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METABOLISM OF GIBBERELLINS IN EARLY IMMATURE BEAN SEEDS

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; identification of gibberellins A_{17}, A_{20}, A_{29} and A_{44}; metabolism of gibberellins A_1, A_4, A_5 and A_{20}.

Abstract—The endogenous free gibberellins in two different stages of immature *Phaseolus vulgaris* seeds were investigated and GA_{17}, GA_{20}, GA_{29} and GA_{44} were isolated and identified in addition to the previously characterized GA_1, GA_4, GA_5, GA_6, GA_8, GA_{37} and GA_{38}. To investigate the metabolic pathways of these endogenous gibberellins in the early immature stage, tritium labelled GA_1, GA_4, GA_5 and GA_{20} were fed to the immature seeds *ca* 10 days after anthesis and their fates were traced. GA_4 was converted to GA_8 via GA_1, and GA_{20}, to GA_1 and GA_{29} and, probably, subsequently to GA_8. The interconversion of GA_5 to known gibberellins was not observed. Apparently gibberellin glucosylating enzymes were not present in the early immature stage of the bean seeds but they appeared in the maturing process. The conversions of GA_4 to GA_4 glucosyl ester and of GA_{20} to GA_{29} were shown in the maturing bean seeds.

INTRODUCTION

It is well-known that the content of endogenous gibberellins in higher plants changes dramatically during seed development, maturation and germination [1–4]. Our objective was to identify the endogenous gibberellins of *Phaseolus vulgaris* seeds at the various developmental stages and to investigate the biosynthetic relationships of these gibberellins.

In previous papers [5–7] we reported the isolation and characterization of endogenous gibberellins and the metabolic pathways of gibberellins in the maturing process of the bean seeds and their behaviour during germination. The present paper describes an investigation of endogenous gibberellins at two different developmental stages of the bean seeds and the metabolism of those in the early immature seeds which corresponds to the period of rapid development of the embryo [1].

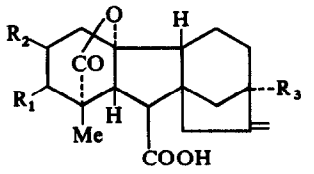
RESULTS AND DISCUSSION

Endogenous gibberellins of immature bean seeds

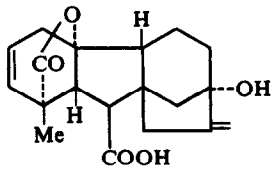
The immature bean seeds at two different developmental stages were collected. One group (group A) consisted of the early immature seeds about 10 days after anthesis (seed length, 8–10 mm; 4.2 kg). The other batch (group B) were the immature seeds about 18 days after anthesis (seed length, *ca* 15 mm; 13.5 kg). The seeds in the two groups were separately extracted and fractionated by standard procedures to give acidic ethyl acetate (AE) fractions.

The AE fraction from group A was purified by charcoal adsorption chromatography (CC), followed by PLC to afford GA_1, GA_5, GA_6, GA_8, GA_{17}, GA_{20}, GA_{29} and GA_{38}. Identifications of gibberellins were carried out by TLC, GLC, MS and GC–MS after derivatization.

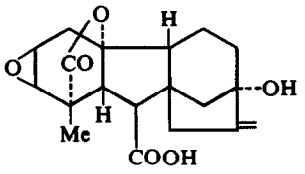
The AE fraction from group B was purified by CC,



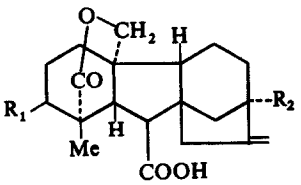
GA_1 $R_1 = R_3 = OH$, $R_2 = H$
GA_4 $R_1 = OH$, $R_2 = R_3 = H$
GA_8 $R_1 = R_2 = R_3 = OH$
GA_{20} $R_1 = R_2 = H$, $R_3 = OH$
GA_{29} $R_1 = H$, $R_2 = R_3 = OH$



