GIBBERELLIN METABOLISM IN SEEDS AND SEEDLINGS OF THE RUNNER BEAN, *PHASEOLUS COCCINEUS* L.

By

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SUMMARY

An investigation was made of GA metabolism in *Phaseolus coccineus* L., cv. Prizewinner using an *in vitro* preparation from immature seeds. GA_{12}-aldehyde underwent non-13-hydroxylated conversion to GA_4 via GA_{12}, GA_{15}, GA_{24}, GA_{36} and GA_{37} and was metabolised to GA_1 via a 13-hydroxylated pathway encompassing GA_{19}, GA_{20}, GA_{44} and GA_{53}. GA_4 was not 13-hydroxylated to produce GA_1. Key incubations were performed using an *in vitro* preparation from immature seeds of a dwarf variety, Hammonds Dwarf Scarlet, but no metabolic differences were observed between dwarf and tall cultivars. GA_1 was not 2β-hydroxylated to form GA_8.

The nature of the 3β-hydroxylating enzyme from immature seeds was investigated by fast protein liquid chromatography (FPLC) separation of the crude cell-free preparations from Prizewinner and Hammonds Dwarf Scarlet. Following assay of the FPLC fractions, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of zones which converted $[^3H]GA_{20}$ to $[^3H]GA_1$ was carried out. Preliminary analysis revealed similarities between the polypeptides of both cultivars.

In order to investigate GA metabolism in seedlings, $[^3H]$-labelled GAs were applied to cvs. Prizewinner and Hammonds Dwarf Scarlet. In contrast to metabolism by the cell-free preparations from immature seeds, $[^3H]GA_4$, as well as $[^3H]GA_{20}$, was converted to $[^3H]GA_1$ which was further converted to $[^3H]GA_8$, in seedlings of each variety. It was concluded that the dwarf growth habit exhibited by seedlings of Hammonds Dwarf Scarlet was not simply a consequence of an inability to synthesise GA_1. A study of the endogenous GA_{20} content of the dwarf and tall seedlings showed mean values of 1.14 ng g^{-1} fresh weight and 0.95 ng g^{-1} fresh weight, respectively.

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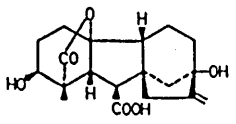
INTRODUCTION

Gibberellins (GAs) are a group of over 70 structurally-related diterpenoid acids (Fig. 1), which occur naturally in plants, in varying quantities and at all stages of the life cycle. Of the species studied to date, none contains all the known GAs and an endogenous complement of 10-15 is quite typical. GAs are implicated as controllers of developmental processes in plants as their exogenous application affects, for example, stem elongation, flowering, the setting of parthenocarpic fruit and senescence (see Crozier, 1983).

The biosynthetic pathway from mevalonic acid (MVA) to *ent*-kaurene produces precursors of sterols, carotenoids, abscisic acid and chlorophyll esters, in addition to those of GAs, (Fig. 2) (see Coolbaugh, 1983). Beyond *ent*-kaurene, a series of oxidation steps occurs to produce *ent*-7α-hydroxykaurenoic acid, the direct precursor of the first formed GA, GA_{12}-aldehyde (Fig. 3) (see Hedden, 1983).

Structurally, GAs are based on the *ent*-gibberellane skeleton (Fig. 4) and have either 19 or 20 carbon atoms. C_{20}-GAs are the metabolic precursors of C_{19}-GAs and, in general, exhibit lower biological activity (Reeve and Crozier, 1974). Metabolic conversions tend to involve oxidation at, or loss of carbon-20, hydroxylations at C-2, C-3, C-11, C-12, C-13, C-15 and C-16, introduction of 1-2 and 2-3 double bonds and C-7 oxidation. Clearly, starting with over 70 related compounds, a large number of theoretically feasible interconversion pathways exist. Figure 5 represents the endogenous GAs of a range of angiosperm species and shows the hypothetical conversions relevant to the following treatise.

Much of this GA biosynthetic information became available as a result of *in vitro* metabolic studies. Preparations are made from plant tissue and incubated with cofactors, buffers, and usually, a radio-labelled or stable, heavy isotope-labelled substrate whose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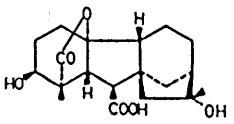
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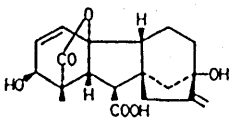
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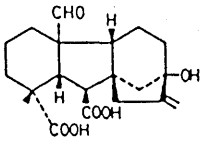
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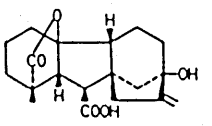
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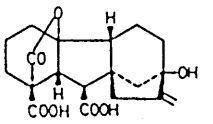
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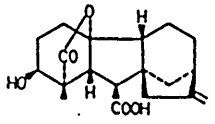
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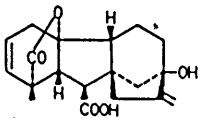
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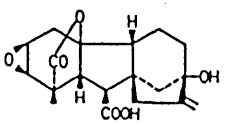
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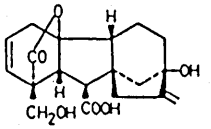
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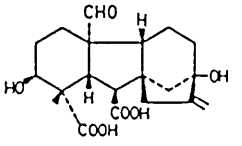
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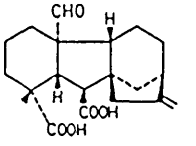
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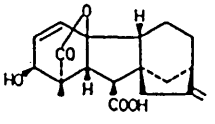
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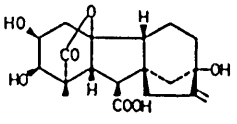
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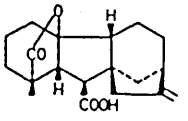
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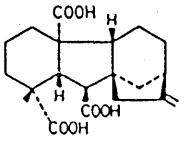
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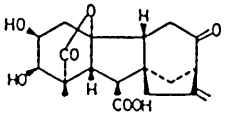
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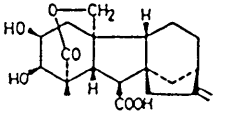
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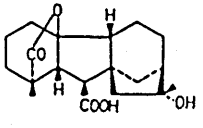
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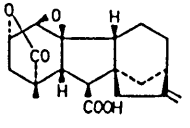
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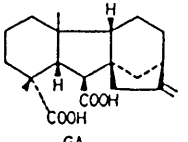
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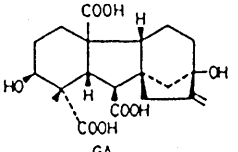
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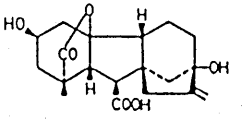
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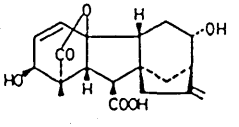
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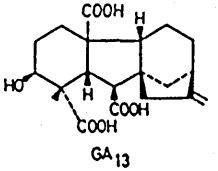
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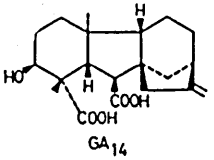
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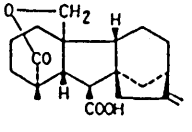
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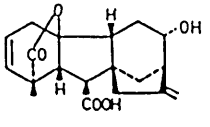
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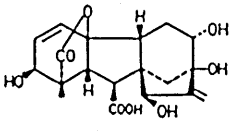
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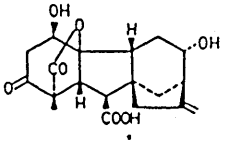
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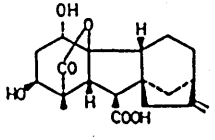
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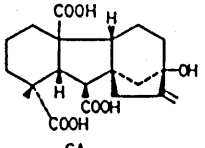
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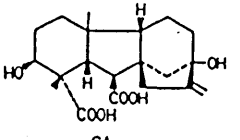
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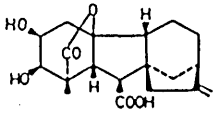
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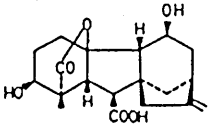
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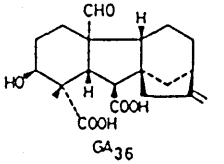
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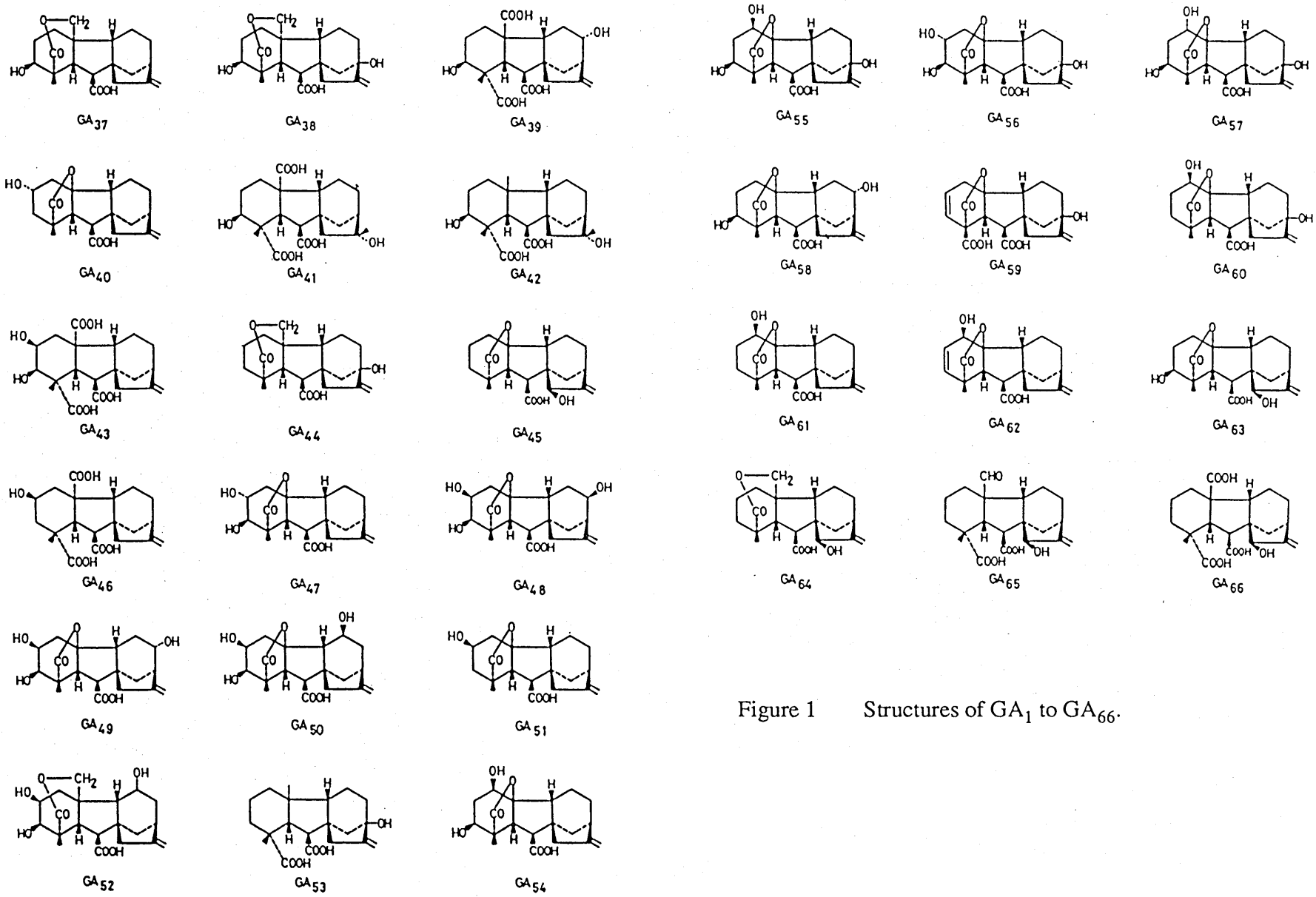


Figure 1 Structures of GA_1 to GA_{66}.

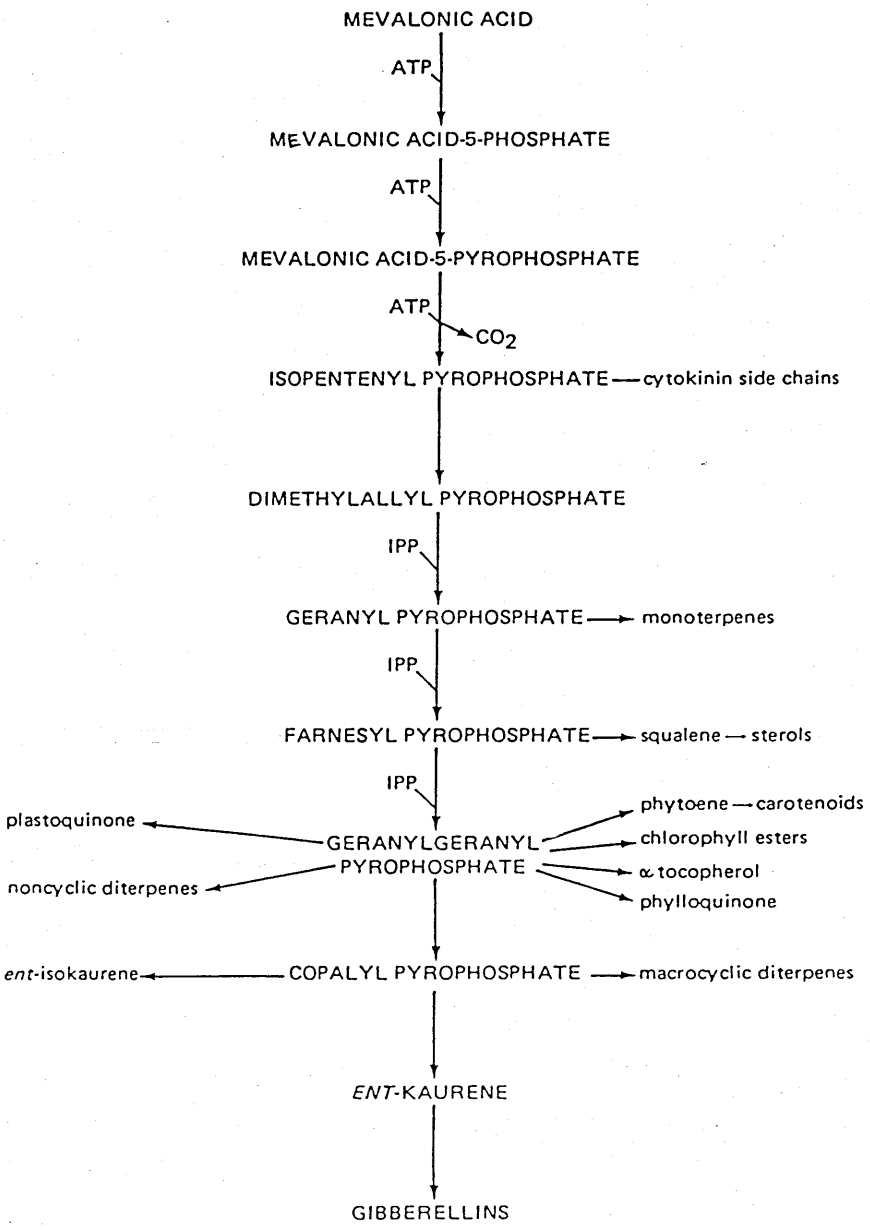


Figure 2 Biosynthesis of the GAs.

