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METABOLISM OF [^{3}H]GIBBERELLIN A_4 IN SOMATIC SUSPENSION CULTURES OF ANISE

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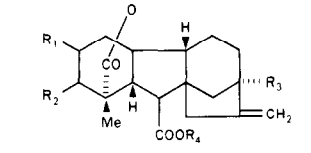
Key Word Index—*Pimpinella anisum*; Umbelliferae; anise; metabolism; [^{3}H]gibberellin A_4; gibberellins; gibberellin glucosyl conjugates.

Abstract—The native gibberellin A_4 (GA_4) was fed as [1, 2-^{3}H]GA_4 (1.3 Ci/mmol) to anise somatic cultures maintained either at a proembryo-like stage with 2,4-dichlorophenoxyacetic acid (2,4-D), or allowed to undergo embryogenic development on a −2,4-D medium. Proembryos, although only 20% of the dry wt of embryos, absorbed 1.4-times more [^{3}H]GA_4/g dry wt than embryos. The [^{3}H]GA_4 was metabolized to GA_1 and GA_8, and at least six conjugates [GA_4-glucoside (GA_4-G), GA_4 glucosyl ester (GA_4-GE), GA_1-0(3)-G, GA_1-0(13)-G, GA_1-GE and a GA_8-glucosyl conjugate]. The major metabolite was GA_4-G at each of two, 204 and 348 hr harvests (56–71%), with GA_8-G increasing from < 1% to 13% with harvest time. The percentage and amount of GA_4-GE was highest at 204 hr (2% and 8%, for embryos and proembryos, respectively), dropping to < 1% at 348 hr, thereby indicating hydrolysis (e.g. reversible conjugation). Embryos had reduced amounts and percentages of biologically active GA_4 and GA_1, and most of their conjugates, but increased amounts and percentages of GA_8 and its conjugate(s). This finding is consistent with the hypothesis (based on present and past work) that high levels of biologically active GAs, especially GA_1, inhibit somatic embryogenesis in anise and carrot. The auxin, 2,4-D, may thus derive, at least in part, its ability to maintain the proembryo-like stage by inhibiting oxidative metabolism and conjugation of biologically active GAs.

INTRODUCTION

Gibberellins (GAs) A_1 (**2**), A_4 (**1**), A_7 (**11**), and the $\Delta^{1(10)}GA_1$ 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 (**10**), and [^{3}H]GA_1-0(3)-glucoside (**8**) has been reported in somatic embryos and proembryos of anise suspension cultures [2], the rapidity of conversion being positively correlated with embryogenic development, as was a lowered level of endogenous GA_1 [2]. The [^{3}H]$\Delta^{1(10)}GA_1$ counterpart was only tentatively identified as a product of [^{3}H]GA_1 metabolism [2].

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 a variety of higher plants, has been reported [1–9]. Since the [^{3}H]$\Delta^{1(10)}GA_1$ counterpart is not a major product of [^{3}H]GA_1 metabolism in anise somatic cultures [2], the possibility existed that it may be produced from [^{3}H]GA_4 by cleavage of the lactone ring followed by dehydration, then C-13 hydroxylation. If so, then such a deactivation mechanism (e.g. $\Delta^{1(10)}GA_1$ counterpart is only 1/500 as active as GA_1 on dwarf rice cv Tan-ginbozu) could be important in regulating the levels of highly biologically active GA_4 and GA_1 (produced from GA_4) by acting as an alternate branch in GA_4 metabolism.



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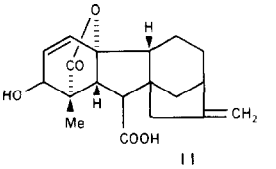
2	R_1 = H	R_2 = OH	R_3 = OH	R_4 = H
3	R_1 = OH	R_2 = OH	R_3 = OH	R_4 = H
4	R_1 = OH	R_2 = OH	R_3 = H	R_4 = H
5	R_1 = H	R_2 = OH	R_3 = H	R_4 = Glu
6	R_1 = H	R_2 = OGlu	R_3 = H	R_4 = H
7	R_1 = H	R_2 = OH	R_3 = OH	R_4 = Glu
8	R_1 = H	R_2 = OGlu	R_3 = OH	R_4 = H
9	R_1 = H	R_2 = OH	R_3 = OGlu	R_4 = H
10	R_1 = OGlu	R_2 = OH	R_3 = OH	R_4 = H



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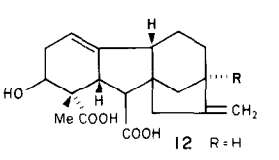
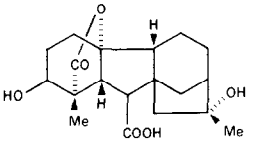


12 R = H
13 R = OH



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Somatic cultures of anise and carrot undergoing embryogenic development (e.g. −2,4-D) have high levels of GA_4 and/or GA_7, and very low levels of GA_1 [2]. Conversely, proembryos (+2,4-D) maintain very high levels of GA_1, and somewhat lower levels of GA_4 and/or GA_7 [2]. Because of this and because of the possibility that the native GA_4 might be a precursor of the $\Delta^{1(10)}GA_1$ counterpart, we have examined the metabolism of [^{3}H]GA_4 in somatic suspension cultures of anise (*Pimpinella anisum* L.) at varying periods of time after transfer of the cells from a maintenance medium (+2,4-D) to either a −2,4-D medium (which allows embryogenic development) or a +2,4-D medium (which maintains the proembryo-like stage of differentiation).