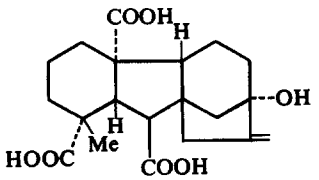
GA_5



GA_6



GA_{37} $R_1 = OH$, $R_2 = H$
GA_{38} $R_1 = R_2 = OH$
GA_{44} $R_1 = H$, $R_2 = OH$



GA_{17}

silica gel partition chromatography and subsequent PLC. Thus GA_1, GA_4, GA_5, GA_6, GA_8, GA_{20}, GA_{29}, GA_{37}, GA_{38} and GA_{44} were isolated and identified. Gibberellins identified from the immature bean seeds in this study and their yields are summarized in Table 1. Of these gibberellins, GA_{17}, GA_{20}, GA_{29} and GA_{44}, were identified for the first time in the *P. vulgaris* seeds, although GA_{17} [8] and GA_{20} [4] have been reported in the seeds of the closely related *P. coccineus*. GA_{44} (13-hydroxy GA_{15}) has been reported recently to occur in the immature seeds of *Pisum sativum* by Frydman *et al.* [9].

The above results suggest the existence of two biosynthetic pathways for gibberellins in the *Phaseolus* seeds. The identification of GA_{44}, GA_{17}, GA_{20} and GA_{29} in the seeds supports the existence of a biosynthetic route which involves C-13 hydroxylation at an early stage of gibberellin biosynthesis, as suggested by MacMillan *et al.* [10]. On the other hand, the occurrence of GA_{37} and GA_4 suggests that another pathway may exist, which does not involve C-13 hydroxylation in the early stages.

Table 1. Gibberellins identified in the immature bean seeds. Figures in parenthesis indicate the weights of isolated material

Group A (*ca* 10 days after anthesis) 4.2 kg	GA_8 (10.2 mg) GA_1 (5.2 mg) GA_{29} (3.2 mg) GA_5* = GA_{17}* (2.0 mg) minor components: GA_6, GA_{20} and GA_{38}
Group B (*ca* 18 days after anthesis) 13.5 kg	GA_8 (13.1 mg) GA_1 (12.6 mg) GA_{38} (7.3 mg) GA_{37} (3.8 mg) GA_{29} (3.3 mg) GA_4* (0.8 mg) minor components: GA_5, GA_6, GA_{20} and GA_{44}

* Weights quantified by GLC analysis.

* As described in the previous paper [7] GA_1-[^{3}H] glucoside-like conjugate and GA_8-[^{3}H] glucoside could be separated by PLC with double development (solvent system: $CHCl_3$–MeOH–HOAc–H_2O (75:20:3:2), although they appeared as a single peak on the radiochromatogram.

Metabolism of gibberellins in early immature seeds

To investigate metabolism of endogenous gibberellins during seed development, feeding experiments were conducted with tritium labelled gibberellins at the early immature stage of the bean seeds, together with some experiments which complemented the previous work using bean seeds in the maturing stage [7].

Tritium labelled GA_1, GA_4, GA_5 and GA_{20} were injected into the seeds 10 days after anthesis and the seeds were cultured aseptically in darkness at 25° on Nitch's medium [11] containing 1% of agar and some vitamins [12]. A half of the seeds treated with the radioactive gibberellins was harvested on the third day after the treatment and the rest, on the tenth day. Each sample was extracted with methanol and fractionated into an acidic ethyl acetate (AE), a neutral ethyl acetate (NE), an acidic *n*-butanol (AB), a neutral *n*-butanol (NB) fraction and an aqueous residue (Aq.). The distribution of radioactivity is summarized in Table 2.

Thin layer radioscanning and GC–RC of the AE fraction from the third day seeds treated with GA_1-[^{3}H] showed that the radioactive components consisted mainly of GA_1-[^{3}H] and GA_8-[^{3}H], the former being the major component. The radiochromatogram of the AB fraction from the third day seeds treated with GA_1-[^{3}H] indicated that the radioactive components were due to unknown products which were less polar than GA_1 glucoside-like conjugates [7] and GA_8 glucoside.