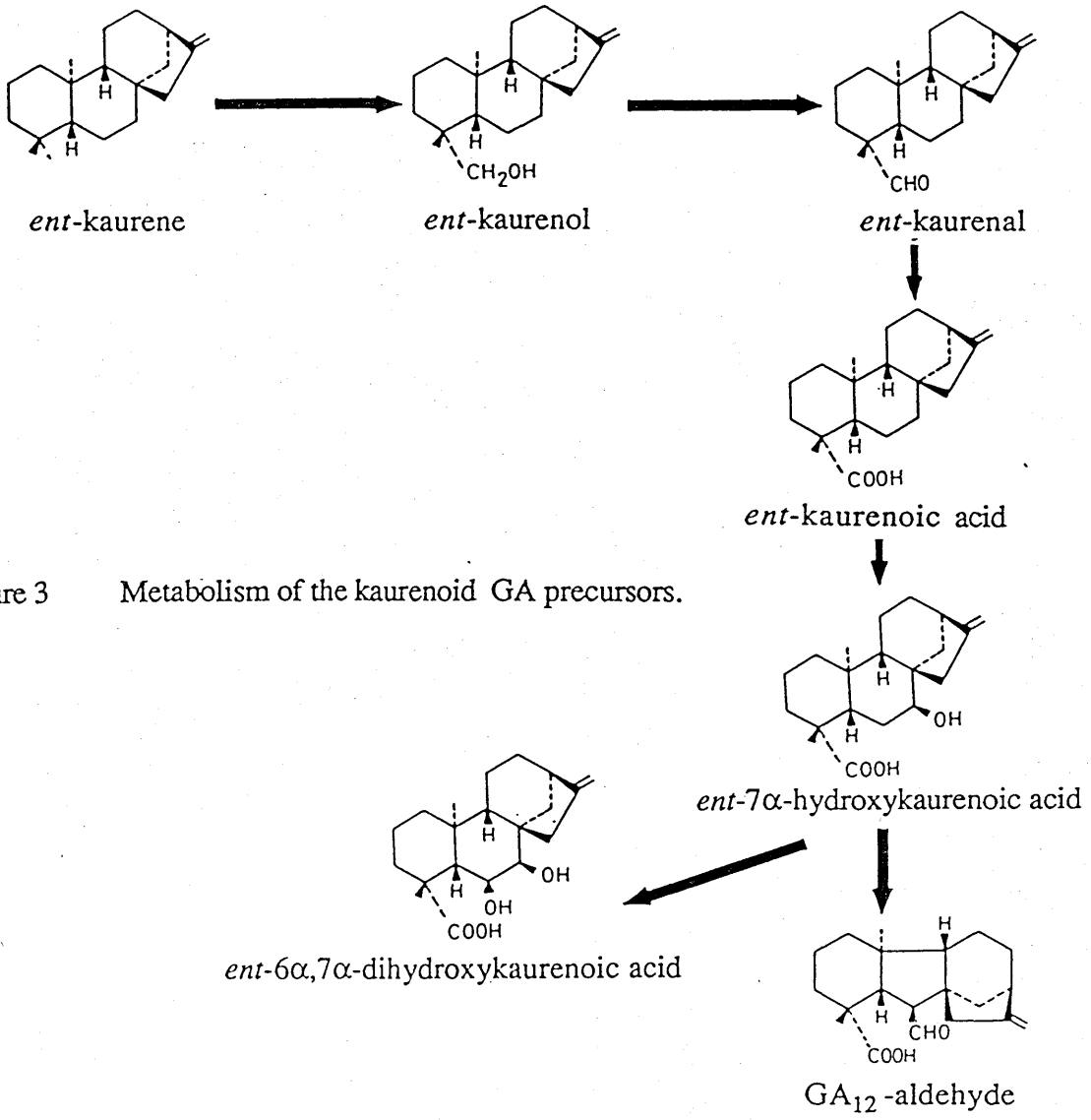
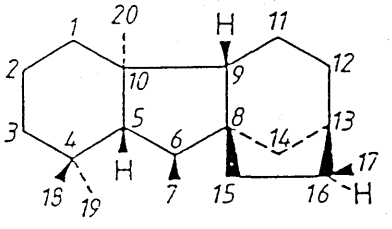


Figure 3 Metabolism of the kaurenoid GA precursors.



ent-gibberellane

Figure 4 The *ent*-gibberellane skeleton.

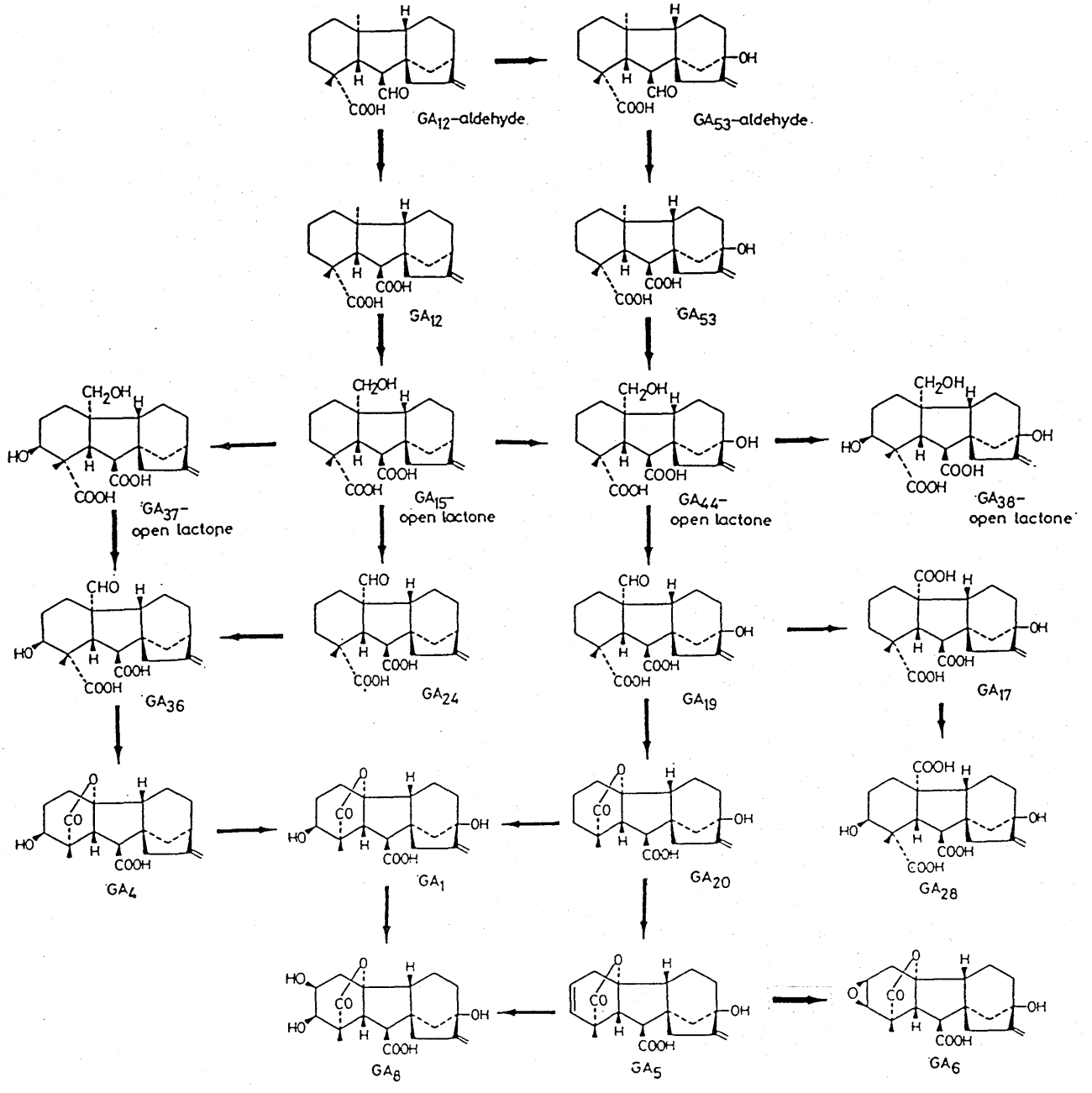


Figure 5 Hypothetical pathways of GA metabolism

products can be identified by high performance liquid chromatography (HPLC) or by combined gas chromatography-mass spectrometry (GC-MS) (see Hedden, 1983). The obvious disadvantage of this technique is the disruption caused to the plant tissue which makes direct correlation between *in vitro* results and the whole plant metabolism no more than tentative. However, when this limitation is borne in mind, cell-free techniques provide the opportunity to optimise conditions for individual metabolic steps, resulting in elucidation of entire metabolic pathways. The researcher then knows the metabolic "capabilities" of a tissue and is in a better position to approach qualitative and ultimately quantitative questions using *in vivo* systems.

The value of *in vitro* studies can be appreciated by considering the work of Graebe *et al.* (1965) who obtained labelled *ent*-kaurene, *ent*-kaurenol and *trans*-geranylgeraniol from MVA (Figs. 2 and 3). This was the first demonstration of cell-free conversions and used a strained homogenised preparation of the Californian wild cucumber (*Marah macrocarpus*[1]) liquid endosperm, with ATP as a cofactor. Further experimentation with this tissue produced labelled *ent*-kaurenol and *ent*-kaurenoic acid from MVA (Dennis and West, 1967), providing supporting evidence for the scheme shown in Fig. 3.

The problems associated with the relatively low conversions of substrate with the cell-free system from *Marah* were overcome with the discovery that liquid endosperm of the pumpkin (*Cucurbita maxima*[2]) metabolised MVA to *ent*-kaurene with a yield of 40% (Graebe, 1969) when incubated with the following cofactors, $MgCl_2$, $MnCl_2$, PEP, pyruvate kinase and ATP (Graebe, 1968). The high enzyme activity of this preparation may reflect the generally rapid turnover and large endogenous GA pools usually associated with seed tissues.

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1 Originally referred to as *Echinocystis macrocarpa*

2 Originally referred to as *Cucurbita pepo*

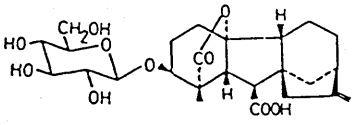
The *in vitro* formation of GA_{12}-aldehyde from *ent*-kaurene was first elucidated by Graebe *et al.* (1972) using the *C. maxima* system and is now known to be common to many species. For example, preparations from suspensors and immature seeds of *Phaseolus coccineus* metabolise [^{14}C]MVA to a number of products, including, *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, *ent*-kaurenoic acid and *ent*-7α-hydroxykaurenoic acid respectively (Ceccarelli *et al.*, 1979, 1981a; Turnbull *et al.*,1986a). This pathway has also been confirmed for *Hordeum distichon* and *Pisum sativum* with germinating grain and immature seed cell-free systems respectively (Murphy and Briggs,1975; Ropers *et al.*,1978).

In vitro systems have been used to investigate GA metabolism as well as biosynthesis in higher plants. Graebe *et al.* (1974a) found that the [^{14}C]GA_{12}-aldehyde produced from [^{14}C]MVA, could be further converted to [^{14}C]GA_{12}, [^{14}C]GA_{15}, [^{14}C]GA_{37}, [^{14}C]GA_{24} and [^{14}C]GA_{36} if Mn^{++} was omitted from the incubation mixture. The same *C. maxima* system was responsible for the production of GA_4, the first C_{19}-GA to accumulate under *in vitro* conditions (Graebe *et al.*,1974b).

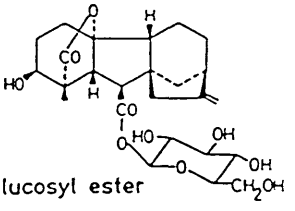
A cell-free preparation of germinating French bean seeds (*Phaseolus vulgaris*) metabolised GA_1 to GA_8 (Patterson and Rappaport, 1974), thus endorsing the view that *in vitro* studies can reflect whole tissue metabolism as this conversion was later found to occur *in vivo* in maturing and germinating *P. vulgaris* seeds (Yamane *et al.*,1975). Recently, seed preparations of this species have been used to demonstrate the conversion of GA_{20} to GA_1, GA_5 and GA_{29} (Kamiya *et al.*,1984) and to provide more information on the endogenous GAs of this tissue. In addition to GA_1, GA_4, GA_5, GA_6, GA_8, GA_{17}, GA_{20}, GA_{29}, GA_{37} and GA_{38} which are known endogenous components of this species (see Sponsel, 1983), GA_9, GA_{12}, GA_{15}, GA_{19}, GA_{23}, GA_{24} and GA_{53} were identified by combined GC-MS (Takahashi *et al.*, 1986). The hypothetical

metabolic relationships between many of these GAs are shown in Fig. 5.