RESULTS

Developmental changes

Cultures maintained on +2,4-D stayed at the 'proembryo-like stage of development' throughout the 348 hr culture period. Cultures transferred to −2,4-D medium, however, underwent rapid somatic embryogenic development yielding, at 204 hr, 34% globular and 66% heart/torpedo stages, and at 348 hr 20% globular and 80% heart/torpedo stages.

Uptake of radioactivity

The total uptake of radioactivity, both as a percentage of radioactivity applied and on a per g dry wt basis is shown in Table 1. Initially (2 hr), proembryos (+2,4-D) tended to take-up more radioactivity on both bases. By 204 hr, however, embryos (−2,4-D) had taken up some five times more radioactivity in total, but proembryos had *ca* 1.4 times more radioactivity per g dry wt. This trend continued through 348 hr. The increased uptake of [^{3}H]GA_4/g dry wt by proembryos, relative to embryos, is consistent with the increased endogenous levels of GAs found in proembryos [2] and with the fact that exogenous GAs will inhibit embryogenic development [2] (and references cited therein).

Separation and identification of metabolites (Table 2)

Each sample was separated and analysed by gradient-eluted C_{18} HPLC-RC after a 'C_{18} Sep-Pak' purification procedure [10, 11] and/or after Si gel partition CC [12], and further investigated by isocratic HPLC-RC and/or GLC-RC. The combined Si gel partition column (Fig. 1) fractions 7–11, 13–16 and 18–23 of each sample were analysed by gradient- and isocratic-eluted C_{18} HPLC-RC and/or GLC-RC (Table 2), yielding [^{3}H]GA_4, [^{3}H]GA_1 and [^{3}H]GA_8, respectively (Table 2). The [^{3}H]$\Delta^{1(10)}GA_1$ counterpart (**13**) [1, 2], a possible metabolite of [^{3}H]GA_1 or [^{3}H]GA_4 (via a postulated intermediate, [^{3}H]$\Delta^{1(10)}GA_4$ counterpart, **12**) was not detected, nor was the postulated intermediate.

Six [^{3}H]GA conjugate-like metabolites, separated from GAs by the combined use of the Sep-Pak procedure and a methanol wash of the Si gel partition column, were observed on gradient-eluted C_{18} HPLC-RC (Fig. 2). Sequential gradient-eluted followed by isocratic-eluted C_{18} HPLC-RC was performed on each conjugate-like metabolite (Table 2). After enzymatic, acid, or base hydrolysis, isocratic-eluted C_{18} HPLC and/or GLC-RC was performed to obtain the R_t of the ^{3}H moiety. Our stocks of authentic GA glucosyl conjugates were insufficient to perform isocratic-eluted C_{18} HPLC. Hence, the information noted in Table 2 with regard to R_ts in isocratic systems I–III is useful only to show that peaks I–VI (Fig. 2) are: (1) distinctly different from GA_1, GA_4 or GA_8; and (2) that the hydrolysis products of peaks I–VI are distinctly different from the original metabolite, and coincidental with either of GA_1, GA_4 or GA_8.

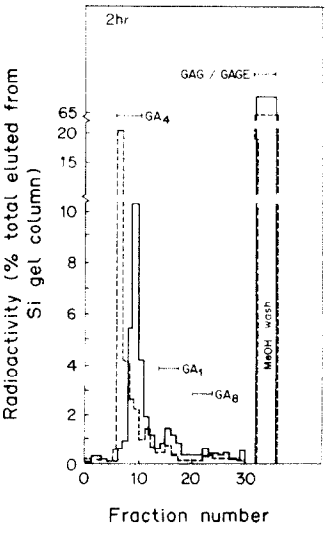

Fig. 1. Elution pattern from a Si gel partition column of [^{3}H]GA_4 (precursor) and ^{3}H metabolites in an extract from anise cells which were incubated for 2 hr in liquid suspension cultures with (——) (proembryos) and without (– – – –) (embryos) 2,4-D. Gibberellin glucosyl conjugates will elute in the methanol wash. Less than 4% of the radioactivity eluted in fractions 1–30 (e.g. 90% eluted in the methanol wash) for extracts of cultures incubated for 204 and 348 hr, hence elution profiles are not shown. Additional details are given in Tables 1 and 3.

Peak I (Fig. 2 and Table 2), when chromatographed on isocratic HPLC system I yielded a single peak, well removed from that of GA_8. Four GA_8 glucosyl conjugates could exist, GA_8-GE, GA_8-0(2)-G, GA_8-0(3)-G and GA_8-0(13)-G, but only the authentic standard of GA_8-0(2)-G was available. It is unlikely that peak I is GA_8-GE since GA glucosyl esters tend to run close to the GA moiety (Table 2) on gradient-eluted C_{18} HPLC, and this tendency would be even more pronounced on isocratic HPLC (see R_t for purported GA_4-GE and GA_4-G, relative to GA_4; Table 2). And, although -0(2) and -0(13) glucosides of GA_1 separate on gradient-eluted C_{18} HPLC (Table 2), and this separation would be further enhanced by the use of isocratic elution, we have no evidence that the -0(2), -0(3), and -0(13) glucosides of GA_8 would separate on either gradient- or isocratic-eluted C_{18} HPLC. Hence, while peak I might be GA_8-0(2)-G, it has been identified only as GA_8-0(?)-G. Upon hydrolysis, peak I yielded at least three ^{3}H products, one of which was coincidental with GA_8 (Table 2), the others probably representing epimers of GA_8 or C/D rearranged GA_8.