TLC of the AE fraction from the tenth day seeds treated with GA_1-[^{3}H] revealed that radioactivity was also found in the zones of GA_1-[^{3}H] and GA_8-[^{3}H]. In this case GA_1-[^{3}H] was the minor radioactive component and GA_8 [^{3}H], the major one. TLC of the AB fraction from the tenth day seeds showed the presence of a GA_1-[^{3}H] glucoside-like conjugate and GA_8-[^{3}H] glucoside* together with the above mentioned unknown products. Enzymatic hydrolysis of the zone corresponding to GA_1-[^{3}H] glucoside-like conjugate and GA_8-[^{3}H] glucoside on the radiochromatogram afforded

Table 2. Distribution (%) of radioactivity extracted from the third day and tenth day seeds treated with GA-[^{3}H]

	GA_1-[^{3}H]		GA_4-[^{3}H]		GA_{20}-[^{3}H]		GA_5-[^{3}H]	
	3rd day	10th day	3rd day	10th day	3rd day	10th day	3rd day	10th day
Total dpm recovered ($\times 10^6$)	2.84	0.56	3.42	2.52	78.8	86.4	96.0	49.2
AE fraction	68.8	68.9	63.1	50.9	68.3	58.3	85.6	8.2
GA_8	21.2	37.5	23.9	37.7	5.5	5.4	—	—
GA_{29}	—	—	—	—	13.5	10.3	—	—
GA_1	47.6	31.4	39.2	13.2	13.1	9.5	—	—
GA_4	—	—	—	—	—	—	—	—
GA_5	—	—	—	—	—	—	23.9	1.7
GA_{20}	—	—	—	—	5.7	1.7	—	—
others	—	—	—	—	30.4	31.4	61.7	6.5
NE fraction	0.6	0.3	1.9	1.3	1.5	0.9	2.5	3.8
GA_4 glucosyl ester	—	—	—	—	—	—	—	—
others	0.6	0.3	1.9	1.3	1.5	0.9	2.5	3.8
AB fraction	27.5	26.2	21.2	32.8	23.0	30.6	6.5	54.1
GA_8 glucoside	—	4.7	—	9.2	—	—	—	—
GA_1 glucoside-like conjugate	—	2.9	—	4.6	—	—	—	—
others	27.5	18.6	21.2	19.0	23.0	30.6	6.5	54.1
NB fraction	2.2	1.5	4.6	7.7	5.1	6.2	3.9	31.7
GA_1 glucosyl ester	—	0.6	—	—	—	—	—	—
others	2.2	0.9	4.6	7.7	5.1	6.2	3.9	31.7
Aq. residue	0.9	3.1	0.2	7.3	2.1	4.0	1.5	2.2

both GA_1-[^{3}H] and GA_8-[^{3}H] as aglycones, which were identified by GA–RC. In the NB fraction from the tenth day seeds, the presence of a small quantity of GA_1-[^{3}H] glucosyl ester was shown by TLC.

TLC of each fraction from the third day and tenth day seeds treated with GA_4-[^{3}H] showed that GA_4-[^{3}H] was immediately converted to GA_1-[^{3}H] after the treatment, and subsequently to GA_8-[^{3}H]. In the tenth day seeds, GA_1-[^{3}H] glucoside-like conjugate and GA_8-[^{3}H] glucoside were also present, although GA_1-[^{3}H] glucosyl ester was not detected. The presence of GA_4-[^{3}H] glucosyl ester was not observed in the NE fraction from either third day or tenth day seeds.