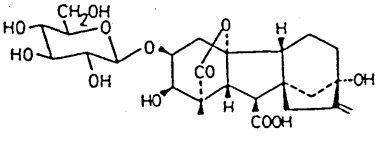
The following are endogenous GAs of seed of the closely related *Phaseolus coccineus* ; GA_1, GA_3, GA_4, GA_5, GA_6, GA_8, GA_8-catabolite, GA_{17}, GA_{19}, GA_{20}, GA_{28}, GA_{29}, GA_{34}, GA_{37}, GA_{38} GA_{44} (see Sponsel 1983; Albone *et al.*, 1984). and GA_8-2-*O*-glucoside, (see Schneider, 1983). In addition, GA_1, GA_8, GA_4-3-*O*-glucoside, GA_4 glucosyl ester, GA_8-2-*O*-glucoside and a GA_{34}-glucoside, probably GA_{34}-2-*O*-glucoside (Turnbull *et al.*, 1986b) have been identified as metabolites of [^{3}H]GA4 in *Phaseolus coccineus* seedlings.



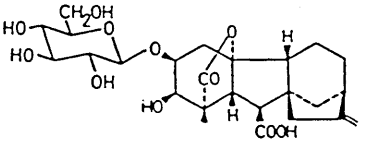
GA_4-3-O-glucoside



GA_4-glucosyl ester



GA_8-2-O-glucoside



GA_{34}-O-glucoside

Metabolism studies with *Phaseolus coccineus* have produced similar results to those obtained with *Phaseolus vulgaris*. Incubation of [^{14}C]*ent*-7α-hydroxykaurenoic acid with a suspensor preparation produced labelled GA_1, GA_5 and GA_8 but gave no indication as to the identities of the C-20 intermediates (Ceccarelli *et al.*,1981b). However, an immature seed preparation of *Phaseolus coccineus* metabolised [^{14}C]GA_{12}-aldehyde to GA_1, GA_4, GA_5, GA_6, GA_{15}, GA_{17}, GA_{19}, GA_{20}, GA_{24}, GA_{37}, GA_{44} and GA_{53}-aldehyde, suggesting that the metabolic scheme in Fig. 5 may also operate in this species (Turnbull *et al.*,1985).

Incubations of a *Pisum sativum* embryo preparation with [^{14}C]GA_{12} produced metabolites that implicated two distinct pathways leading to the C_{19}-GAs (Kamiya and Graebe, 1983). 13-Hydroxylation of [^{14}C]GA_{12} produced [^{14}C]GA_{53}, [^{14}C]GA_{44}, [^{14}C]GA_{19}, [^{14}C]GA_{20} and [^{14}C]GA_{29} sequentially, while C-20 oxidation of the substrate formed [^{14}C]GA_{15}, [^{14}C]GA_{24}, [^{14}C]GA_9 and [^{14}C]GA_{51}. It is interesting to note that despite being endogenous to pea seedlings (Gaskin *et al.*,1985), GA_1 was not observed in this study.

In summary, many angiosperm species have GAs in common with each other and evidence from *in vitro* metabolic studies suggests common routes of conversion. The possibility that similar metabolic enzymes exist in a variety of species is being investigated in many laboratories using *in vitro* systems but the underlying developmental role of the resultant GAs has yet to be confirmed.

Cell-free experiments have provided details on the nature and cofactor requirements of the enzymes responsible for GA biosynthesis and metabolism. The enzymes catalysing the conversions of MVA to *ent*-kaurene are soluble while those involved in *ent*-kaurene oxidation, leading to GA_{12}-aldehyde are microsomal (see Coolbaugh, 1983; Hedden, 1983). Most of the GA metabolic steps are associated with soluble enzymes but 13-hydroxylation is membrane-bound. This information came from assays of *in vitro* preparations after they had been subjected to varying degrees of centrifugation. Activity that is retained after centrifugation at 200,000-g is regarded as soluble while that retained after a 2,000-g, but prior to a 200,000-g spin is microsomal (see Hedden, 1983).

[^{14}C]GA_{12}-aldehyde is maximally converted in *C. maxima* incubations that contain Fe^{++}, 2-oxoglutarate, NADPH, O_2 and ascorbate (Hedden and Graebe, 1982). One possible explanation for these cofactor requirements is that an enzyme-bound $Fe{\cdot}O_2$ complex oxidises 2-oxoglutarate to form an oxonium species which then oxidises the GA (see Hedden and Graebe, 1982). Ascorbate, which enhances GA turnover but which is not stringently required, may

prevent oxidation of SH groups at the active site or may reduce Fe^{+++} to Fe^{++} which is the form required for enzyme activity.

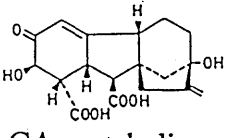
It is interesting to note that Mn^{++} is a cofactor for MVA incubations but inhibits the metabolism of GA_{12}-aldehyde by *C. maxima* preparations (Graebe *et al.*,1974a). This inhibition can be reversed by the addition of Fe^{++} and may demonstrate competition for binding sites. The pH of the incubation medium also exerts an effect on the metabolism of GA_{12}-aldehyde in *C. maxima*. Incubations carried out below pH 6.5 produce 12α-hydroxy GA_{12}-aldehyde and a series of 12α-hydroxylated GAs, while preparations above pH 6.5 yield GA_{12} (Hedden *et al.*,1984).

Kamiya and Graebe (1983) incubated [^{14}C]GA_{12} with microsomal and with soluble preparations of *Pisum sativum* immature seed. The soluble fraction displayed the Fe^{++}, 2-oxoglutarate and ascorbate requirements observed with *C. maxima* and gave rise to a series of GAs oxidised at C-20 and hydroxylated at C-2, while the microsomal incubation required O_2 and NADPH and produced C-20 oxidised, 2β- and 13-hydroxylated GAs. The conclusion that the 13-hydroxylation step is membrane-associated led Takahashi *et al.* (1986) to suggest that *in vivo*, this conversion is likely to occur early in GA metabolism, as steps prior to GA_{12}-aldehyde production are also microsomal. Further studies with this system revealed that loss of carbon-20 is from a C-20 aldehyde, directly as CO_2 rather than via formic acid (Kamiya *et al.*,1986).

Kamiya *et al.* (1984), studied the mechanism of metabolism of GA_{20} to GA_1, GA_5 and GA_{29} with respect to the standard cofactor requirement, using an *in vitro* system from immature seed of *P. vulgaris*. They propose that an Fe-O-C complex is formed at carbon-3 of GA_{20}, which, when cleaved between Fe and O, produces GA_1. In contrast, cleavage between O and C introduces a 2-3 double bond and yields GA_5. Patterson and Rappaport (1974), demonstrated the conversion of GA_1 to GA_8 in *Phaseolus vulgaris*, by an enzyme that

remained in the supernatant after centrifugation at 95,000-g. Inhibition of this reaction by the cation-binding EDTA concurs with the general findings of an Fe^{++} requirement. Further study of the 2β-hydroxylase from mature seed revealed that the enzyme has a molecular weight of 36 kilo daltons (kd) and the characteristics of a dioxygenase (Smith and MacMillan, 1984). This enzyme was active on a number of substrates, namely, GA_1, GA_4, GA_9 and GA_{20} but showed a higher conversion rate when fed 3β-hydroxylated substrates.

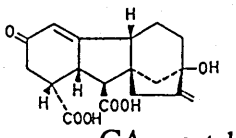
Although little is known about the compartmentation of GAs on a cellular level, there is some evidence for the predominance of 2β-hydroxylated GAs in the testas of seeds. Takahashi *et al.* (1986) reported an absence of 2β-hydroxylation by *in vitro* systems of embryos from immature seed of *Phaseolus vulgaris*. However, when endogenous GAs of embryos and testas were examined, GA_8 and GA_{29} were present in both, but the testas contained much higher levels. Similarly, GA_8 and a GA_8-catabolite are the major GA components of the testas of maturing *Phaseolus coccineus* seeds (Albone *et al.*, 1984).



GA_8-catabolite

Further evidence for GA compartmentation came from the finding that GA_{20} and GA_{29} were present in the cotyledons of mature *Pisum* seeds (see Sponsel, 1983). After synthesis in the cotyledons, GA_{29} is transported to the testa and is converted to a GA_{29}-catabolite which is then transported to the embryo during germination. Conjugates, typically GA glucosyl esters and ethers are of widespread occurrence (see Schneider, 1983). Although the biological activity of conjugates and GA catabolites is much lower than that of free GAs, these

compounds may regulate physiological processes by altering the pool of available plant growth substance.



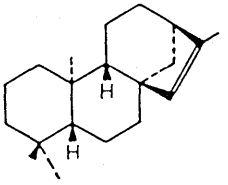
GA_{29}-catabolite

As can be appreciated from this account, the likely GA metabolic conversions for a given set of endogenous compounds are fairly well understood but little is known of the nature of the responsible enzymes. However, having attained a certain information level in the field of GA metabolism, it is of interest to attempt to correlate this knowledge with the regulation of a particular developmental process.

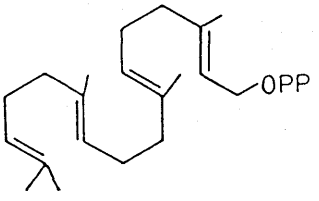
Exogenous GA applications have long been known to increase stem elongation rate but the biochemistry of tissue perception and subsequent growth is not understood. Research in this area has centred on comparison of GA metabolism, endogenous content and effect of exogenous application in tall and dwarf cultivars of several crop species.

Phinney and Spray (1982), hypothesised that in maize (*Zea mays*) and possibly other angiosperms, GA_1 might be the only GA active *per se* in promoting shoot growth. That GA_{17}, GA_{19}, GA_{20}, GA_{44}, GA_{53} and in lower quantities, GA_1, GA_8 and GA_{29} are among the endogenous compounds of tall maize tassels (Hedden *et al.*,1982) led to the assumption that the early 13-hydroxylation pathway (see Fig. 5) might be occurring in this species. An investigation of GA metabolism in several maize mutants, in which dwarfism is expressed from germination onwards, suggested that these cultivars are dwarfed as a consequence of blocks in this biosynthetic pathway leading to GA_1. The four dwarves considered here have non-allelic recessive genes, namely, *d*5, *d*2, *d*3 and *d*1 (Emerson *et al.*, 1935)

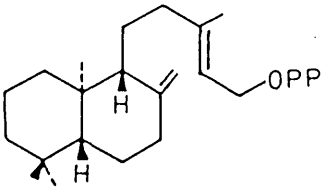
There is strong evidence that *d*5 causes dwarfism by preventing synthesis of *ent*-kaurene and hence GAs. This was first proposed because of the observations that GA_1, GA_{20} and kaurenoid GA precursors promoted growth of *dwarf*-5 seedlings while pre-*ent*-kaurene GA precursors were inactive (Katsumi *et al.*,1964; Phinney and Spray, 1982). More direct evidence has been obtained with cell-free preparations from etiolated shoots of *dwarf*-5 and tall plants which produce different metabolites when fed [^{14}C]MVA, [^{14}C]geranylgeranyl pyrophosphate and [^{3}H]copalyl pyrophosphate (Hedden and Phinney, 1979). All the substrates were converted to *ent*-kaurene by tall extracts while *dwarf*-5 extracts yielded predominantly *iso*-kaurene, which, unlike *ent*-kaurene, is not a GA precursor. The mutation would appear to result in proton loss from carbon-15 in the dwarf, leading to alteration of the double bond position. However, it should be noted that the tall extract did produce some *iso*-kaurene while the dwarf also yielded *ent*-kaurene, albeit less than 25% of the tall. Although the *d*5 gene is "leaky", it is not sufficiently so to result in endogenous dilution of the [^{3}H,^{13}C]GA_1 and [^{3}H,^{13}C]GA_{29} extracted from *dwarf*-5 seedlings fed with [^{3}H,^{13}C]GA_{20} (Spray *et al.*, 1984).



ent-isokaurene



Geranylgeranyl pyrophoshate



Copalyl pyrophosphate

The conclusion that the *d*5 mutation blocks *ent*-kaurene

production implies that application of GAs beyond the block will result in a normal growth habit due to their conversion to GA_1. However, the only metabolites present in detectable amounts after feeding $[^{14}C]GA_{53}$ to tassels of *dwarf*-5 plants were $[^{14}C]GA_{44}$, $[^{14}C]GA_{19}$, $[^{14}C]GA_{29}$ and conjugated $[^{14}C]GA_{53}$ (Heupel *et al.*,1985). One explanation may lie in the very low endogenous levels of GA_1 and GA_{20} in normal tassels (Hedden *et al.*, 1982), implying turnover rates so slow that metabolism of exogenous GAs would be negligible.

The growth of dwarves with the *d*2 gene is enhanced by treatment with GA_1, GA_{20} and GA_{53} but not GA_{53}-aldehyde and GA_{12}-aldehyde. This implies that the mutation blocks oxidation of the 7-CHO group (Phinney and Spray, 1982).

The *d*3 gene appears to prevent 13-hydroxylation of GA_{12}-aldehyde to GA_{53}-aldehyde and/or GA_{12} to GA_{53}. This is shown by the promotion of growth of *d*2 mutants by GA_{53}-aldehyde, GA_{53}, GA_{20} and GA_1 and the inactivity of GA_{12}-aldehyde and *ent*-kaurene (Phinney and Spray, 1982).

