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Formation of GA_{20} Glucosyl Conjugates in Seedlings of *Vicia faba*

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Abstract. [^{3}H]GA_{20} (1)[1], fed to *Vicia faba* seedlings, was converted to [^{3}H]GA_{20} glucosyl ester (5) and [^{3}H]GA_{20}-13-0-glucoside (6). The GA_{20} glucosyl ester (5) was identified by HPLC-RC and by GC-MS of GA_{20}-Me formed by transesterification of (5). The [^{3}H]GA_{20}-Me was crystallized to constant specific radioactivity with authentic GA_{20}-Me. On HPLC-RC the GA_{20}-13-0-glucoside (6) was shown to have the same retention time as an authentic sample. Subsequent enzymic hydrolysis gave a product with an HPLC retention time identical to that of authentic GA_{20} (1).

For a number of years the biochemistry of GAs has been investigated, and at present much information is available about the different pathways of GA biosynthesis that exist in various plants (Graebe and Ropers 1978, Sembdner et al. 1980, Sponsel 1983). It has become apparent that only a single GA may be active in its own right in each species—e.g., GA_1 (4) in *Zea mays* (Phinney and Spray 1982). This leads to the question of how the level of the active GA might be regulated, in relation to its role in plant development. Besides deactivation by 2β-hydroxylation—e.g., GA_{20} (1) → GA_{29} (2) (Sponsel 1983)—conjugation is another potential mechanism for regulating GA pool sizes, probably operating in conjunction with the biosynthetic pathway (Schneider 1983).

Furthermore, it has been argued that the taxonomic background of a plant may determine whether 2β-hydroxylation or conjugation acts as the favored process (Sponsel 1983). In *Vicia faba*, 2β-hydroxylation is thought to be a major metabolic route. However, the occurrence of GA conjugates has been postulated because hydrolysis of polar fractions of extracts gave rise to traces of free GAs (Sponsel et al. 1979, Dathe and Sembdner 1984). This paper is part of a more detailed study of the role of GA conjugation in *Vicia faba*, and

[1] Numbers in parentheses refer to formulae in Fig. 1.

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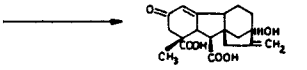

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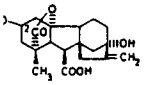

A_{29}-2-O-G (8)

GA glucosyl conjugates,

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Table 1. GC-MS data of some relevant gibberellin methyl esters (for conditions see Materials and Methods).

Gibberellin methyl ester	Retention time (min)	Characteristic ions (m/e)			
A_{20}-Me	5.2	346	314	303	286
A_4-Me	4.7	346	314	300	224
A_1-Me	9.5	362	344	330	316
A_5-Me	5.3	344	312	301	284
Transesterified neutral Fraction of Exp. 1	5.2	346	314	303	286

eluted isocratically using MeOH–0.1% H_3PO_4 mixtures. For preparative work, a Merck Lobar RP 8 column (40–63 μm particles, 25 × 250 mm) was used with MeOH-H_2O mixtures. Radioactivity in the column eluent was analyzed by fraction collection followed by scintillation counting.

Gas Chromatography–Mass Spectrometry

GC-MS analysis of GA methyl esters was performed on a Varian Mat 111 equipped with a 3% QF-1 column (Gaschrom Q, 125–160 mesh, 180 × 0.2 cm) operating isothermally with helium (10 ml/min, 230°C). Mass spectra were obtained using a 300°C source temperature at 80 eV.

Radiocounting (RC)

Samples were mixed with a POPOP/PPO/toluene cocktail and measured in a Tricarb 2660 (Packard) liquid scintillation counter. TLC plates were scanned on a Bertold TL-scanner.

Experiment Details

Experiment 1. Two and one-half milligrams of GA_{20} (1) was administered to 50 *Vicia faba* seedlings. After 24 h, cotyledons and roots were removed, and the remaining parts were homogenized and extracted twice with 80% MeOH at 4°C. The extract was evaporated, and the resulting aqueous phase was treated with PVP overnight. After filtering off the PVP and extracting with n-hexane, the aqueous phase was acidified to pH 2.5 with HCl and partitioned three times against ethyl acetate, then three times against water-saturated n-butanol. The butanol extract was neutralized with NH_3 solution (1 N) and taken to dryness *in vacuo* (310 mg). The sample was loaded onto a DEAE-Sephadex A 25 column and eluted with a stepwise gradient of acetic acid in MeOH (Gräbner et al. 1976), giving a neutral fraction (0–0.25 N acetic acid, 170 mg), a nonpolar acidic fraction (0.75–1.0 N acetic acid, 50 mg), and a polar acidic fraction (3.0–