Peaks II–IV (Fig. 2 and Table 2) were further chromatographed on isocratic C_{18} HPLC system II and eluted at different R_ts, all of which were appreciably different from [^{3}H]GA_1 (Table 2). Upon hydrolysis they

Table 1. The uptake of radioactivity by somatic suspension cultures of anise incubated with [1,2-^{3}H]GA$_4$ for varying periods in the presence or absence of 2,4-D, and the distribution of radioactivity after Si gel partition CC

Time of incubation after transfer onto new medium (hr)	Cell density* (10^{-4} cells/ml)	2,4-D	Tissue dry wt at harvest (g)	Uptake of radioactivity into cells at time of harvest			Recovery of [^{3}H]GA$_4$ and metabolites from C_{18} Sep-Pak step (% activity in 80% MeOH)			
				(μCi/g dry wt)	(μCi/culture flask)	(% of radioactivity applied)	[^{3}H]GA$_4$	[^{3}H]Acidic metabolities	[^{3}H]Conjugates	Total
2	157.0	–	1.28	0.225	0.287	3.9	23.4	11.6	60.5	95.5
204	8.6	–	1.965	1.864	3.662	50.6	0.2	2.3	85.2	87.7
348	8.6	–	5.50	0.810	4.455	59.6	0.4	2.1	86.4	88.9
2	157.0	+	1.36	0.254	0.344	4.6	21.2	11.2	63.4	95.8
204	8.6	+	0.50	2.522	1.260	17.0	1.1	1.5	83.6	86.2
348	8.6	+	1.03	0.918	0.946	12.7	0.4	2.7	85.1	88.2

*In 500 ml of medium for 2 hr, and 510 ml for 204 or 348 hr cultures, at start of incubation.

Cultures were incubated with 16.49×10^6 dpm of [^{3}H]GA$_4$ (1.3 Ci/mmol) for 2, 204 and 348 hr in the presence (+ 2,4-D) or absence (– 2,4-D) of 2,4-D (5×10^{-6} M). Transfer from medium with 2,4-D onto medium free of 2,4-D allowed embryogenic development to proceed, whereas transfer onto medium with 2,4-D maintained the 'proembryo' stage of differentiation.

Table 2. Separation and identification of [^{3}H]GA_4 and its metabolites by gradient and isocratic HPLC-RC after 2, 204 and 348 hr incubation of [^{3}H]GA_4 with suspension cell cultures of anise

Unknown compounds and authentic standards	R_t (min) Gradient-eluted HPLC-RC	Isocratic HPLC-RC (for conditions see Experimental)	Identity*
From Si gel fractions of Fig. 1			
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 of Fig. 2			
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	21–22	17–18 (II)	GA_1-0(13)-G (**9**)
Hydrolysate of II‡	25–26	26–27 (II)	GA_1 (**2**)
Peak III	23–24	21–22 (II)	GA_1-0(3)-G (**8**)
Hydrolysate of III‡	25–26	26–27 (II)	GA_1 (**2**)
Peak IV	24–25	25–26 (II)	GA_1-GE (**7**)
Hydrolysate of IV‡	25–26	26–27 (II)	GA_1 (**2**)
Peak V§	33–34	15–16 (III)	GA_4-G (**6**)
Hydrolysate of V†‡	37–38	35–37 (III)	GA_4 (**1**)
Peak VI	35–36	29–31 (III)	GA_4-GE (**5**)
Hydrolysate of VI†‡	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.

‡These compounds were hydrolysed by 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 a 3% OV-101 column.

each yielded ^{3}H products which were coincidental with GA_1 (Table 2), 3-epi GA_1 [13], or C/D rearranged GA_1 [13].

Peaks V and VI (Fig. 2 and Table 2) were further chromatographed on isocratic HPLC system III and eluted at distinctly different R_ts, both of which differed from the R_t of GA_4 (Table 2). Upon hydrolysis they each yielded one or two ^{3}H products, one of which was coincidental with GA_4 (Table 2), the other of which was probably 3-epi-GA_4.

Metabolism of [^{3}H]GA_4 *(common trends)*

At 2 hr after feeding [^{3}H]GA_4 both cell types had produced a high percentage of GA conjugate (*ca* 60–63% of the extractable radioactivity; Table 1), mainly GA_4-G (Table 3). The major acidic metabolites were GA_1 and GA_8 (2% and 1% of the extractable radioactivity, respectively, Table 3).

By 204 hr GA conjugate levels had increased to *ca* 97% of the extractable radioactivity (Table 4), being composed mainly of GA_4-G (e.g. 65–73%; Table 4). However, GA_4-GE had increased appreciably, both in amount and percentage (Table 4). Conjugates of GA_1 increased in amounts, but their percentages remained low (Table 4). The GA_8 conjugate(s) increased appreciably in amount and percentage (8–13%), as did other conjugate-like substances (Table 4).