Dwarf-1 plants respond by normal growth to applications of GA_1, whereas GA_{20} does not elicit a marked response (Phinney and Spray, 1982). These results led to the conclusion that only GA_1 is biologically active *per se* and that other GAs are active only via their conversion to GA_1. The probability that the *d*1 mutation affects the plants' capacity for 3β-hydroxylation was further investigated by Spray *et al.* (1984). $[^3H,^{13}C]GA_{20}$ fed to *dwarf*-1 plants was converted to $[^3H,^{13}C]GA_{29}$ and to a $[^3H,^{13}C]GA_{29}$ catabolite but not to $[^3H,^{13}C]GA_1$, as in tall plants. The presence of ^{12}C dilution in the products of these feeds suggests that these findings reflect endogenous GA metabolism.

The presence of GA_1 (Ingram *et al.*,1983), GA_{17}, GA_{19}, GA_{20}, GA_{29} and a GA_{29}-catabolite (Davies *et al.*, 1982), as endogenous compounds of pea (*Pisum sativum*) seedlings infers the operation of the early 13-hydroxylation pathway. Initial work on stem elongation

in *P. sativum* concentrated on the *Le* gene locus; plants with the genotypes *Le/le* and *Le/Le* being tall, while *le/le* plants are dwarfed from the third internode onwards. Extracts of both tall and dwarf were found to contain GA_{20}-like activity (Potts *et al.*,1982). It was therefore suggested that the *Le* gene codes for the 3β-hydroxylation of GA_{20}, leading to the biologically active GA_1. *Le* and *le* plants, both of which were extreme dwarves (*compactum*) due to possession of an additional dwarfing allele *na*, were fed with [^{3}H,^{13}C]GA_{20} and the resultant extracts subjected to bioassay. The *Le*-containing line showed a polar peak with GA_1-like biological activity while the *le* line did not (Potts and Reid, 1983). This supported the proposed role for *Le* in the enzymatic conversion of GA_{20} to GA_1. Further positive evidence appeared with the identification, by combined GC-MS, of endogenous GA_1 from *Le* plants but not from *le* plants (Ingram *et al.*,1983).

The role of the *Na* gene was investigated and that of the *Le* gene further clarified by Ingram *et al.* (1984). Tall (*Le*) plants converted [^{3}H,^{13}C]GA_{20} to [^{3}H,^{13}C]GA_1 while dwarf (*le*) plants did not. Plants with the *na* allele were hypothesised to contain no endogenous GAs due to their inability to complete successfully an early step in synthesis. [^{3}H,^{13}C]GA_{20} fed to *Na* and to *na* plants was converted to [^{3}H,^{13}C]GA_1 in both but analysis confirmed that there was no dilution of the isotopic label by endogenous GA_1 in the *na* extract. Work by Ingram and Reid (1987), suggests that *na* plants may be unable to synthesise GA_{12}-aldehyde from *ent*-7α-hydroxykaurenoic acid (seeFig. 3). When this compound is fed, extracts yield side branch products, for example, 6α,7α-dihydroxykaurenoic acid, rather than GAs. Applications of labelled GA_{12}-aldehyde to *na* plants produce a range of biologically active GAs including GA_{20} and GA_1.

The evidence, so far, shows an analogous situation in peas and maize. Both species possess a gene whose homozygous recessive allele blocks 3β-hydroxylation of GA_{20} to GA_1 and a gene which controls a step in GA biosynthesis prior to the formation of GA_{12}-

aldehyde. However, further investigation revealed that the situation is not as clear as anticipated in *Pisum sativum*.

Two commercial pea cultivars, Alaska (*Le*) and Progress No. 9 (*le*) were grown in darkness and received applications of exogenous GA_{20} (Sponsel,1986). When dark-grown, both varieties displayed the tall habit and both metabolised GA_{20} to GA_1. Furthermore, the endogenous GA_1 contents of dark-grown Alaska and Progress No. 9 seedlings were similar (Gaskin *et al.*,1985). These findings are in contrast to those of Reid (1983) who studied the same gene but in different varieties and who found no loss of dwarf characteristics in the dark. This discrepancy suggests that possession of the dwarf genotype is not in itself sufficient to reduce the rate of stem elongation and that light may also exert an influence. It has been proposed that possession of the *le* allele results in a P_{fr}-mediated inhibition of 3β-hydroxylation (Campbell and Bonner, 1986). This inhibition is not manifested in darkness due to the predominance of the P_r form of phytochrome. The situation is further complicated by the results of GA_1 quantitation in red light-grown seedlings of Alaska and Progress No. 9 with both tall and dwarf varieties containing endogenous GA_1, but in levels too low to quantify (Sponsel,1986). This concurs with the findings of Ingram *et al.* (1986) who obtained [^{3}H,^{13}C]-labelled GA_1, GA_8 and GA_8-catabolite from *na/Le* and *na/le* seedlings fed with [^{3}H,^{13}C]GA_{20}. This suggests that either the *Le* gene is "leaky", but effective, or that any red light control of stem elongation is associated with the response to, rather than at the production of GA_1. It is possible that the tall growth habit and capacity for GA_1 synthesis demonstrated by dark-grown Progress No. 9 seedlings may be due to the expression of alleles other than *le*. The value of using isogenic lines where the only variable between cultivars is the allele under study is stressed by Reid (1987).

Shoots of GA-deficient dwarves of the genotype *na/Le* were fed with [^{13}C]GA_{20} and, on extraction, yielded [^{13}C]GA_1, [^{13}C]GA_8,

[^{13}C]GA_{29} and [^{13}C]GA_{29}-catabolite (Ingram *et al.*, 1985). Identifications of the products were by GC-MS and only the [^{13}C]GA_{29}-catabolite contained dilution with the endogenous ^{12}C isotope, said to have arisen from the mature seed. When the same feed was applied to root tissue, small quantities of [^{13}C]GA_1 and [^{13}C]GA_8 accumulated, while [^{13}C]GA_8- and [^{13}C]GA_{29}-catabolites were more abundant and showed a strong endogenous contribution in the mass spectra. These data suggest that the *na* allele is operational only in the shoots as the roots contain endogenous GA pools. It can also be deduced that no root to shoot GA transport is occurring. If it were, GA_1 or its precursors, originating from the roots, could be exported to the shoots and *na/Le* plants would be tall.

A third *Le* allele, namely le^d, has recently been described (Ross and Reid, 1987). These plants are extreme dwarves, phenotypically indistinguishable from *compactum* (*nana*) individuals and it has been suggested that the le^d allele further inhibits the GA_{20} to GA_1 step.

In peas, a number of other genes also influence stem elongation (Jolly *et al.*,1987). Their identities are as follows: *Le, Lh, Ls, Na, La, Cry, Lk, Lm, Lw.* Interactions of the different alleles of these genes result in variable phenotype which makes it difficult to conceive of an explanation of their regulatory role, in terms of GA metabolism. The three familiar phenotypes of tall (*Le*), dwarf (*le*) and *compactum* (*na*) are altered by their combination with recessive alleles of these genes.

Possession of *la/cry*^{c}, the so-called "*crypto*" phenotype, makes both tall and dwarf plants taller, while *la/cry*^{s} results in extremely tall, "*slender*" mutants, irrespective of whether *Le* or *le* is present. An *Le* plant which also has *lm* becomes a "*microtall*" while *le/lm* is a "*microdwarf*". The combination of *le /la /cry*^{c} with *lm* produces a "*microcryptodwarf*" while *la /cry*^{s}*/lm* is a "*microslender*".

Na in the form *nana*, is epistatic to *Le* and is partially epistatic

to *Lw*. *Na/Lw* plants are tall, *Na/lw* plants are dwarf, *na/Lw* and *na/lw* plants are both *compactum* with *na/lw* being smaller than *na/Lw*. The combination of *le* with *lw* seems to have a cumulative effect as Le/Lw is tall, *Le/lw* and *le/Lw* are both dwarf but *le/lw* is *compactum*. The probable effects of *le* and *na* have been discussed and the little that is known of the biochemical influence of the other genes will be dealt with later.

GA_1 and GA_{19} were identified by combined GC-MS as endogenous compounds of tall rice (*Oryza sativa*) plants, (Kurogochi *et al.*,1978, 1979; Murofushi, 1983). It is therefore possible that the early 13-hydroxylation pathway leading to GA_1 is operational and that the importance of this GA for stem elongation, first hypothesised for maize, may also apply in this species.

Two rice cultivars, *Tanginbozu* and *Waito-C* have dwarfing genes d_x and d_y respectively. Like the maize mutants, these plants are considered GA dwarves but in this case, phenotype is expressed from early stem elongation onwards. *Tanginbozu* seems comparable to the *dwarf*-5 of maize, as GAs and GA precursors beyond *ent*-kaurene cause an increase in height, with GA_1 being most effective (Murakami, 1972). Bioassay data suggested that *Tanginbozu* contained no GA-like compounds (Murakami, 1972) but more recent analyses of d_x dwarf extracts by GC-MS revealed the presence of GA_1 with minor quantities estimated by bioassay (Suzuki *et al.*,1981). This is in agreement with a set of preliminary data where *Tanginbozu* was found, by HPLC-immunoassay, to contain about 20% of the endogenous GA_1 level of *Ginbozu*, the tall cultivar (Martin,1985).

Waito-C is similar to *Zea dwarf*-1 and to Alaska as it responds by normal growth to GA_1 but not to GA_{20} application. Extracts of d_y plants were active when assayed on *Tanginbozu* but not when tested on *Waito-C* seedlings, thus supporting the claim that the d_y mutation prevents GA_1 synthesis (Murakami, 1972). However, GA_1

was found in extracts of *Waito-C* analysed by HPLC-radioimmunoassay and feeds of [2,3-^{3}H]GA_{20} to this dwarf resulted in GA_1 production (Martin, 1985). These contradictory data imply that either the hypothesised role of GAs and in particular GA_1, is inappropriate for *Oryza sativa* or that d_x and d_y are "leaky" but still have sufficient metabolic effect to induce dwarfism.

Of the dwarfing genes so far discussed, the maize evidence best upholds the hypothesis that dwarfism is simply a consequence of lack of endogenous GA_1 (Phinney and Spray, 1982). While studies of the *le* and *na* genes of pea lend some credence to this proposal, the situation for *le* dwarves and for the d_x and d_y dwarves of rice requires further explanation. These three genes do not totally prevent GA_1 synthesis and for *le*, the control of dwarfism is further complicated by the influence of red light.

There are several dwarfing genes of wheat (*Triticum aestivum*), for example, *Rht*1, *Rht*2, *Rht*3 which, in contrast to maize, pea and rice, have dominant mutant alleles. In the case of *Rht*3, seedlings are insensitive to exogenous GA application, yet take up and metabolise GA_3 like tall plants (Ho *et al.*,1981). *Rht*3 dwarves were reported to contain ten times the concentration of free GAs as talls, as determined by bioassay, (Radley, 1970) and thirteen times the content of endogenous GA_1, as measured by radioimmunoassay (Stoddart, 1984).

Evidence from the *slender* mutant i.e. *la/cry^S* of pea, is hard to reconcile with the current hypothesis for stem elongation control. Extracts of plants with the genotypes *le/la/Cry/Na/Lm* (dwarf) and *le/la/cry^S/Na/Lm* (*slender*) were tested in the lettuce hypocotyl and *Tanginbozu* rice bioassays (Potts *et al.*, 1985) and the large *slender* mutant yielded much less GA-like activity than the dwarf. The fact that *slender* plants seem to have low levels of endogenous GAs and do not show marked response to exogenous application, yet are taller than normal plants, does not support a correlation

between high GA concentration and growth. In an attempt to explain this, Graebe (1987) offered the hypothesis that the accumulation of GA_1 to a certain unspecified concentration, triggers increased 2β-hydroxylation which reduces the GA_1 content due to its conversion to GA_8. He cites *slender* as an example of this accelerated turnover and suggests that it then leads to a low estimate of apparent endogenous GA_1, but an increased stem elongation rate. That dwarf plants demonstrate reduced internode length relative to tall plants, he explains by their failure to synthesise sufficient GA_1 to attain the target concentration. However, when it is remembered that *slender* plants, which also have the *na* allele, are without detectable endogenous GA_1(Potts *et al.*, 1985), this hypothesis does not appear to withstand scrutiny. The conclusion of Ingram and Reid (1987), who confirmed the biochemical expression of *na* and *le* with *la/cry*^{S}, that "GA levels do not affect internode extension in *slender*", would seem the more feasible viewpoint.

Just as it was proposed that GAs are active only via their conversion to GA_1, it can also be suggested that GA_1 is capable of promoting growth only if it triggers a series of biochemical steps, the first being its interaction with the "correct" receptor. As yet, the nature of this sequence of events is not known, but Jolly *et al.* (1987) have developed this idea to explain the effects of the numerous pea mutant genes that do not fit the simplest GA hypothesis. They propose three possible sites at which these alleles can exert their effects. They suggest that *lh, le, na* and *ls* affect GA synthesis/metabolism due to the low levels of endogenous GA_1 in plants of these genotypes. In comparison, they propose that *la/cry*^{S} may affect sensitivity by reducing tissue perception of the growth substance. Alternatively, the *slender* and giant mutations may act by mimicking the effect of GA saturation at the binding sites. The fact that slender plants which also have *na* (no extractable biological activity) are taller than tall plants, would tend to support this idea.

The third possibility is partial or total suppression of the response to GA_1, at the post receptor stage, with *lw* and *lk* possibly acting in this way. Like the *rht*3/*Rht*3 of wheat, *lw* plants do not have lower GA levels than *Lw* plants, implying that lack of GA is not the cause of dwarfism.