The AE fraction from the third day seeds treated with GA_{20}-[^{3}H] contained GA_1-[^{3}H], GA_{29}-[^{3}H] and GA_8-[^{3}H] together with an unidentified product (X_1). X_1 and GA_8-[^{3}H] showed the same R_f value on TLC even as their methyl esters, but would be separated by GC–RC as the TMS ethers of their methyl esters. Each fraction from the tenth day seeds treated with GA_{20}-[^{3}H] also contained metabolites similar to those from the third day seeds. Neither the third day nor the tenth day seeds contained the glucosyl derivatives such as GA_1-[^{3}H] glucosyl ester, GA_8-[^{3}H] glucoside and glucoside-like conjugates of GA_1-[^{3}H] and GA_{20}-[^{3}H]. Thus GA_{20}-[^{3}H] in the early immature bean seeds might be mainly metabolized to GA_1-[^{3}H] and GA_{29}-[^{3}H] and, probably, subsequently to GA_8-[^{3}H], although the conversion of GA_{29}-[^{3}H] to GA_8-[^{3}H] is not yet confirmed.

The AE fractions from both the third and tenth day seeds treated with GA_5-[^{3}H] showed three peaks on TLC. The R_f value of the least polar peak corresponded to that of authentic GA_5 and the middle peak, to that of GA_1 or GA_3, and the third peak was more polar than GA_8. GC–RC analysis of these components, separated by PLC, as the TMS ethers of the methyl esters indicated that the former peak consisted of GA_5-[^{3}H] and did not contain any GA_6-[^{3}H] and that the second peak, whose R_f value was similar to that of GA_1 or GA_3, was not in fact either of these compounds but an unknown product (X_2). The most polar peak was also due to another unknown product (X_3). It was further demonstrated by TLC that the radioactivity in the AB and NB fractions was due to unknown products. It is interesting to note that the interconversion of GA_5-[^{3}H] to other gibberellins was not observed in the early immature stage, while it was shown to be converted to GA_8-[^{3}H] in the maturing period.

It must be pointed out that in the early immature stage of the bean seeds, gibberellin glucosylating enzyme activity could not be observed and in the further culture for an additional 7 days, such enzyme activity was only shown in GA_1-[^{3}H] and GA_4-[^{3}H] treatments.* This result contrasts with the observation that in the maturing stage there is conversion of free gibberellins to the corresponding glucosyl ethers and/or esters [7]. These facts suggest that gibberellin glucosylating enzyme may develop in the maturing period.

* Gibberellin glucosyl derivatives such as GA_1-[^{3}H] glucosyl ester, GA_1-[^{3}H] glucoside-like conjugate and GA_8-[^{3}H] glucoside were not observed in the tenth day seeds treated with GA_{20}-[^{3}H], although GA_{20}-[^{3}H] was metabolized to GA_1-[^{3}H] and subsequently to GA_8-[^{3}H] in the seeds. This may be due to the slight difference of developmental stage of the seeds and/or the quantity of their precursors.

Table 3. Distribution (%) of radioactivity extracted from immature and mature seeds treated with GA-[^{3}H]

	GA_4-[^{3}H] Immature	GA_4-[^{3}H] Mature	GA_{20}-[^{3}H] Immature
Total dpm recovered ($\times 10^6$)	1.90	1.69	18.1
AE fraction	61.1	24.9	77.0
GA_8	7.2	2.1	10.2
GA_{29}	—	—	5.0
GA_1	34.3	7.4	41.8
GA_4	—	—	—
GA_{20}	—	—	13.8
others	19.6	15.4	6.2
NE fraction	6.9	3.4	1.2
GA_4 glucosyl ester	1.0	1.1	—
others	5.9	2.3	1.2
AB fraction	13.9	51.3	16.2
NB fraction	2.8	4.5	1.6
Aq. residue	15.3	15.9	4.0