By 348 hr the overall distribution of radioactivity had not changed appreciably (from 204 hr) between acidic and conjugate fractions (Tables 1, 3 and 4), although changes were apparent within the conjugate fraction (Table 4). The amounts and percentages of GA_4-G were maintained at a high level (Table 4), but GA_4-GE levels had dropped drastically (Table 4). The conjugates of GA_1 remained at relatively low amounts and percentages (Table 4). Conjugates of GA_8 remained high, and 'other conjugate-like substances' increased (Table 4).

Metabolism of [^{3}H]GA_4 *(differences between embryos and proembryos)*

There were no appreciable differences in [^{3}H]GA_4 metabolism for the two cell types by the 2 hr harvest

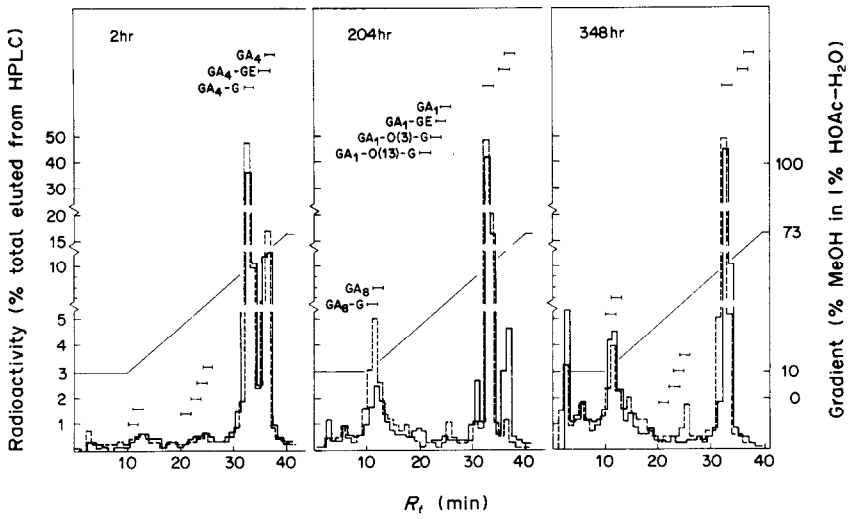

Fig. 2. Elution patterns from gradient-eluted reverse phase C_{18} HPLC columns of [^{3}H]glucosyl conjugate-like substances in the methanol wash of the Si gel partition column (e.g. Fig. 1 and Tables 1 and 4) from extracts of anise cells that had been incubated for 2, 204 and 348 hr in liquid suspension cultures, with (——) (proembryos) and without (– – – –) (embryos) 2,4-D, for varying periods of time. R_fs of standard GA glucosyl esters (GA-GE) and GA glucosides (GA-G) are shown as (——). Additional details are given in the legends of Tables 1–4. Estimates of radioactivity within each peak are given in Table 4.

Table 3. Levels of [^{3}H]GA$_1$, GA$_4$, GA$_8$ and other acidic substances (e.g. unknowns and tailing) [nCi/g dry wt tissue* and as a percentage of radioactivity extracted in 80% methanol* (in parentheses)] in anise cells incubated in liquid suspension cultures, with and without 2,4-D, for varying periods of time

	2 hr		204 hr		348 hr	
	Embryos (−2,4-D)	Proembryos (+2,4-D)	Embryos (−2,4-D)	Proembryos (+2,4-D)	Embryos (−2,4-D)	Proembryos (+2,4-D)
[^{3}H]GA$_4$	55.1 (24.5)	56.1 (22.1)	3.7 (0.2)	32.8 (1.3)	4.1 (0.5)	4.6 (0.5)
[^{3}H]GA$_1$	4.1 (1.8)	4.8 (1.9)	3.7 (0.2)	12.6 (0.5)	4.1 (0.5)	5.5 (0.6)
[^{3}H]GA$_8$	2.3 (1.0)	3.3 (1.3)	26.1 (1.4)	5.0 (0.2)	0.8 (0.1)	4.6 (0.5)
^{3}H other, including tailing	20.9 (9.3)	21.6 (8.5)	20.5 (1.1)	2.5 (1.0)	13.8 (1.7)	17.4 (1.9)
Total nCi	82.4	85.9	54.1	75.7	22.7	32.1
Total (%)	(36.6)	(33.8)	(2.9)	(3.0)	(2.8)	(3.5)

*Calculations normalized for work-up and chromatography losses

$$\left(\text{e.g. percentage in peak eluted from Si gel column} \times \frac{\text{nCi extracted initially in 80\% MeOH}}{\text{dry wt celles}}\right).$$

Somatic embryos (−2,4-D cultures) were 34% and 20% globular stage, and 66% and 80% heart/torpedo stages, by 204 and 348 hr, respectively.

(Tables 1, 3, 4 and Figs. 1, 2, 4). However, by 204 hr distinct trends between embryos and proembryos had become apparent. Proembryos maintained 3–9 times more GA$_4$ and GA$_1$ (both highly biologically active GAs) than did embryos, both per g dry wt and as a percentage (Table 3). Conversely, embryos had 5–7 times more GA$_8$ than did proembryos (Table 3). This tendency for proembryos to have higher levels of the two biologically active GAs (GA$_4$ and GA$_1$) was also reflected by higher amounts per g dry wt of GA$_4$-G, GA$_4$-GE and GA$_1$-G (Table 4).