In summary, the role of GAs in stem elongation is far from clear and further studies of their biochemical effects at the cellular level are required. It will also be of interest to ascertain whether GAs have a universal role by investigating their status in as many species as possible.

The existence of a dwarf mutant of the runner bean (*Phaseolus coccineus*) has allowed the range of species under study to be extended. This dwarf (Hammonds Dwarf Scarlet), has a single mutation from the tall variety (Prizewinner) and responds to exogenous GA_4 application with normal growth (Malcolm, unpublished data). Results of $[^{14}C]GA_{12}$-aldehyde feeding experiments to an *in vitro* seed preparation of Prizewinner suggested that both "early" and "late" 13-hydroxylation pathways may occur in this species. This is supported by reports that tall runner bean seedlings contain endogenous GA_1, GA_4, GA_5 and GA_{20} (Bowen *et al.*,1973) and convert $[^3H]GA_4$ to $[^3H]GA_1$ (Crozier and Reeve, 1977). The possibility that production of, or response to, GA_1 is important for stem elongation in *Phaseolus coccineus* prompted investigation of the GA status in seeds and seedlings of tall and dwarf cultivars.

MATERIALS AND METHODS

Plant material

The runner bean, *Phaseolus coccineus* L., varieties Prizewinner (Charles Sharpe and Co., Sleaford) and Hammonds Dwarf Scarlet (Thomas Daggs and Son, Glasgow) were field-grown at Garscube Estate, Glasgow during the summers of 1984, 1985 and 1986. Pods containing immature seeds, *ca.* 15-25 mm in length, were harvested at weekly intervals between September and November of each year.

Seeds of Prizewinner and Hammonds Dwarf Scarlet were imbibed in aerated running tap water for 24 h after which the testas were removed and the seeds placed on moist paper under a 16 h photoperiod at 20°C. GA metabolism experiments were carried out with 7-day-old seedlings while estimates of endogenous GA content utilised 10-day-old material. Light was supplied by "warm white" and "daylight" fluorescent tubes (Osram Ltd., Glasgow, UK) with a radiation flux of *ca.* 50 W m^{-2} at plant height.

In vitro GA metabolism in seeds

Crude enzyme preparations

Immature beans were removed from their pods, size graded (5-10 mm, 10-20 mm, >20 mm) and chilled on ice after removing and discarding the testas. The seeds were ground in chilled phosphate buffer (0.05 M, pH 8.0, 1 ml g^{-1}) with a mortar and pestle. After filtration through four layers of muslin, the total enzyme preparation was centrifuged at 2,000-g for 10 min at 2°C. The supernatant was frozen in liquid nitrogen and freeze-dried. The powdered total

enzyme preparation, (S-1), was stored over silica gel at -20°C prior to screening of individual preparations for activity by incubation with a radio-labelled GA substrate. In general, preparations from seed 10-20 mm in length yielded highest enzyme activity and were, therefore, used routinely .

In vitro incubations

Incubations contained 5-10 mg of S-1 enzyme preparation suspended in 100µl phosphate buffer (0.05 M, pH 7.4). Cofactors were Fe^{++} (0.5 mM), ascorbate (5 mM), 2-oxoglutarate (5 mM) and NADPH (1 mM). Bovine serum albumin (BSA), (20 mg ml^{-1}) was also added as protection against protease activity. Mixtures were stored on ice prior to the addition of the isotopically-labelled GA substrate. After gentle mixing, incubation was carried out in a shaking water bath at 30°C, typically for a 2 h period.

Isotopically-labelled GAs

[^{14}C]GA_{12} (5.5 x 10^{12} Bq mol^{-1}) and [^{14}C]GA_{12}-aldehyde (5.7 x 10^{12} Bq mol^{-1}) were produced from incubation of R-[2-^{14}C]MVA (1.96 x 10^{12} Bq mol^{-1}) (Amersham International, Amersham, UK) with cell-free preparations from *C. maxima* liquid endosperm. [^{14}C]GA_{53} (4.6 x 10^{12} Bq mol^{-1}) was produced from incubation of [^{14}C]GA_{12} (5.5 x 10^{12} Bq mol^{-1}) with cell-free preparation from *Phaseolus coccineus* seed. [^{3}H]GA_{15} was also produced by the *C. maxima* cell-free system.

[1,2-^{3}H]GA_1 (1.1 x 10^{15} Bq mol^{-1}) and [1,2-^{3}H]GA_4 (1.4 x 10^{15} Bq mol^{-1}) were supplied by Amersham International, (Amersham, UK) while [1-^{3}H]GA_5 (1.9 x 10^{14} Bq mol^{-1}) and [2,3-^{3}H]GA_{20} (5.3 x 10^{13} Bq mol^{-1}) were a gift from Professor R.P. Pharis. [17-^{3}H]GA_{14} (2.2 x 10^{12} Bq mol^{-1}) was synthesised in Glasgow by Dr. T. Yokota. [15,17-^{2}H$_3$]GA_{19} (34% enrichment) was a gift from Dr. Y. Kamiya while [^{2}H$_2$]GA_{20} was donated by Mr. E. Jensen and Dr. G. Schneider.

[^{3}H]GA_{24} and [^{3}H]GA_{36} were produced from incubation of [^{3}H]GA_{12}-aldehyde (from [^{3}H]MVA, 2.32 x 10^{16} Bq mol^{-1}) with the cell-free system from *C. maxima*. Following thin-layer chromatography, these [^{3}H]GAs were identified by reversed- and normal-phase HPLC of the free acids and their methoxycoumaryl ester derivatives. GAs were further purified by reversed-phase HPLC prior to use.

Extraction

At the end of the incubation period, HPLC-grade methanol (Rathburn Chemicals, Walkerburn, Scotland) was added up to 40% total volume and the mixture centrifuged at 13,000-g for 2 min. The supernatant was decanted and the pellet resuspended in 50 µl of 50 % methanol and re-centrifuged. The supernatants were mixed and acidified with 10 µl glacial acetic acid prior to further centrifugation at 13,000-g for 2 min, after which the supernatant was subjected to HPLC.

Cellulase hydrolysis

Cellulase from *Aspergillus niger* (Sigma Chemical Company Ltd, Poole, UK), 10 mg ml^{-1}, suspended in sodium citrate buffer (0.1 M, pH 4.5) was dialysed (dialysis tubing-visking size 2, Medicell International Ltd., London, UK), for 24 h at 4°C, then decanted and stored at 4°C. To hydrolyse suspected GA conjugates, the sample was dissolved in 100 µl distilled H_2O and 200 µl cellulase preparation added prior to incubation at 37°C for 24 h. Methanol was added and the sample was acidified with 10 µl glacial acetic acid before analysis by HPLC.

Fast protein liquid chromatography

Prior to fast protein liquid chromatography (FPLC) separation, *ca.* 150 mg of S-1 preparation were taken up in10 ml, 20mM Tris-HCl buffer (pH 7.5), gently shaken and passed through a 0.22 µm Millipore filter (Millipore Corporation, Massachusetts, USA). The FPLC instrumentation comprised a Pharmacia Gradient Programmer GP-250, (Pharmacia, Uppsala, Sweden) and two Pharmacia P-500 pumps which delivered mobile phase at a flow rate of 1 ml min^{-1}, with samples being introduced off-column via a Pharmacia V-7 valve with a 10 ml Superloop. A column 5 x 50 mm i.d. packed with a 10 µm Mono-Q support, eluted in the gradient mode with varying ratios of Na Cl (1 M) in Tris-HCl buffer (20 mM, pH 7.5), was used for anion-exchange separations. Both buffer and salt solutions contained 1.0 mM benzamidine and 0.4 mM dithiothreitol which respectively inhibit protease activity and prevent oxidation. Column eluates were directed to a Pharmacia Single Path UV-1 Monitor, set at 280 nm, before being collected as 0.5-1.0 ml fractions by a Pharmacia Frac-100 Collector. Aliquots of each fraction were then incubated with cofactors and radio-labelled GA substrate at 30^0C for 2 h prior to HPLC analysis, to assess post-FPLC enzyme activity.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

After FPLC separation and assay for 3β-hydroxylation capacity, aliquots of active regions from the semi-purified S-1 preparations from tall and dwarf cultivars were examined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The general method was as described by De Martini *et al.* (1978), employing slab gels.

Glass plates (200 x 185 mm) are clamped together with a 1.5 mm

space between and the edges sealed with 2% agarose. A "plug" of 18 % polyacrylamide gel solution is poured to 1 cm from the base to prevent leakage. Above this, the resolving gel mixture (11.5 % polyacrylamide), is poured to within 4 cm of the top and covered with a layer of the stacking gel. Samples are prepared by addition of an equal volume of boiling solution before treatment at 100^0C for 1.5 min.

Standard protein mixtures were run adjacent to samples to provide an indication of the relationship between molecular weight and mobility in each gel system. The standard mixture comprised, bovine serum albumin (BSA) 68 kd (Fraction V, Sigma Chemical Company, Poole, UK), alcohol dehydrogenase 41 kd (baker's yeast, BDH Chemicals, Poole, UK), myoglobin 17.2 kd (horse heart, salt-free, BDH Chemicals, Poole, UK) and cytochrome C 12.2 kd (horse heart type ll-A, Sigma Chemical Company, Poole, UK). After loading the wells, the apparatus was connected by leads to a power pack such that the proteins migrated towards the anode ie. the lower reservoir. The gel is electrophoresed at 9 mA for 16 h. When the dye front reached the base of the plate, gels were placed in staining solution for over 2 h, then in destaining solution.

Buffers and gels

Lower gel buffer: 1.5 M Tris-HCl, 0.4 % SDS, pH 8.8.

Plug Gel (18% polyacrylamide): 1.6 ml lower gel buffer, 3.0 ml acrylamide, 1.8 ml H_2O, 0.16 ml 20 % NaCl, 50 µl 10% ammonium persulphate, 12.5 µl temed.

Resolving gel (11% polyacrylamide): 9.8 ml lower gel buffer , 11.2 ml acrylamide, 3.0 ml 65 % sucrose, 0.98 ml 20 % NaCl, 15.0 ml H_2O, 100µl 10 % APS, 25µl temed.

Stacking gel (6% polyacrylamide): 2.5 ml upper gel buffer, 1.5 ml acrylamide, 6.0 ml H_2O, 80 µl10 % ammonium persulphate, 10µl temed.

Upper gel buffer: 0.5 M Tris-HCl, 0.4% SDS, pH 6.8.

Boiling solution: 62.5 mM Tris-HCl, pH 6.8. 10% glycerol, 2% SDS, 0.01% bromophenol blue, 5% ß-mercaptoethanol or 5 mM dithiothreitol (DTT).

Running buffer: 6.0 g Tris base, 28.8 g glycine, 1.0 g SDS, pH 8.3, final volume of 1 l.

Staining solution: 0.25% coomassie blue, 50% methanol, 10% acetic acid.

Destaining solution: 10% methanol, 10% acetic acid.

In vivo GA metabolism in seedlings

Application of radio-labelled GAs

7-Day-old seedlings were injected with [^{3}H]-labelled GA in 2 µl 50% aqueous methanol at the base of the epicotyl. After 5 h, whole seedlings were immersed in liquid N_2 prior to storage at -20°C.

Extraction and partitioning

Seedlings, minus cotyledons, in which little or no radioactivity accumulates (Turnbull and Crozier, 1988) were macerated in methanol (10 ml g^{-1} fresh weight) in a Waring blender, before filtration. The methanolic extract was reduced to *ca.* 20 ml *in vacuo* and an equal volume of phosphate buffer (0.5 M, pH 8.0) added. The extract was partitioned three times with an equal volume of petroleum ether. The aqueous phase was passed through cellulose powder and adjusted to pH 2.5. The extract was

partitioned five times with 2/5 volumes of ethyl acetate and the organic layer retained. After freezing and filtration to remove water, the organic phase was reduced to dryness *in vacuo.*

C_{18}Sep-Pak

A C_{18} Sep-Pak (Waters Associates, Massachusetts, USA) cartridge was primed by elution with 5 ml HPLC-grade methanol followed by 5 ml phosphate buffer (0.1 M, pH 2.5). The acidic ethyl acetate extract was loaded onto the cartridge in 5 ml 20% methanol in phosphate buffer (0.1 M, pH 2.5) and the GAs were eluted with 5 ml of 70% methanol.

Estimation of endogenous GA content

Extraction and partitioning

Plant tissue was harvested and weighed then immersed in methanol and deuterated $[^2H_2]GA_{20}$ was added prior to homogenisation. The methanolic extract was reduced to the aqueous phase prior to addition of an equal volume of phosphate buffer (0.1 M, pH 8.0). The aqueous extract was then partitioned three times against equal volumes of hexane, before being slurried with polyvinylpoly-pyrrolidone (50 mg ml^{-1}), for 1h. After filtration the extract was adjusted to pH 2.5 with 50% aqueous H_2SO_4 and partitioned five times with 2/5 volumes of ethyl acetate. The combined ethyl acetate fractions were frozen, filtered and reduced to dryness *in vacuo* prior to purification by Sephadex DEAE-25.

Sephadex DEAE-25 anion-exchange chromatography

Purification by anion-exchange chromatography was carried out on a 40 x 100 mm i.d. column containing Sephadex DEAE-25 (Pharmacia, Sweden) in the acetate form, equilibrated by elution

with 4 bed volumes (320 ml) 0.2 N acetic acid/methanol (1:1). Samples were loaded on to the column in 40 ml 0.2 N acetic acid/methanol (1:1) and washed in with a further 80 ml of this mixture before GAs were eluted with 3-4 bed volumes (240-320 ml) 2 N acetic acid/methanol (1:1). GA-containing eluate was reduced to dryness *in vacuo* prior to preparative HPLC.