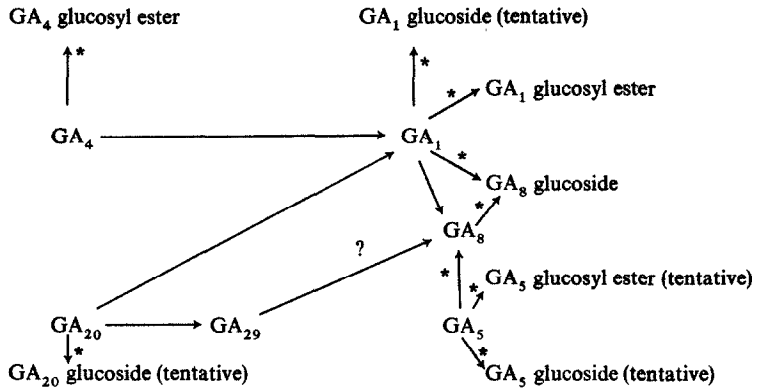
In the previous work [7], we found that GA_{20}-[^{3}H] was metabolized to GA_1-[^{3}H], GA_8-[^{3}H] and some glucosyl derivatives together with unknown products in the maturing process, but the conversion of GA_{20}-[^{3}H] to GA_{29}-[^{3}H] was not confirmed. Since a biosynthetic pathway from GA_{20} to GA_{29} was confirmed in the early immature stage and the occurrence of GA_{29} in the immature seeds was also indicated, this conversion in the seeds in the maturing period was reinvestigated. GA_{20}-[^{3}H] was directly injected into the bean seeds 18 days after anthesis. After 2 days the bean seeds treated with GA_{20}-[^{3}H] were harvested and extracted with methanol, followed by fractionation in the usual way.

TLC of each fraction exhibited almost the same results as reported previously [7]. However, GC–RC of the AE fraction showed the presence of a small quantity of GA_{29}-[^{3}H] (Table 3).

The conversion of GA_4-[^{3}H] into GA_4-[^{3}H] glucosyl ester [7] was not confirmed but this might have been due to interference by other radioactive components in the TLC analysis. Therefore the possibility of this conversion was reinvestigated. The bean seeds 18 days after anthesis were treated with GA_4-[^{3}H], and a half of the seeds were harvested on the second day after treatment (immature

Table 4. The medium used for the culture of the early immature bean seeds

$MgSO_4 \cdot 7H_2O$	125 mg/l.
$Ca(NO_3)_2 \cdot 4H_2O$	500
KH_2PO_4	125.0
KNO_3	125.0
$MnSO_4 \cdot 4H_2O$	3.0
$ZnSO_4 \cdot 7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4 \cdot 5H_2O$	0.025
$Na_2MoO_2 \cdot 2H_2O$	0.025
Fe-Citrate	10.0
Thiamine	0.25
Ca-Pantothenate	0.25
Pyridoxin	0.25
Nicotinic acid	1.25
Glycine	7.50
Sucrose	50.0 g/l.
Agar	10.0



Scheme 1. Metabolic pathways of radioactive gibberellins fed to the immature bean seeds. Conversion of GA_{29} to GA_8 has not been confirmed. The conversions indicated with an asterisk (*) were not observed in the early immature seeds.

seeds) and the rest were grown to maturity (mature seeds). The NE fractions from the immature and mature seeds thus obtained were examined on TLC developed with EtAc–$CHCl_3$–HOAc (20:8:1). The zones of R_f 0–0.1 from the chromatograms were rechromatographed developing with $CHCl_3$–MeOH (3:1). These chromatograms were divided into 10 equal zones and the radioactivity associated with each zone was determined by a liquid scintillation counting. Maximum radioactivity was observed in the zone with R_f 0.5–0.6 on each chromatogram, which indicated the occurrence of GA_4-[^{3}H] glucosyl ester in the immature and mature bean seeds. However, as shown in Table 3, the conversion of GA_4-[^{3}H] into its glucosyl ester was very low. This may be because of the rapid conversion of GA_4-[^{3}H] to GA_1-[^{3}H].