Table 2. Partition of label from the [^{3}H]GA_{20} feed (Exp. 3)

	Label (Bq)	Percentage (%)
1. Total feed	1.5×10^5	
2. Methanolic extract	4.9×10^4	Set for 100
3. Aqueous phase after PVP and hexane	4.5×10^4	92.8
4. Hexane extract	2.8×10^3	5.7
5. Total radioactivity of the fractions from DEAE-Sephadex	4.6×10^4	93.5
6. Neutral fraction of DEAE-Sephadex	5.4×10^3	11.0
7. GA_{20}GE in 6. determined by HPLC	1.7×10^3	3.5
8. Polar acidic fraction of DEAE-Sephadex	2.5×10^3	5.1
9. GA_{20}-13-0-G in 8. determined by HPLC	1.8×10^2	0.4

10.0 N acetic acid, 10 mg), containing GA glucosyl esters, free GAs, and GA glucosides, respectively. The neutral fraction was transesterified with 0.5 N sodium methoxide (1 ml/10 mg extract) (Schneider and Lattke 1985), then partitioned between water and ethyl acetate. The resulting organic phase (33 mg) was subjected to preparative HPLC on RP 8 (MeOH/H_2O, 2:5). HPLC fractions were analyzed by TLC, and a GA_{20} methyl ester-like compound was found. This compound was identified as GA_{20}-Me by GC-MS. The acidic fractions from DEAE-Sephadex have not yet been analyzed further.

Experiment 2. Twenty-seven micrograms of [^{3}H]GA_{20} (2.33×10^6 Bq) was applied to ten seedlings, incubated for 48 h, then extracted as in Exp. 1. After the hexane step, the aqueous phase was taken to dryness (250 mg) and then subjected directly to DEAE-Sephadex A 25 chromatography, giving neutral (0–0.25 N acetic acid) and acidic fractions (1.0–10 N acetic acid). The neutral fraction (1.1×10^4 Bq) was transesterified as above, and the products were purified by TLC ($CHCl_3$/ethyl acetate/acetic acid, 70:30:5). The GA_{20}-Me zone (R_f 0.66) was eluted with MeOH and mixed with 15 mg unlabeled GA_{20}-Me. Crystallization four times from ethyl acetate/hexane resulted in constant specific radioactivity after the first recrystallization.

The polar acidic fraction (1.65×10^4 Bq) from the DEAE-Sephadex was purified by TLC ($CHCl_3$/MeOH/acetic acid/H_2O, 80:20:4:2). The GA_{20}-13-0-glucoside zone (R_f 0.61) was eluted and rechromatographed on DEAE-Sephadex. By HPLC (MeOH/0.1% H_3PO_4, 2:5) the sample was shown to contain [^{3}H]GA_{20}-13-0-glucoside (6), as coinjection with the authentic compound resulted in coincident RC and UV peaks (Fig. 3, recovered radioactivity 4.2×10^2 Bq). Part of the sample was hydrolyzed with cellulase (2 mg, 30°C, 24 h, pH 3; Schliemann and Schneider 1979). The free GAs liberated were extracted with ethyl acetate at pH 2.5 and analyzed by HPLC-RC (MeOH/0.1% H_3PO_4, 2:5; Fig. 4).

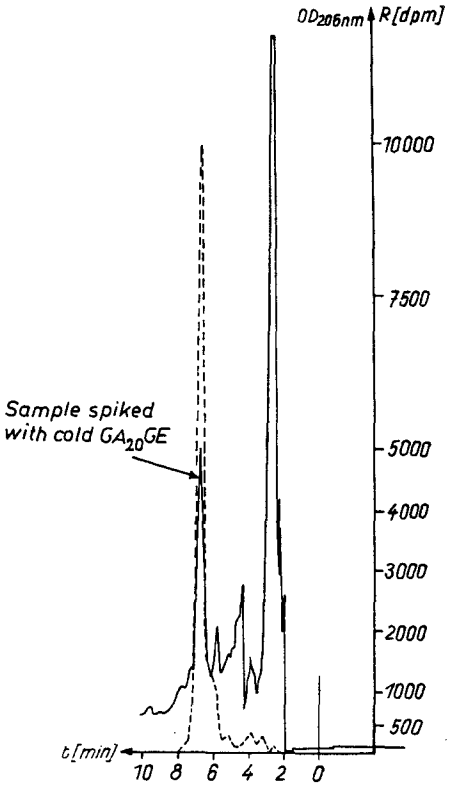

Fig. 2. HPLC-RC chromatogram of the neutral fraction from Exp. 3. Si 100 Polyol RP 18, isocratic elution with 45% MeOH/H_3PO_4 (0.1%). Sample was cochromatographed with 5 μg of cold authentic GA_{20}GE: optical density (206 mm) ——, radioactivity – – –.