DISCUSSION

Thus, as noted above, the two acidic ^{3}H metabolites of [^{3}H]GA$_4$ were identified as GA$_1$ (**2**) and GA$_8$ (**3**); the six

Table 4. Levels of glucosyl ester- and glucoside-like conjugates of [^{3}H] gibberellins A_1, A_4 and A_8 and other conjugate-like ^{3}H substances (e.g. unknowns and tailing) expressed as nCi/g dry wt tissue*, and as a percentage of radioactivity extracted in 80% methanol* (in parentheses) in anise cells incubated in liquid suspension cultures, with and without 2,4-D, for varying periods of time

	2 hr		204 hr		348 hr	
	Embryos (−2,4-D)	Proembryos (+2,4-D)	Embryos (−2,4-D)	Proembryos (+2,4-D)	Embryos (−2,4-D)	Proembryos (+2,4-D)
[^{3}H]GA_4 glucoside	137.7 (61.2)	143.0 (56.3)	1323.4 (71.0)	1588.9 (63.0)	494.1 (61.0)	573.8 (62.5)
[^{3}H]GA_4 glucosyl ester	0.7 (0.3)	11.7 (4.6)	31.7 (1.7)	196.7 (7.8)	5.7 (0.7)	6.4 (0.7)
[^{3}H]GA_1 glucosyl ester	— (—)	— (—)	33.6 (1.8)	30.3 (1.2)	23.5 (2.9)	5.5 (0.6)
[^{3}H]GA_1 glucoside(s)	2.0 (0.9)	2.8 (1.1)	13.0 (0.7)	55.5 (2.2)	13.0 (1.6)	11.0 (1.2)
[^{3}H]GA_8 glucoside(s)	1.8 (0.8)	— (—)	242.3 (13.0)	206.8 (8.2)	85.1 (10.5)	114.8 (12.5)
^{3}H other conjugate-like substances, including tailing	0.2 (0.1)	10.7 (4.2)	165.9 (8.9)	368.2 (14.6)	166.1 (20.5)	174.4 (19.0)
Total nCi	142.4	168.1	2198.3	2446.3	787.3	885.9
Total (%)	(63.3)	(66.2)	(97.1)	(97.0)	(97.2)	(96.5)

*Calculations normalized for work-up and chromatography losses

$$\left(\text{e.g. percentage in peak eluted from HPLC column} \times \frac{\text{nCi extracted initially in 80\% MeOH}}{\text{dry wt cells}}\right).$$

^{3}H conjugate-like metabolites were identified as GA_4-G (**6**), GA_4-GE (**5**), GA_1-0(3)-G (**8**), GA_1-0(13)-G (**9**), GA_1-GE (**2**), and GA_8-0(?)-G [probably GA_8-0(2)-G (**10**)].

Possible pathways of [^{3}H]GA_4 metabolism in anise somatic suspension cultures are shown in Fig. 3.