The results obtained in this work, taken together with those reported [7] in the previous paper, may be summarized as follows: (1) Radioactive gibberellins fed to the immature bean seeds were metabolized along the pathways illustrated in Scheme 1. (2) The conversions of free gibberellins in the seeds at the early immature and maturing stages are similar. However, in the early immature seeds, gibberellin glucosyl ether and/or ester synthetases are not present and they are thought to develop during the maturing process of the seeds. This was further supported by the facts that GA_8 accumulated in the early immature seeds and its quantity in the mature seeds was relatively small. For example, Hiraga *et al.* [5] isolated 10 mg of GA_1 and 1 mg of GA_8 from 100 kg of the mature seeds of *Phaseolus vulgaris* although GA_8 was the main endogenous gibberellin in the early immature seeds. (3) The interconversions of GA_1, GA_5 and GA_6 in the bean seeds, which might be expected to occur in view of their structural relationship, have not yet been observed.

EXPERIMENTAL

MS were obtained by a direct inlet system at 70 eV and 150–200°. GLC was conducted on glass columns (1 m × 3 mm) packed with 2% OV-1, SE-30, XE-60 or QF-1, detection by FID. GC–MS was carried out on a glass column packed with 2% SE-30 (1 m × 3 mm). The He flow was 30 ml/min and a Watson–Biemann separator was employed. Bioassay was carried out using a dwarf rice, *Oriza sativa* L., Tan-ginbozu by the water culture method.

Isolation and identification of gibberellins in the immature seeds. Plant materials. Two groups of the immature seeds of *P. vulgaris* cv. Kentucky Wonder were used. One group (group A, 4.2 kg) consisted of the seeds *ca* 10 days after anthesis, the others (group B, 13.5 kg) *ca* 18 days after anthesis. *Extraction and fractionation.* Each group was separately extracted × 3 with MeOH. Extracts were evaporated *in vacuo* to give aq. residues, which were fractionated by the procedure described previously [7]. *Purification of the AE fraction from group A.* The AE fraction (3.3 g) was dissolved in 50 ml of 20% aq. Me_2CO and applied to a charcoal column (35 g), which was eluted with increasing amounts of Me_2CO in H_2O by 5% steps for every 1 l. from 30–70%. Each fraction from the charcoal column was purified by PLC (adsorbent; Si gel G, solvent; C_6H_6–Me_2CO–HOAc, 70:25:5). Thus from the 50% aq. Me_2CO fraction, GA_1, GA_{29} and GA_8 were isolated and from the 55% aq. Me_2CO fraction, GA_1 and GA_{38}: from the 60% aq. Me_2CO fraction, GA_6 and GA_{20}; from the 65% aq. Me_2CO fraction, GA_5 and GA_{20}; from the 70% aq. Me_2CO fraction, GA_{17} (Table 1). Identification of these gibberellins was carried out by GLC (2% SE-30, 2% QF-1 and 2% XE-60) and MS after methylation and subsequent trimethylsilylation.

Purification of the AE fraction from group B. Charcoal adsorption chromatography. The AE fraction (3.5 g) was dissolved in 50 ml 20% aq. Me_2CO and applied to a charcoal column (40 g), which was eluted with increasing amounts of aq. Me_2CO by 5% steps for every 1 l. from 20–70%, and by 10% steps for every 1 l. from 70–100%. Based on the biological activity on rice seedling and TLC, active eluates were combined to afford two fractions; fraction *a* (426 mg) was the eluate with 35–50% aq. Me_2CO and fraction *b* (499 mg) was the eluates with 50–100% aq. Me_2CO. *Purification of fraction a.* Fraction *a* was adsorbed on Celite (1 g) and placed on a column packed with Si gel (8 g) impregnated with 0.5 M aq. HCOOH [13]. The column was eluted with EtOAc–*n*-hexane, increasing the EtOAc content by 5% steps for every 30 ml from 0–30% and 60–100%. From 30–60%, the column was eluted with increasing amounts of EtOAc in hexane by 2.5% steps for every 30 ml. The combined fractions (47.5:52.5 to 52.5:47.5) were purified by PLC to give GA_1, which was identified by MS after methylation. The fractions (55:45 to 65:35) were subjected to repeated PLC to give GA_{38} and GA_{29} which were identified by GLC (SE-30 and XE-60) and MS after methylation and subsequent trimethylsilylation. GA_8 was obtained by PLC from the fractions eluted with EtOAc–hexane (70:30 to 80:20), and identified by MS of its methyl ester and its methyl ester TMS ether. *Purification of fraction b.* Fraction *b* was purified by silicic acid partition column chromatography by the same procedure as before. The column was eluted with EtOAc–hexane, increasing the EtOAc content by 3% steps for every 50 ml from 0–30% and 5% steps from 30–60%. From the 9–15% EtOAc fractions GA_4 was identified and quantified by