Experiment 3. The volume of 13.2 μg [^{3}H]GA_{20} (1.5×10^5 Bq) was administered to ten seedlings and treated as in Exp. 2. The neutral fraction (5.4×10^3 Bq) from DEAE-Sephadex was purified by TLC (propan-2-ol/ 1 N NH_3, 5:1). The GA_{20} glucosyl ester zone (R_f 0.58) was eluted and subjected to HPLC-RC (MeOH/0.1% H_3PO_4, 9:11; Fig. 2, recovered radioactivity 1.68×10^3 Bq). The GA_{20}-13-0-glucoside fraction was treated as in Exp. 2 and confirmed the results of that experiment (recovered radioactivity 1.83×10^2 Bq). The partition of the radioactivity is shown in Table 2.

Results

Formation of GA_{20} Glucosyl Ester (5)

In the initial experiment, a very high dose of GA_{20} (1) (50 μg/seedling) was fed, and the metabolites were extracted after incubation for 24 h. From acidic butanol fraction a neutral fraction was obtained using DEAE-Sephadex. This fraction was subjected to sodium methoxide treatment which transforms presumptive esters like GA glycosyl esters into GA methyl esters (Schneider and Lattke 1985). The formation of a GA methyl ester therefore provides the first evidence for the presence of a corresponding ester—e.g., GA glycosyl ester—in the fraction. The transesterified sample was subjected to HPLC, and the fraction eluting at the time of GA_{20}-Me was analyzed by GC-MS without further

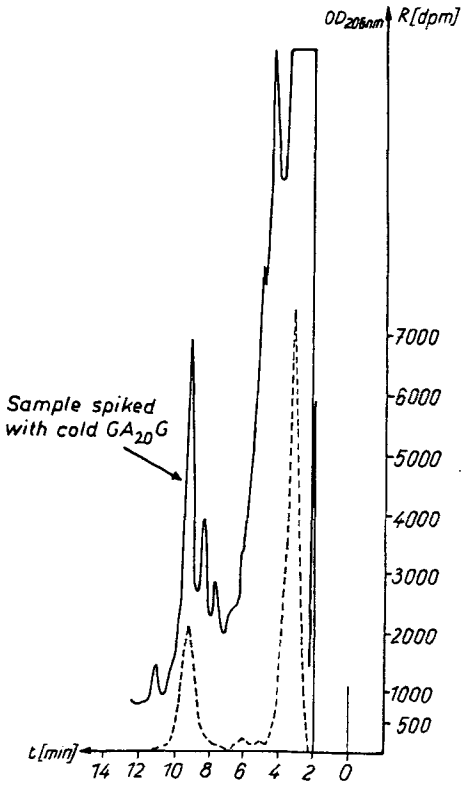

Fig. 3. HPLC-RC chromatogram of the acidic fraction from Exp. 2. Sample was cochromatographed with 5 μg of cold authentic $GA_{20}G$. Si 100 Polyol RP 18, isocratic elution with 40% MeOH/H_3PO_4 (0.1%), optical density (206 mm) ——, radioactivity – – –.

derivatization. Retention time and characteristic ions of the spectrum (Table 1) were found to be identical with those on authentic GA_{20}-Me.

This result was confirmed by Exp. 2 where [^{3}H]GA_{20} (2.33×10^2 Bq) was fed to *Vicia* seedlings for a 48-h incubation period. The neutral fraction was transesterified and then purified by TLC. To the GA_{20}-Me fraction (R_f 0.66), cold GA_{20}-Me was added and crystallized from ethyl acetate/hexane. The specific radioactivity remained constant after the first recrystallization.

Finally, the presumptive GA_{20}-glycosyl ester was identified in another feeding experiment with [^{3}H]GA_{20} (1) (1.54×10^5 Bq). In this case, the neutral fraction was purified by TLC (R_f 0.58 for GA_{20} glucosyl ester) and then analyzed by HPLC-RC. Fig. 2 shows clearly that the peak of radioactivity coincides with the UV peak of authentic GA_{20} glucosyl ester (5) added to the purified extract. The radioactivity associated with the isolated GA_{20} glucosyl ester (5) represents about 3.5% of the total extracted radioactivity.