High performance liquid chromatography

A Spectra Physics (San Jose, California, USA) SP 8700 liquid chromatograph was used to deliver mobile phase at a flow rate of 1 ml min^{-1}, with samples being introduced off-column via a Rheodyne (Berkeley, California, USA) 7125 valve with a 500 µl loop. A 250 x 5 mm i.d. column packed with a 5 µm ODS Hypersil support (Shandon, Runcorn, UK), eluted in both isocratic and gradient modes with varying ratios of methanol in 10 mM aqueous acetic acid, was used for reversed-phase separations. With analytical runs of samples containing radio-labelled GAs, column eluate was mixed with liquid scintillant (10 g l^{-1}, 2,5-diphenyloxazole in Triton X-100/xylene/methanol [11:22:5 , v/v/v]) pumped at a flow rate of 3 ml min^{-1} via a Reagent Delivery unit (Reeve Analytical, Glasgow, UK) and directed to a radioactivity monitor comprising a manual scintillation counter (ICN, Tracerlab, Mechelen, Belgium) with a 300 µl coiled glass flow cell (see Reeve and Crozier,1977; Sandberg *et al.*, 1987).

Normal-phase HPLC was carried out on a 250 x 5 mm i.d. 5 µm CN nitrile Spherisorb column (Phase Separation, Deeside, Clwyd, UK) eluted isocratically with varying ratios of either dichloromethane/hexane or ethyl acetate/hexane, containing 0.5% acetic acid. For the analysis of radio-labelled GAs, column eluate was mixed with liquid scintillant containing 12 g 2,5-diphenyloxazole, 50 ml Triton X-100 and 1 l of distilled toluene to give a 2:1

scintillant-eluate ratio prior to being directed to the radioactivity monitor.

The retention properties of unlabelled GA standards subjected to reversed-phase HPLC were determined with an LC 871 absorbance monitor (Pye Unicam, Cambridge, UK) operating at 206nm. GA methoxycoumaryl esters (GACEs) were detected after both reversed- and normal-phase HPLC with an LS-3 spectrofluorimeter (excitation 320 nm, emission 400nm) fitted with a 16µl flow cell (Perkin-Elmer, Norwalk, Conn., USA).

Derivatisation

Following preparative HPLC of underivatised samples, aliquots of appropriate peaks were converted to methoxycoumaryl ester derivatives prior to further analysis by analytical HPLC. Dry samples were dissolved in 20 µl acetone to which was added a crystal of potassium carbonate, bromoethylmethoxy-coumarin (10 mM, 20 µl in acetone) and 18-Crown-6 catalyst (10 mM, 2 µl in acetone). After incubation at 60°C for 2 h in sealed tubes, mixtures were dried under nitrogen, 100 µl distilled water added and the GACEs partitioned into chloroform (Crozier and Durley, 1983).

Prior to analysis by GC-MS, underivatised HPLC fractions were methylated with 100µl methanol/diazomethane (1:1, v/v) at room temperature for 15 min (Schlenk and Gellerman, 1960). The methylated samples were dried under N_2 in a desiccator before silylation with 50-150 µl bis-trimethylsilyltrifluoroacetamide/ acetonitrile (1:1, v/v) at 60°C for 10 min.

Gas chromatography-mass spectrometry

Metabolites of [^{14}C]GA_{12} and [^{14}C]GA_{53} after cell-free incubation

Analyses of HPLC-purified [^{14}C]GA_{12}- and [^{14}C]GA_{53}-products from incubations with Prizewinner S-1 preparation were carried out by combined GC-MS. Identification of [^{14}C]GA_{53}, [^{14}C]GA_{15} and [^{14}C]GA_{44} was performed on a Finnigan 4015 GC/MS/data system at the University of Göttingen, West Germany. Derivatised samples were injected (260°C) in 1 µl aliquots into a fused silica capillary column (SE-30) chemical bonded phase, 25 m x 0.32 mm internal diameter, using a Grob splitless injector. The column temperature was maintained at 50°C for 1 min then programmed at 15°C min^{-1} to 200°C and at 4°C min^{-1} to 260°C. The helium carrier gas flow rate was 1 ml min^{-1}. The split was opened 1 min after injection. The column effluent was led directly into the ion source at 260°C. Electron energy was 30 eV and emission current was 0.24 mA.

Estimation of endogenous GA_{20} content of seedlings

A Hewlett Packard 5970 series Mass Selective Detector was utilised for estimation of endogenous GA_{20} content in Prizewinner and Hammonds Dwarf Scarlet seedlings and identification of [$^{2}H_3$]GA_{19}-incubation products with S-1 preparation from Prizewinner seed. These analyses were carried out at the University of Tromsö, Norway.

Derivatised samples were injected in 1-3 µl aliquots with the injector at 275°C and the oven at 60°C for 2 min after injection. The column was of fused silica capillary type (cross linked methyl silicone), 25 m long, 0.31 mm internal diameter. The column temperature was maintained at 60°C for 2 min, then programmed at 30°C min $^{-1}$ to 180°C and at 10°C min^{-1} to 240°C. The helium

carrier gas flow rate was 1 ml min^{-1} and the split was opened 1 min after injection. The column effluent was led directly into the ion source at 240°C and the electron energy was 70 eV.

RESULTS

GA metabolism in cell-free preparations from immature seeds.

Cell-free preparations of immature seed from Prizewinner (tall) and Hammonds Dwarf Scarlet (dwarf) were incubated with radio-labelled GA substrates to establish i) the authenticity of the pathway illustrated in Fig. 5 for Prizewinner and ii) whether Hammonds Dwarf Scarlet displays differences in GA metabolism from Prizewinner, at this stage of the life cycle.

Metabolism of [^{14}C] GA_{12}-aldehyde

[^{14}C]GA_{12}-aldehyde (200,000 dpm, 183 ng) was incubated with cofactors and S-1 preparations of Prizewinner and Hammonds Dwarf Scarlet. After incubation at 30°C for 2 h, the reaction mixtures were extracted and analysed by gradient elution reversed-phase HPLC-RC. Typical traces are illustrated in Fig. 6. The HPLC profiles are similar to each other and the major peaks are tentatively identified on the basis of their HPLC retention times (R_ts) and the data of Turnbull *et al.* (1985), where, following the incubation of [^{14}C]GA_{12}-aldehyde with S-1 preparation from Prizewinner seed, GC-MS was used to identify [^{14}C]-labelled GA_1, GA_4, GA_5, GA_6, GA_{15}, GA_{17}, GA_{19}, GA_{20}, GA_{24}, GA_{37}, GA_{38}, GA_{44} and GA_{53}-aldehyde. In the current investigation, with preparations from both Prizewinner and Hammonds Dwarf Scarlet , a range of C_{19}- and C_{20}-GAs was observed, with GA_1, GA_4 and the putative intermediates GA_{15}, GA_{19} and GA_{44} accumulating in largest amounts (Fig. 6).

Having detected no obvious differences in GA metabolism with the cell-free systems from the dwarf and tall material, elucidation of

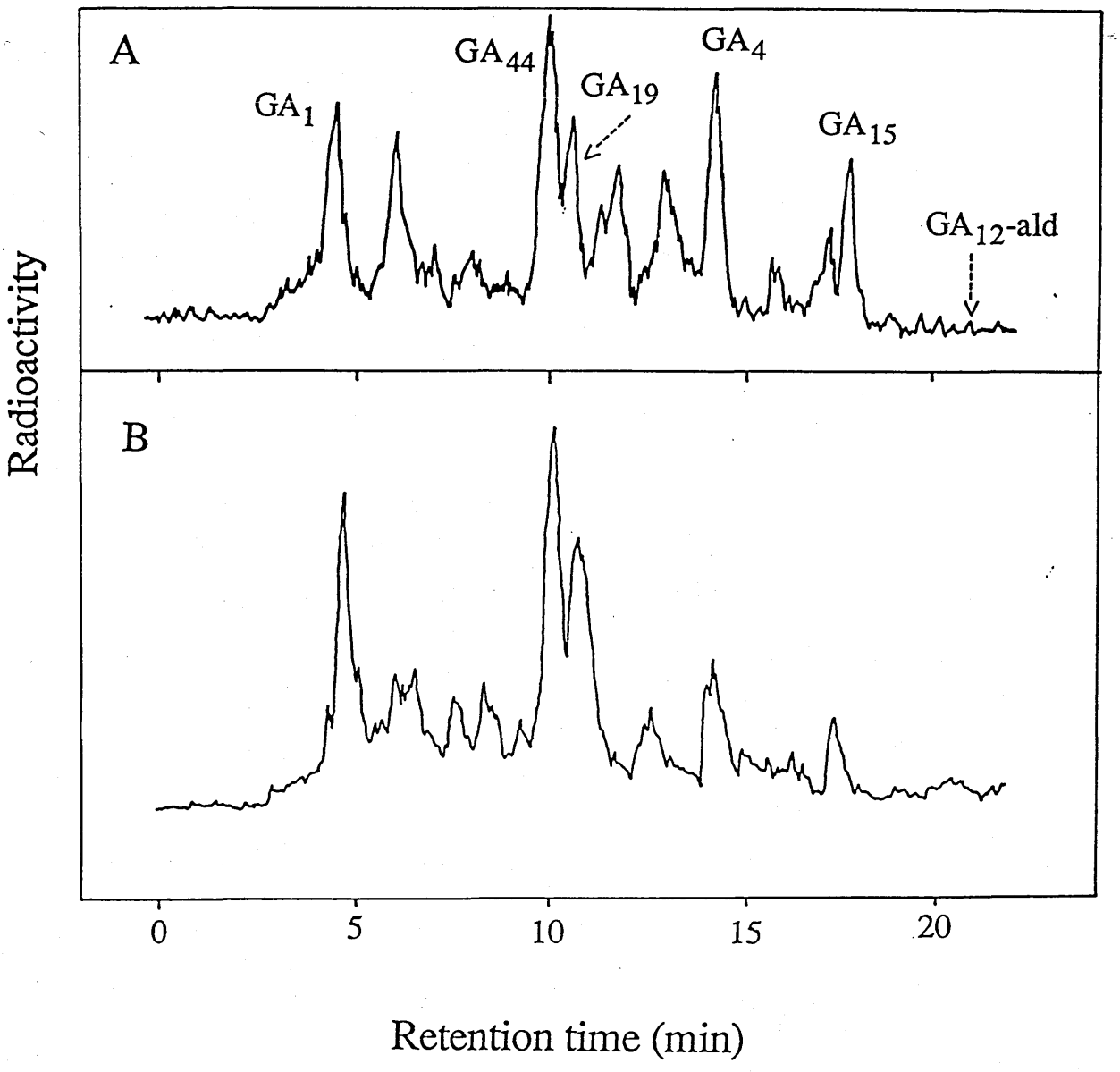


Figure 6 Metabolism of [^{14}C]GA_{12}-aldehyde by cell-free preparations from immature seed of *Phaseolus coccineus* A) cv. Prizewinner and B) cv. Hammonds Dwarf Scarlet. Reversed-phase HPLC: 250 x 5 mm (i.d.), 5µm ODS Hypersil support, gradient elution with methanol in 1% acetic acid (0-15 min, 50-80% MeOH; 15-19 min, 80-100% MeOH; 19-24 min, 100% MeOH). Sample: 50,000 dpm. Detector: radioactivity monitor.

the metabolic pathway leading from GA_{12}-aldehyde to GA_1 was undertaken using the Prizewinner S-1 preparation. Subsequently, however, key incubations were performed with the Hammonds Dwarf Scarlet cell-free system in order to examine in detail the capacity of the dwarf for GA_1 synthesis. Theoretically, GA metabolism in *Phaseolus coccineus*, from GA_{12}-aldehyde onwards, can proceed via two main routes, namely 13- and non-13-hydroxylated pathways (Fig. 5). Appropriate isotopically-labelled GAs from each route were therefore incubated with the Prizewinner S-1 preparation.

Metabolism of [^{14}C]GA_{12} and [^{14}C]GA_{53}

[^{14}C]GA_{12} (600,000 dpm, 597 ng) and 60,000 dpm [^{14}C]GA_{53} (74 ng) were incubated with enzyme preparation from Prizewinner seed. After a 2 h incubation period, extraction was followed by gradient elution reversed-phase HPLC-RC. The traces obtained are shown in Fig. 7. Preliminary identities of the metabolites were based on reversed-phase HPLC retention times. This indicated metabolism of [^{14}C]GA_{12} to [^{14}C]GA_{53} and [^{14}C]GA_{15} and the conversion of [^{14}C]GA_{53} to [^{14}C]GA_{44}. In order to investigate further the identities of the metabolites, the samples were fractionated and individual peaks collected, methylated and silylated prior to analysis by combined GC-MS. The data obtained are summarised in Table 1 and confirm the presence of [^{14}C]GA_{53} and [^{14}C]GA_{15} in the [^{14}C]GA_{12} incubation and conversion of [^{14}C]GA_{53} to [^{14}C]GA_{44}.