Both proembryos and embryos metabolized [^{3}H]GA_4

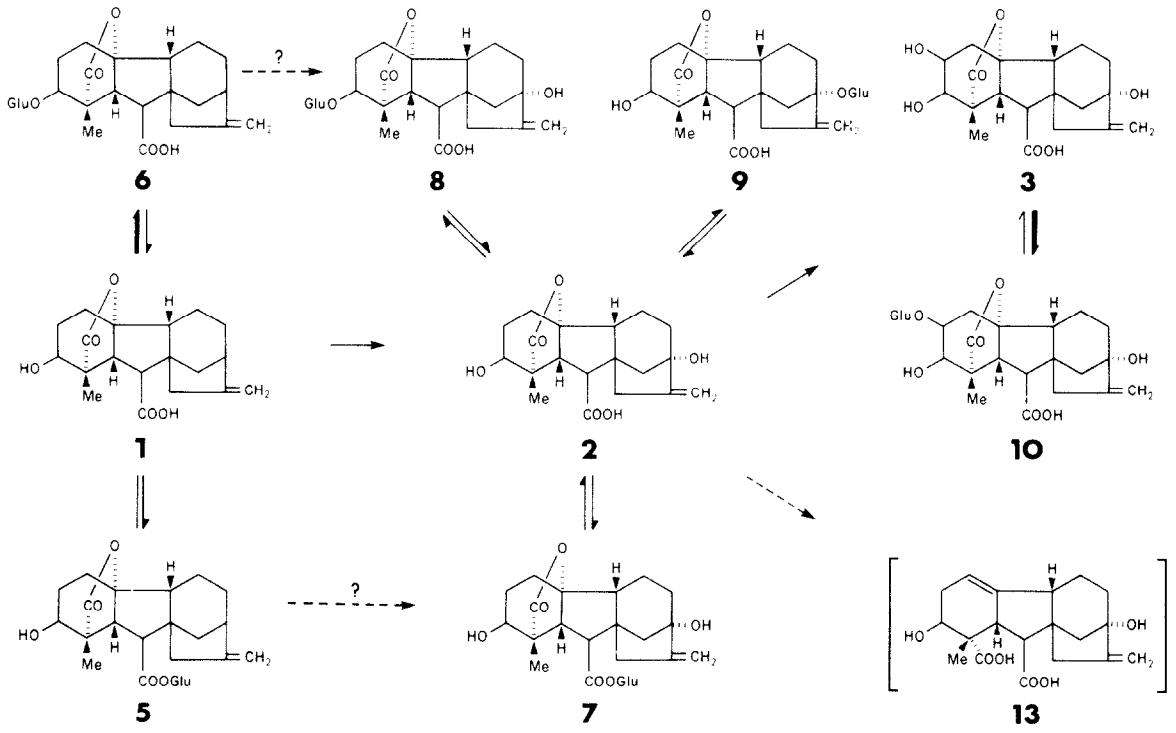

Fig. 3. Possible pathway of [^{3}H]GA_4 metabolism (**2** → **13**) in anise somatic cell suspension cultures based on Noma *et al.* [1, 2].

very rapidly, mainly to GA$_4$-G, but with increasing time of incubation to GA$_8$-G and other conjugate-like substances (Tables 1, 3 and 4). Since formation of GA$_4$-GE continued to increase through 204 hr, but declined sharply between 204 and 348 hr, hydrolysis is implied (e.g. reversible conjugation).

The fact that the non-embryogenic (+2,4-D) system maintained higher levels of biologically active GAs and their conjugates, but had lowered levels of GA$_8$ and GA$_8$-G (Table 4 and Fig. 4), thereby implies that proembryos have a diminished ability for oxidative metabolism at the GA$_1$ to GA$_8$ step. Somatic proembryos of anise and carrot were found to have high levels of GA$_1$ and a greatly reduced rate of GA$_1$ to GA$_8$ metabolism, relative to somatic embryos [2]. The trends noted above for the present study are, thus, confirmatory of work by Noma *et al.* [2] and consistent with the known inhibitory effects of exogenous application of GAs on embryogenesis (e.g. after 2,4-D has been withdrawn) [2] (and references cited therein). Taken *in toto*, the above results suggest strongly that embryogenic development in somatic cultures of anise is prevented by high levels of biologically active GAs, especially GA$_1$, and that one basis for the maintenance of these high levels in +2,4-D cultures is a possible inhibition of the GA$_1$ to GA$_8$ hydroxylation step.

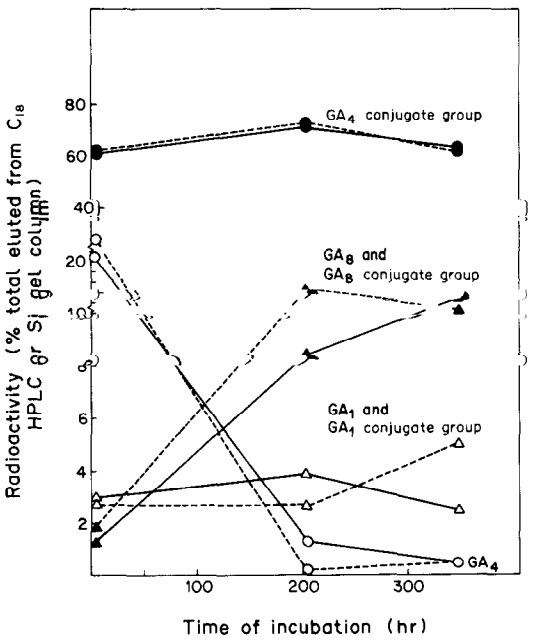

Fig. 4. Diagrammatic representation of changes in levels of precursor [^{3}H]GA$_4$ (as a percentage of total radioactivity eluted from the Si gel partition column and C$_{18}$ HPLC) and three metabolite groupings, GA$_4$ conjugates, GA$_1$/GA$_1$ conjugates, and GA$_8$/GA$_8$ conjugates. The reciprocal change in precursor GA$_4$ and product GA$_8$/GA$_8$ conjugate grouping suggests that free GA$_4$, present in the early hours of incubation, is a major source of GA$_8$/GA$_8$ conjugates, and that the kinetics of GA$_1$ metabolism favour GA$_8$ formation in preference to GA$_1$ conjugation (see also Tables 2 and 3). Embryos tend to remove more precursor GA$_4$ and produce more GA$_8$/GA$_8$ conjugates than proembyos. Estimates for GA$_8$ and GA$_8$ conjugates may be low due to partial or total loss of ^{3}H at C-2 (e.g. precursor was [1,2-^{3}H]GA$_4$). Actual radioactivity (shown here as a percentage) can be calculated from data provided in Table 1, and is given on a per g dry wt basis in Tables 3 and 4.

It has also recently been noted [14] that in +2,4-D somatic cultures of carrot where embryo formation is suppressed, abscisic acid (ABA) levels are increased significantly. Although exogenously applied ABA has been shown to increase metabolism of [^{3}H]GA$_1$ and [^{3}H]GA$_4$ in two systems (barley half-seeds and *Pinus radiata* xylem/phloem/cambial cells [15, 16]), ABA reduced metabolism of [^{3}H]GA$_4$ to [^{3}H]GA$_1$ in a third system (lettuce hypocotyls [17]). Reduced metabolism of GAs, effected by increased levels of ABA, would, thus, offer another mechanism by which 2,4-D might prevent embryogenesis.