GC–MS (SE-30, column temp. 210°) after purification by PLC and subsequent methylation. The 18–21% fractions were purified by PLC and analysed by GLC (SE-30 and XE-60) and MS after methylation. The presence of GA_{37}, GA_5, GA_6 and GA_{20} in these fractions was confirmed. The 35–45% EtAc fractions were subjected to PLC to give GA_{44}, which was identified by GLC (SE-30 and XE-60) and MS after methylation. The yields of gibberellins identified are summarized in Table 1.

Feeding experiments. Plant materials. P. vulgaris cv. Kentucky Wonder was used. Ammonium salts of tritium labelled gibberellins were dissolved in H_2O. A 0.5 μl aliquot of the soln was injected by microsyringe into husked seeds *ca* 10 days after anthesis which were then cultured aseptically in darkness at 25° on Nitch's medium containing 1% agar and some vitamins (Table 4). Preparations of GA_1-[^{3}H], GA_5-[^{3}H], GA_8-[^{3}H] and GA_{20}-[^{3}H] had already been reported [14]. GA_4-[^{3}H] was kindly supplied by Professor R. P. Pharis of the University of Calgary. Sp. radioactivities and quantities of radioactive gibberellins fed to the bean seeds were as follows: GA_1-[^{3}H], 2.4 mCi/mM, 0.23 μCi/seed; GA_4-[^{3}H], 0.6 Ci/mM, 0.38 μCi/seed; GA_5-[^{3}H], 5.3 Ci/mM, 14 μCi/seed; GA_{20}-[^{3}H], 3.2 Ci/mM, 14 μCi/seed. A half (5 seeds) of the seeds treated with radioactive gibberellins was harvested on the third day after injection and the rest, on the tenth day. Complementary feeding experiments using maturing bean seeds 18 days after anthesis were carried out by the procedure described previously [7]. GA_4-[^{3}H], 0.6 Ci/mM, 0.4 μCi/seed; GA_{20}-[^{3}H], 3.2 Ci/mM, 14 μCi/seed). *Extraction and fractionation.* 5 seeds from each treatment (4 seeds for the complementary feeding experiment) were extracted with MeOH and fractionated into 5 fractions (AE, NE, AB, NB and Aq.) by the procedure described above. TLC was on Si gel G, the solvent systems employed were: for fraction AE, EtOAc–$CHCl_3$–HOAc (20:8:1); for fractions NE and NB, $CHCl_3$–MeOH (3:1); for fraction AB, $CHCl_3$–MeOH–HOAc–H_2O (45:15:3:2). Radioactivity was determined by liquid scintillation counting using Bray's scintillator [15]. The location of radioactivity on TLC plates was determined by a radiochromatogram scanner. GC–RC was carried out on a glass column packed with 2% SE-30 or QF-1. Mass peaks were detected by a FID and the resulting effluents were trapped directly into nonione–toluene scintillator [7] and subsequently assayed by liquid scintillation counting. *Enzymatic hydrolysis.* Gibberellin glucoside-like conjugates from the AB fractions were hydrolysed with cellulase (Sigma) in 0.2 M acetate buffer (pH 4) by the procedure described previously [16].

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