Metabolism of [^{3}H]GA_{15}

[^{3}H]GA_{15} (150,000 dpm) was incubated for 2 h with Prizewinner S-1 enzyme preparation prior to extraction and analysis by gradient elution reversed-phase HPLC. Figure 8 shows the routinely observed single product with an R_t similar to that of [^{3}H]GA_{37}, the identity of

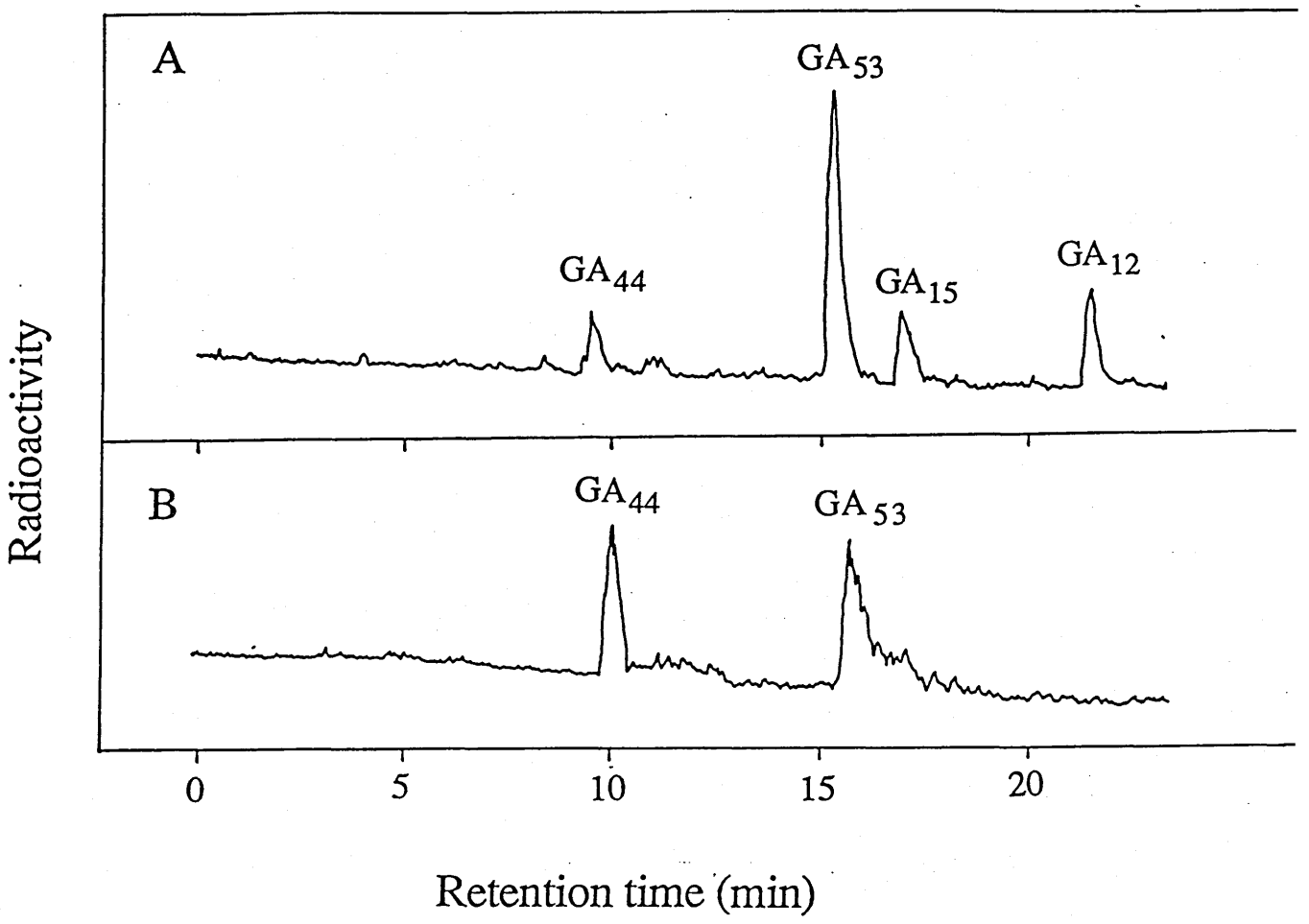


Figure 7 Metabolism of A) [^{14}C]GA_{12} and B) [^{14}C]GA_{53} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. Reversed-phase HPLC conditions as for Fig. 6. Sample: 60,000 dpm. Detector: radioactivity monitor.

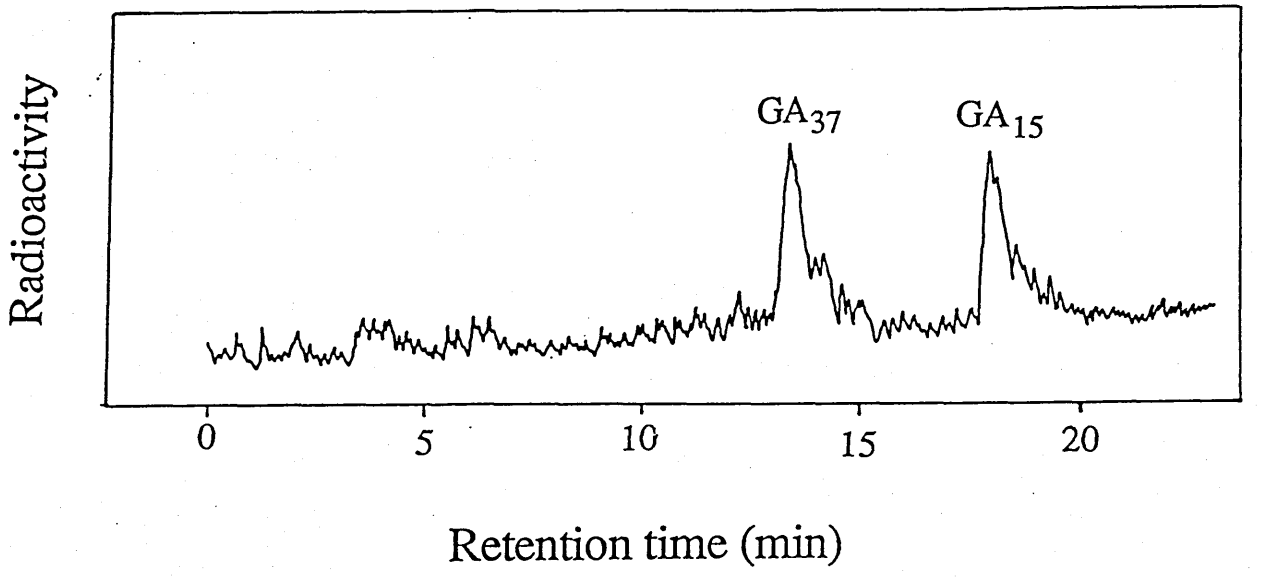


Figure 8 Metabolism of [^{3}H]GA_{15} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. Reversed-phase HPLC conditions as for Fig. 6. Sample: 30,000 dpm. Detector: radioactivity monitor.

which was confirmed by analysis of the underivatised and derivatised metabolite by reversed- and normal-phase HPLC (Table 2).

Table 1. GC-MIM data of metabolites from [^{14}C]GA_{12} and [^{14}C]GA_{53} incubation with cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner.

Substrate (Bq mol^{-1})	Putative product	m/z (rel. int.) [M$^+$]	[M+8]$^+$	Other ions	Sp. Act. (Bq mol^{-1})
[^{14}C]GA_{12} (5.5×10^{12})	[^{14}C]GA_{53}	448	456	- -	4.6×10^{12}
	[^{14}C]GA_{15}	344	352	239 245	2.8×10^{12}
[^{14}C]GA_{53} (4.6×10^{12})	[^{14}C]GA_{44}	432	440	373 379	1.9×10^{12}

GA_{15} is a δ-lactone which is believed to be formed spontaneously from the C-20 alcohol during extraction (see Hedden, 1983). In order to open the lactone ring and return to a C-20 alcohol structure, it is necessary to treat [^{3}H]GA_{15} with 1M KOH at 100°C prior to incubation (Hedden and Graebe, 1982). However, despite repeated attempts, this procedure proved unsuccessful as [^{3}H]GA_{15} incubations yielded only its 3β-hydroxyl analogue, [^{3}H]GA_{37}.

Metabolism of [^{3}H]GA_{14} and [^{3}H]GA_{37}

The S-1 preparation from Prizewinner did not further metabolise [^{3}H]GA_{37}. Treatment of the δ-lactone with KOH does not yield GA_{37}-open lactone because the 3β-hydroxyl group results in a reversible retroaldol arrangement (MacMillan and Pryce, 1973). In an attempt to overcome this problem, incubations were carried out using [^{3}H]GA_{14} as a substrate. Although not an endogenous constituent of *Phaseolus coccineus* seed, it was thought that oxidation of GA_{14} at C-20 might yield GA_{37}-open lactone, *in situ*, which would then be metabolised in a similar manner to the endogenous constituent. In

practice, this approach proved successful. The S-1 preparation completely metabolised [^{3}H]GA_{14} (165,000 dpm, 427 ng) to a range of metabolites with HPLC R_ts corresponding to GA_4, GA_{36} and GA_{37}. In addition, a polar peak was detected which did not co-chromatograph with either GA_1 or GA_8 (Fig. 9).

Table 2. Metabolism of [^{3}H]GA_{14} and [^{3}H]GA_{15} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. HPLC-RC data of product based on co-chromatography with [^{3}H]-labelled GA standards. R-P; reversed-phase HPLC, N-P; normal-phase HPLC, underiv.; underivatised GA, deriv.; methoxycoumaryl ester derivative.

		%		Isocratic HPLC R_t min		
Substrate	Putative product	Recovered radioactivity	GA standard	R-P underiv.	R-P deriv.	N-P deriv.
[^{3}H]GA_{15}	[^{3}H]GA_{37}	52		10.1[b]	15.1[c]	11.5[f]
			[^{3}H]GA_{37}	10.1[b]	15.1[c]	11.5[f]
[^{3}H]GA_{14}	[^{3}H]GA_{37}	56		9.2[b]	9.2[d]	12.2[f]
			[^{3}H]GA_{37}	9.2[b]	9.2[d]	12.2[f]
	[^{3}H]GA_{36}	7		6.8[b]		
			[^{3}H]GA_{36}	6.8[b]		
	[^{3}H]GA_4	29		10.5[b]	14.1[c]	13.4[e]
			[^{3}H]GA_4	10.5[b]	14.1[c]	13.4[e]
	[^{3}H]GA_{23}	8		8.5[a]		
			GA_{23}	8.5[a]		

Mobile phase: a) 35% MeOH in 1% acetic acid, b) 60% MeOH, c) 70% MeOH; d) 75% MeOH e) 20% dichloromethane in hexane, f) 25% dichloromethane

Confirmation of the identity of GA_4, GA_{36} and GA_{37} was obtained by reversed- and normal-phase HPLC analyses of the free acids and their methoxycoumaryl ester derivatives (Table 2).

Metabolism of [^{3}H]GA_{24} and [^{3}H]GA_{36}

Incubation of [^{3}H]GA_{36} (300,000 dpm) with S-1 preparation from Prizewinner produced a peak with the same HPLC R_t as the unidentified product of [^{3}H]GA_{14} incubation, (Fig. 10). When used as a substrate for incubation with Prizewinner S-1, 250,000 dpm,

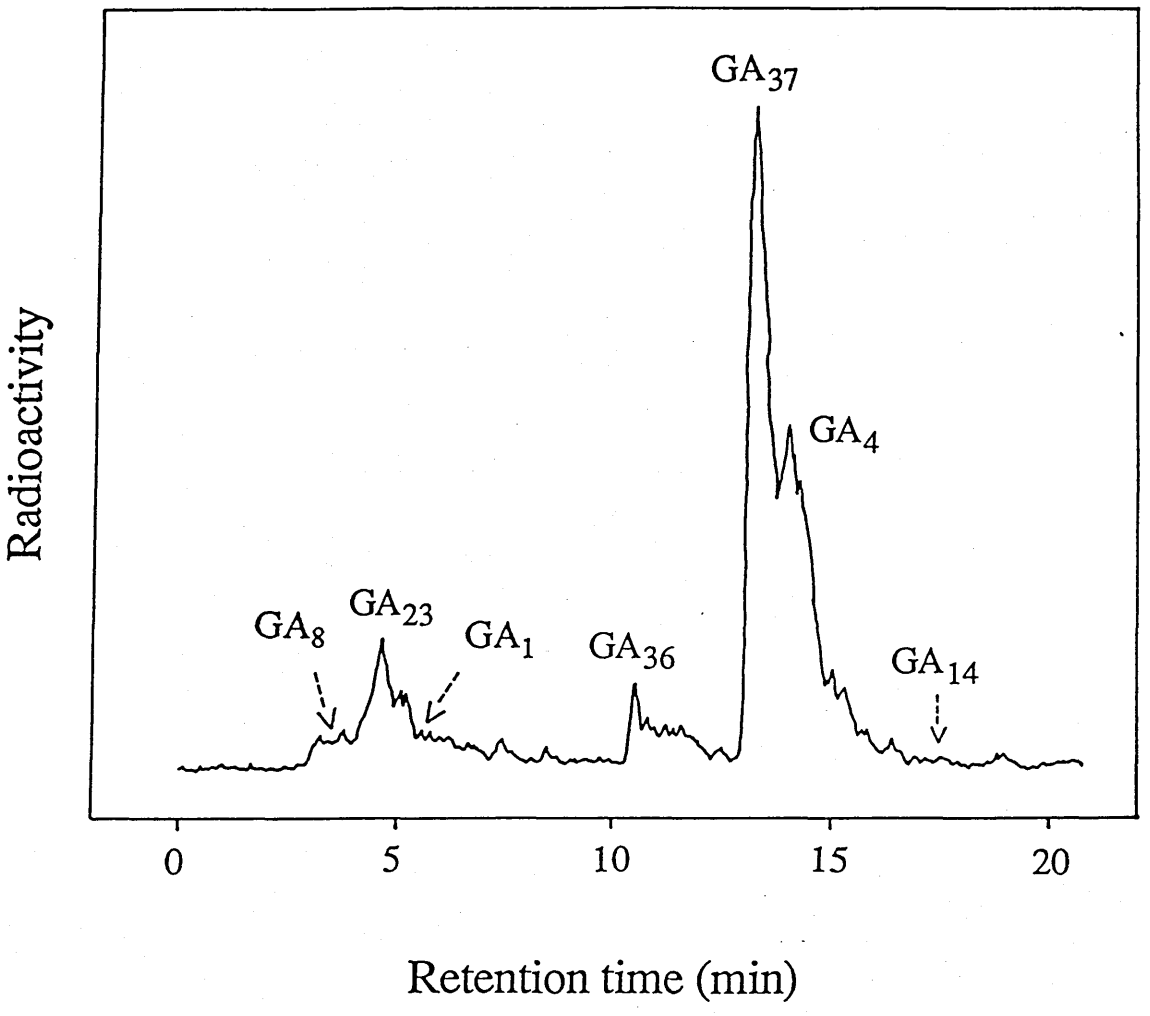


Figure 9 Metabolism of [^{3}H]GA_{14} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. Reversed-phase HPLC conditions as for Fig. 6. Sample: 80,000 dpm. Detector: radioactivity monitor.