EXPERIMENTAL

Plant material. An embryogenic strain of cells (when grown in liquid suspension cultures) was isolated initially from hypocotyl explants from etiolated seedlings of anise (*Pimpinella anisum* L.).

Cell conditions. Cell suspension cultures, composed of cell aggregates of varying degrees of complexity but not showing any microscopic signs of morphological organization or cell differentiation, were termed 'proembryos', and were developed and maintained on OB5 liquid medium [18] containing 5×10^{-6} M 2,4-D. Somatic embryogenesis was obtained by transferring the cells to a 2,4-D-free (e.g. −2,4-D) medium. The culture conditions were as described earlier [19] except that samples of cells for the short-term expt (e.g. 2 hr incubation) were placed immediately after transfer into their new medium (+/−2,4-D, + [^{3}H]GA$_4$), whereas samples of cells for the long-term expt (e.g. 204 and 348 hr incubation) were washed for 2 hr in −2,4-D medium before transfer onto their new medium (+/−2,4-D, + [^{3}H]GA$_4$). Culture after transfer took place in 500 ml (2 hr incubation) or 510 ml (204 and 348 hr incubations) of B5 medium in Fernbach flasks.

Applications of [^{3}H]GA$_4$. [1,2-^{3}H]GA$_4$ (1.3 Ci/mmol, [3]) was dissolved in a small amount of 95% EtOH and added to the OB5 medium for a final concn of 1.649×10^{-8} g/ml (4.1×10^{-8} M GA$_4$) for [^{3}H]GA$_4$, and 0.07% EtOH, in 500 or 510 ml OB5 medium. Neither of these concns of GA$_4$ or EtOH affected embryogenesis or growth of the anise cell cultures.

(1) Short-term cultures (2 hr incubation). Cells from the maintenance cultures were passed through a sieve (400 μm) and collected by sedimentation, the old medium decanted and the new medium (+/−2,4-D, + [^{3}H]GA$_4$) added, the cell cultures (1.57×10^6 cells/ml) being shaken (150 rpm at 28° on a gyratory shaker in continuous fluorescent light at 800 lx) for 2 hr until harvest. Harvest was accomplished by sedimenting the cells at 100 *g*, washing with 100 ml fresh B5 medium and again sedimenting the cells. The cells were then frozen with liquid N_2 and lyophilized.

(2) Long-term cultures (204 and 348 hr incubation). Cells from the maintenance cultures were sieved and collected as above, except that they were washed for 2 hr in −2,4-D medium before transfer to new medium (+/−2,4-D, + [^{3}H]GA$_4$). Cell cultures (8.6×10^4 cells/ml) were maintained and harvested as above for 204 and 348 hr.

Extraction of tissue. 1 g samples of dried cells were extracted with 80 ml aq. 80% MeOH. The MeOH extract was forced through a first column (2.5 cm i.d.) of C$_{18}$ 'Sep-Pak' (Waters Associates) material (3 g of C$_{18}$ material/g dry wt tissue) for removal of pigments [20]. 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 additional non-polar substances while retaining GAs and GA glucosyl conjugates [10]. The effluent off the second column was dried *in vacuo*, the residue

being dissolved in either (a) a small amount of 50% MeOH, or (b) successively with EtOAc–MeOH (1:1) (40 ml) or H_2O-satd EtOAc (40 ml), followed by MeOH (40 ml) and followed, finally, by H_2O (40 ml), depending upon the type of chromatography (see below) to which the sample, or an aliquot thereof, was to be subjected.

Si gel partition column chromatography. Gradient elution Si gel partition CC was as described in ref. [12], acidic, EtOAc-soluble GAs being eluted in the first 30 fractions, highly H_2O soluble GAs/GA conjugates being eluted by washing the column with MeOH. It was used for the residue soluble in 50% MeOH [e.g. (a) above]. Aliquots of each fraction were dissolved in 1 ml MeOH and assayed for radioactivity by liquid scintillation spectrometry. Acidic, EtOAc-soluble GAs, and the MeOH wash fraction were chromatographed, subsequently, on C_{18} reverse phase HPLC-RC (with radiocounting) and/or GLC-RC.

High pressure/performance liquid chromatography. Gradient and isocratic elution C_{18} reverse-phase HPLC [21] was accomplished for Si gel partition column fraction groupings or for the residues soluble in (b) above. A Waters Associates ALC/GPC R-401 liquid chromatograph with two Model 6000 pumps, Model 660 solvent flow programmer, and Model U6K universal injector was used. A Berthold HPLC radioactivity monitor (LB503) was used as a detector. Conditions: column, Waters Associates μBondapak (3.9 × 300 mm); solvents: Pump A, 10% MeOH in 1% HOAc–H_2O; Pump B, 100% MeOH. (1) Standard linear gradient programme: 0–10 min (Pump A, 100%), 10–40 min (Pump B, 0–70%), 40–50 min (Pump B, 70%), 50 80 min (Pump B, 100%), temp. 22–25°; (2) isocratic programme I: 7% MeOH in 1% HOAc–H_2O; (3) isocratic programme II: 17.2% MeOH in 1% HOAc–H_2O; (4) isocratic programme III: 41.5% MeOH in 1% HOAc–H_2O.