Formation of GA_{20}-13-0-glucoside (6). Besides GA glucosyl esters, GA glucosides are likely products of GA metabolism (Schneider 1983). These compounds are known to elute in the polar acidic fraction from DEAE-Sephadex. Therefore this fraction from extracts of [^{3}H]GA_{20} (1) feeding experiments was separated by TLC. The GA_{20}-glucoside fraction (R_f 0.61) was analyzed by

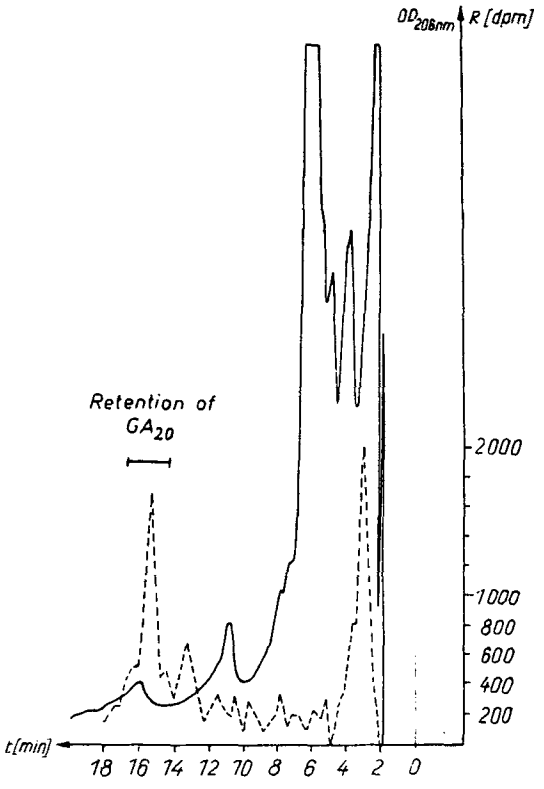

Fig. 4. HPLC-RC chromatogram of the hydrolysate obtained from the acidic fraction of Exp. 2 (see Fig. 3). Si 100 Polyol RP 18, isocratic elution with 40% MeOH/H_3PO_4 (0.1%), optical density (206 mm) ——, radioactivity – – –.

HPLC-RC (Fig. 3) and demonstrated that the radioactivity was located exactly in the peak of authentic GA_{20}-13-0-glucoside (6).

For further confirmation, an aliquot of the fraction was hydrolyzed with cellulase. By HPLC-RC analysis, the liberated GA was indeed found to be GA_{20} (1, Fig. 4). About 0.4% of the extracted radioactivity was incorporated into GA_{20}-13-0-glucoside (6).

As shown in Fig. 3, there is at least one more radioactive metabolite of high polarity, but this has yet to be identified. The metabolite was susceptible to cellulase hydrolysis, but the aglycone was found to be different from GA_{20} (1) and monohydroxy-GA_{20} derivatives—e.g., GA_{29} (2) or GA_1 (4).

Discussion

GA_{20} (1) attracts special interest because of its special position in the context of GA-biosynthesis. In *Zea mays* and possibly other plants possessing the 3β-hydroxylation pathway, GA_{20} is considered to be the last biosynthetic precursor before activation to GA_1 (4) (Phinney and Spray 1982). In *Vicia* tribes, 3β-hydroxylated GAs have not yet been found (Sponsel 1983). The occurrence of GA_{29} (2) and GA_{29}-catabolite (3) in *Vicia faba* seeds indicates the presence of one potential route of GA_{20} deactivation (Fig. 1). From the results described

above, it appears that transformation of GA_{20} (1) into the temporarily deactivated glucosyl conjugates also plays a role in controlling the level of GA_{20} (1) in *Vicia faba*.

The identification of GA_{20} glucosyl ester (5) and GA_{20}-13-0-glucoside (6) by reference to standard compounds is the first definitive evidence for the occurrence of GA_{20} glucosyl conjugates in plants, although some indications of their presence or formation have previously been reported (Yamane et al. 1977, Sponsel et al. 1979, Rood et al. 1983). Moreover, GA_{20}-13-0-glucoside (6) is, as yet, the only evidence that GA-13-0-glucosides may occur endogeneously.

At present, the comparatively low incorporation of the GA_{20} label into (5) and (6), representing 3.5% and 0.4% activity respectively, is difficult to evaluate because of losses during the purification procedure and because of the radioactivity being split into several other fractions which have not been considered here. With respect to the highly polar metabolite present in the glucoside fraction (Fig. 3), it should be remembered that GA_{20} conjugates may undergo further metabolism. Processes other than 2β-hydroxylation should also not be discounted, as the failure to detect their products could be due to small pool sizes. These questions clearly merit more detailed investigations.

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