II) | $GA_1$-0(13)-G(**9**) |
| Hydrolysate of III‡ | 25–26 | 26–27(II) | $GA_1$(**2**) |
| Peak IV | 23–24 | 21–22(II) | $GA_1$-0(3)-G(**8**) |
| Hydrolysate of IV‡ | 25–26 | 26–27(II) | $GA_1$(**2**) |
| Peak V | 24–25 | 25–26(II) | $GA_1$-GE(**7**) |
| Hydrolysate of V‡ | 25–26 | 26–27(II) | $GA_1$(**2**) |
| Peak VI§ | 33–34 | 15–16(III) | $GA_4$-G(**6**) |
| Hydrolysate of VI†‡ | 37–38 | 35–37(III) | $GA_4$(**1**) |
| Peak VII | 35–36 | 29–31(III) | $GA_4$-GE(**5**) |
| Hydrolysate of VII‡ | 37–38 | 35–37(III) | $GA_4$(**1**) |
| Standards | | | |
| $GA_8$(**3**) | 12–13 | 22–23(I) | — |
| $GA_8$-0(2)-G(**10**) | 11–12 | — | — |
| $GA_1$(**2**) | 25–26 | 26–27(II) | — |
| $GA_1$-0(13)-G(**9**) | 21–22 | — | — |
| $GA_1$-0(3)-G(**8**) | 23–24 | — | — |
| $GA_1$-GE(**7**) | 24–25 | — | — |
| $GA_4$(**1**) | 37–38 | 35–37(III) | — |
| $GA_4$-G(**6**) | 33–34 | — | — |
| $GA_4$-GE(**5**) | 35–36 | — | — |

*Identified by co-chromatography with known standards on HPLC and/or GLC-RC.

†These compounds were also identified as [$^3$H]$GA_4$ by GLC-RC: The $R_t$ on three columns were 7.4 min, 1% XE-60; 7.6 min, 2% QF-1; and 9.1 min, 3% SE-30, respectively.

‡Obtained on hydrolysis with β-glucosidase.

§This compound was also identified as [$^3$H]$GA_4$-G by GLC-RC as the permethylated derivative. The $R_t$ was 7.9 min on 3% OV-101 column.

acetate- and acidic, butanol-soluble phases, Table 1), the metabolites were the same as in extracts of the suspension cell cultures except that no [$^3$H]$GA_1$-0(3,13) diglucoside-like compound was detected. The quantities of [$^3$H]$GA_4$ metabolite levels in the medium of immobilized cultures were similar to those in the medium of cell suspension cultures. Possible pathways of the metabolism of [$^3$H]$GA_4$ in carrot cell suspension cultures are shown in Fig. 3.

The similarity between types of metabolites and their amount (Table 2) in the medium of cultured cells and immobilized cells indicates that the immobilized cell system may be a useful technique for biosynthetic production of GAs from labelled (e.g. $^3$H, $^2$H, $^{13}$C) and unlabelled precursors since the level of sucrose in the immobilized cell system medium need be only 25% of that in the suspension cell system. The immobilized cell system is now being tested for production of a variety of [$^3$H]GA metabolites from several [$^3$H]GA precursors.

## EXPERIMENTAL

*Culture origin and maintenance.* The Ca68 culture of *Daucus carota* was derived from root tissues of germinated seeds [10] and obtained from the National Research Council of Canada Culture Collection, Ottawa. The suspension culture was maintained by weekly passages on 71V medium [11] at 26° in continuous light on a shaker (150 rpm). In expts using immobilized carrot cells, a 5-day-old culture was taken and the cells entrapped within a calcium alginate matrix by a procedure described previously [12]. Immobilized cells were incubated in either complete 71V medium or a MES buffer mixture [12].

*Metabolism of [$^3$H]gibberellin $A_4$ by the cells.* Suspension cultures of *D. carota* (5-day-old inoculum) or beads of immobilized cells were pre-incubated for 3 days in 50 ml 71V medium or MES buffer prior to addition of sterile (by filtration) [1,2-$^3$H]$GA_4$ (2.6 μCi, 1.06 Ci/mmol [3] dissolved in 200 μl, diluted to 5.2 ml with medium) to 50 ml medium. The incubation was continued for a further 2 days.

Suspension cultures were harvested by filtration, the cells washed with $2 \times 20$ ml $H_2O$, frozen and freeze-dried. The medium and washings were combined and similarly freeze-dried. For immobilized cells the incubation medium was decanted from the beads. The beads were then washed with $2 \times 20$ ml $H_2O$ and these washings were combined with the incubation medium, frozen and freeze-dried.

*Extraction of cells and medium. Cells.* Freeze-dried cells (0.38 g) were extracted with 40 ml aq. 80% MeOH. The MeOH extract was forced through a first column (2.5 cm i.d.) of Waters Associates $C_{18}$ 'Sep-Pak' material (3 g of $C_{18}$ material per g dry wt tissue) for removal of pigments [16]. This effluent was then diluted with $H_2O$ to a 50% MeOH concn and forced through a second column of $C_{18}$ material for removal of relatively nonpolar substances (e.g. kaurene (**15**), kaurenoic acid (**16**) and a significant portion of $GA_{12}$-aldehyde (**12**) will be retained while GAs and GA glucosyl conjugates will be eluted [17]]. The effluent of the second column was dried *in vacuo* and the residue dissolved in a small amount of MeOH–EtOAc–$H_2O$ (50:50:1), leaving only a minor amount of radioactivity undissolved. The soluble radioactivity was subjected to Si gel partition CC [18].

*Medium.* Extraction was accomplished according to the method of Kamienska *et al.* [4]. The dried medium was dissolved in 100 ml 0.25 M Pi buffer, pH 8.0, and partitioned against $Et_2O$ (discarded after sampling for radioactivity), the $H_2O$ phase being adjusted to pH 3.0 with 1 M HCl prior to partitioning ($\times$ 4) with equal vols. of EtOAc. This fraction was then subjected to Si gel partition chromatography [18]. The acidic, aq. phase was further extracted ($\times$ 3) with equal vols. of BuOH. This acidic, BuOH-soluble fraction was then subjected to $C_{18}$ HPLC.

*Si gel CC, HPLC, radio-GLC, derivitization, and hydrolysis of GA glucosyl conjugate-like substances.* These procedures were carried out as described in ref. [15].

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