Gas chromatography. A Packard Model 430 gas chromatograph with a FID detector and a Packard Model 894 gas proportional counter (radio-counter) were used. Conditions: For GAs: columns, 1% XE-60 (2.5 mm × 2 m), 2% QF-1 (2 mm × 2 m) and 3% SE-30 (2 mm × 2 m), column temp. 205°, detector temp. 250°, injector temp. 230°, carrier gas, He 50 ml/min, RC split ratio, 25:1 (RC:FID); for GA glucosyl conjugates: column, 3% OV-101 (2.5 mm × 2 m), column temp. 285°, detector temp. 300°, injector temp. 295°, carrier gas, He 50 ml/min RC split ratio, 10:1.

Derivatization. GAs were derivatized to the Me esters by using CH_2N_2 then to the trimethylsilyl ethers (TMS) with *N,O*-bis(trimethylsilyl)trifluoroacetamide (1% trimethylchlorosilane). Gibberellin glucosyl conjugates were derivatized to permethylated derivatives according to the method of Rivier *et al.* [22] modified as noted below [personal communication from J. MacMillan via M. Noma]. The GA glucosyl conjugate which was already derivatized to the Me ester of metabolized to the glucosyl ester, was dissolved in a mixture of 100 μl *N,N*-dimethyl formamide (dried over CaH_2 and distilled under dried N_2) and 100 μl distilled MeI. This soln was transferred to a Reacti-vial which contained 25 mg NaH (prewashed with dried petrol) and allowed to stand for 4 hr at room temp. After evaporation of the solvent under dry N_2 at 50°, 200 μl MeOH was added and evaporated under the same conditions. 300 μl H_2O and 300 μl EtOAc were added and partitioned. The EtOAc phase was used for GLC-RC.

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 soln. The mixture was then left to stand at 37° for 16 hr [23]. (2) Sample dissolved in 0.5 ml 0.1 M HCl and heated at 100° for 1 hr [13], or dissolved in 0.5 ml 0.5 M H_2SO_4 and heated at 100° for 4 hr [23]. (3) Sample dissolved in 0.5 ml 0.1 M NaOH and heated at 100° for 1 hr [13].

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REFERENCES

1. Noma, M., Huber, J. and Pharis, R. P. (1979) *Agric. Biol. Chem.* **43**, 1793.
2. Noma, M., Huber, J., Ernst, D. and Pharis, R. P. (1982) *Planta* **155**, 369.
3. Durley, R. C. and Pharis, R. P. (1973) *Planta* **109**, 357.
4. Kamienska, A., Durley, R. C. and Pharis, R. P. (1976) *Plant Physiol.* **58**, 68.
5. Looney, N. E., Kamienska, A., Legge, R. L. and Pharis, R. P. (1978) *Acta Hortic.* **80**, 105.
6. Pharis, R. P., Legge, R. L., Noma, M., Kaufman, P. B., Ghosheh, N. S., LaCroix, J. D. and Heller, K. (1981) *Plant Physiol.* **76**, 892.
7. Reeve, D. R., Crozier, A., Durley, R. C., Reid, D. M. and Pharis, R. P. (1975) *Plant Physiol.* **55**, 42.
8. Wample, R. L., Durley, R. C. and Pharis, R. P. (1975) *Physiol. Plant.* **35**, 273.
9. Yamane, H., Murofushi, N., Osada, H. and Takahashi, N. (1977) *Phytochemistry* **16**, 831.
10. Koshioka, M., Takeno, K., Beall, F. D. and Pharis, R. P. (1983) *Plant Physiol.* (in press)
11. Barendse, G. W. M., Van De Werken, P. H. and Takahashi, N. (1980) *J. Chromatogr.* **198**, 449.
12. Durley, R. C., Crozier, A., Pharis, R. P. and McLaughlin, G. E. (1972) *Phytochemistry* **11**, 3029.
13. Hiraga, K., Yokota, T., Murofushi, N. and Takahashi, N. (1974) *Agric. Biol. Chem.* **38**, 2511.
14. Kamada, H. and Harada, H. (1981) *Plant Cell Physiol.* **22**, 1423.
15. Nadeau, R., Rappaport, L. and Stolp, C. F. (1972) *Planta* **107**, 315.
16. Pharis, R., Jenkins, P., Aoki, H. and Sassa, T. (1981) *Aust. J. Plant Physiol.* **8**, 559.
17. Durley, R. C., Bewley, J. D., Railton, I. D. and Pharis, R. P. (1976) *Plant Physiol.* **57**, 699.
18. Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) *Exp. Cell Res.* **50**, 148.
19. Huber, J., Constabel, F. and Gamborg, O. L. (1978) *Plant Sci. Letters* **12**, 209.
20. Eskins, K. and Button, H. J. (1979) *Analyt. Chem.* **51**, 1885.
21. Koshioka, M., Harada, J., Takeno, K., Noma, M., Sassa, T., Ogiyama, K., Taylor, J. S., Rood, S. B., Legge, R. L. and Pharis, R. P. (1983) *J. Chromatogr.* **256**, 101.
22. Rivier, L., Gaskin, P., Albone, K. S. and MacMillan, J. (1981) *Phytochemistry* **20**, 687.
23. Yokota, T., Murofushi, N. and Takahashi, N. (1970) *Tetrahedron Letters* **18**, 1489.