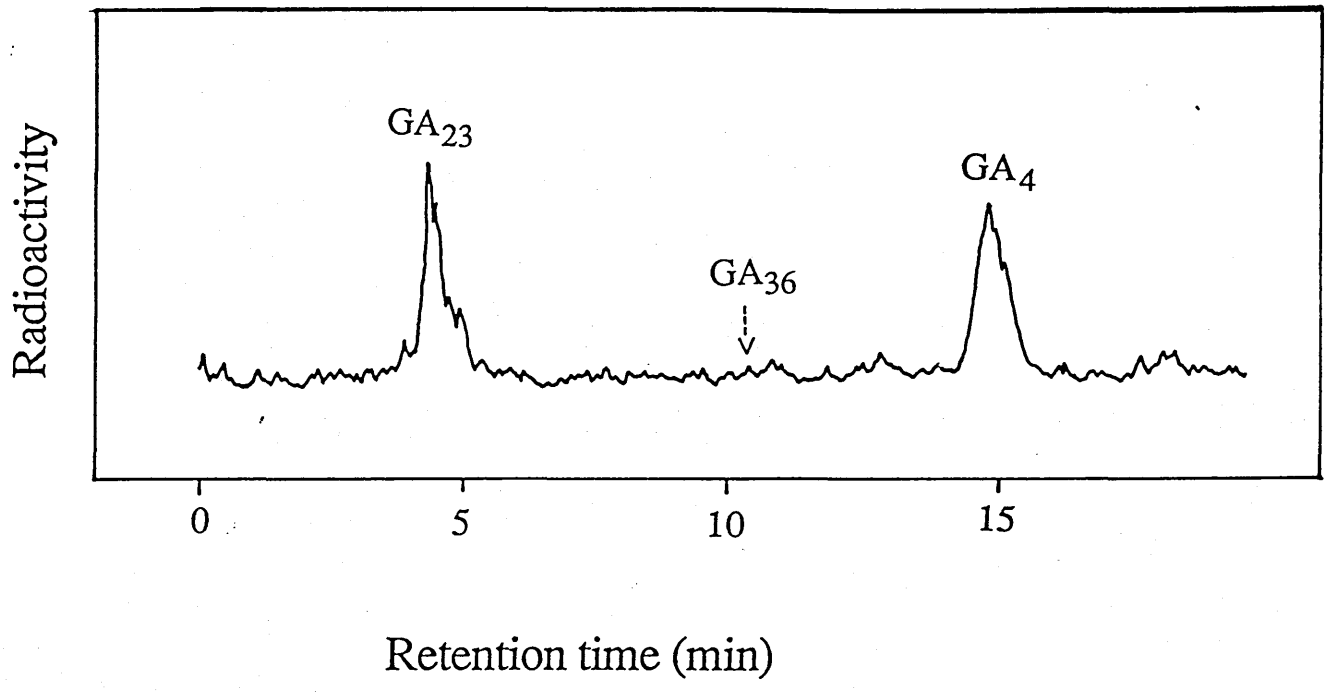


Figure 10 Metabolism of [^{3}H]GA_{36} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. Reversed-phase HPLC conditions as for Fig. 6. Sample: 40,000 dpm. Detectors: radioactivity and variable wavelength UV monitors.

[^{3}H]GA_{24} were metabolised to a peak with the HPLC characteristics of [^{3}H]GA_4 and to a number of other unidentified minor products none of which was GA_1, GA_8 or GA_{36} (Fig. 11). The provisional identifications of [^{3}H]GA_4 from [^{3}H]GA_{24} and [^{3}H]GA_{36} were confirmed and the polar product of [^{3}H]GA_{36} and [^{3}H]GA_{14} incubations identified tentatively as [^{3}H]GA_{23}. Analyses were by reversed- and/or normal-phase HPLC of the free acids and/or the corresponding methoxycoumaryl ester derivatives with the data summarised in Table 3.

Table 3. Metabolism of [^{3}H]GA_{24} and [^{3}H]GA_{36} by cell-free preparation from immature seeds of *Phaseolus coccineus* cv. Prizewinner. HPLC-RC data of product based on co-chromatography with [^{3}H]-labelled standards. GA_{23} standard detected with absorbance monitor operating at 206 nm

Substrate	Putative product	% Recovered radioactivity	GA standard	Isocratic HPLC $R_t$min R-P underiv.	R-P deriv.	N-P deriv.
[^{3}H]GA_{24}	[^{3}H]GA_4	11		8.8[b]	12.8[c]	
			[^{3}H]GA_4	8.8[b]	12.8[c]	
[^{3}H]GA_{36}	[^{3}H]GA_4	49		8.4[c]	7.5[d]	10.1[e]
			[^{3}H]GA_4	8.4[c]	7.5[d]	10.1[e]
	[^{3}H]GA_{23}	51		8.4[a]		
			GA_{23}	8.4[a]		

Mobile phase: a) 35% MeOH in 1% acetic acid, b) 60% MeOH, c) 70% MeOH, d) 80% MeOH, e) 20% dichloromethane in hexane.

Incubation of [^{3}H]GA_{24} (400,000 dpm) with S-1 preparation from Hammonds Dwarf Scarlet produced a peak with the HPLC R_t of [^{3}H]GA_4 without the accumulation of a [^{3}H]GA_{36}-like product (Fig. 12). When used as a substrate with S-1 preparation from Hammonds Dwarf Scarlet, [^{3}H]GA_{36} (300,000 dpm) was completely converted to a [^{3}H]GA_4-like metabolite and to two very polar peaks, one of which had a similar R_t to that of GA_{23} (Fig. 13). Table 4 shows the confirmation of both the [^{3}H]GA_4 identifications by HPLC analyses of the free acid and/or the methoxycoumaryl ester derivative.

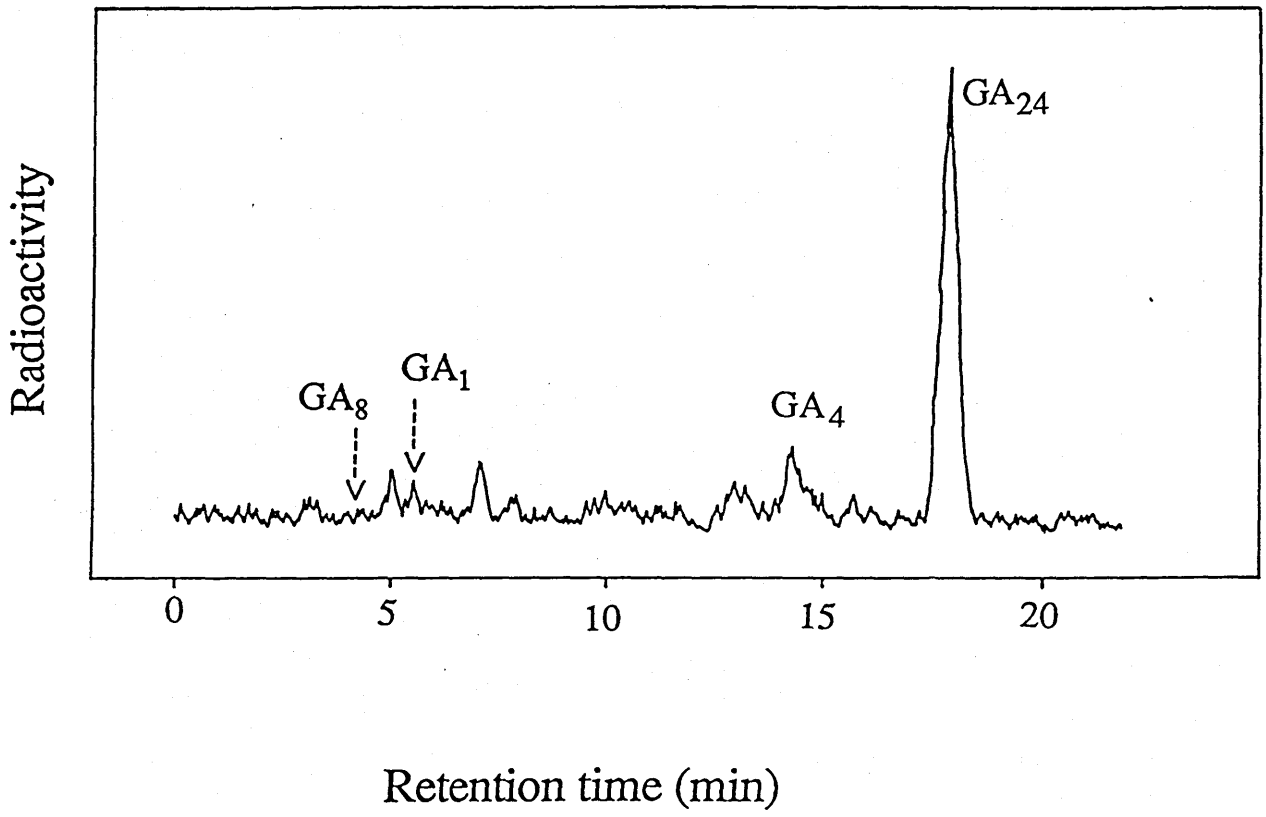


Figure 11 Metabolism of [^{3}H]GA_{24} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. Reversed-phase HPLC conditions as for Fig. 6. Sample: 40,000 dpm. Detector: radioactivity monitor.

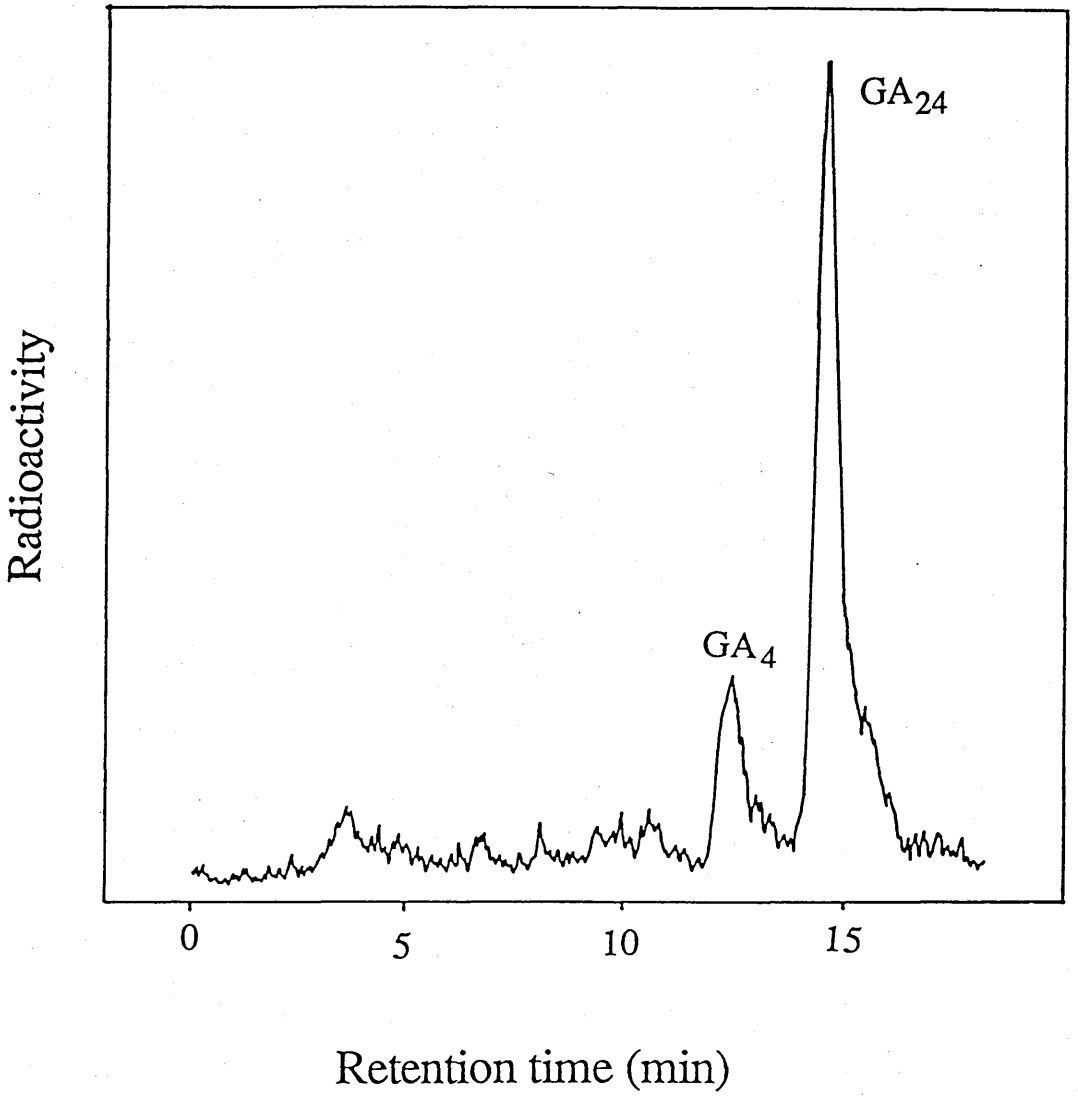


Figure 12 Metabolism of [^{3}H]GA_{24} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Hammonds Dwarf Scarlet. Reversed-phase HPLC conditions as for Fig. 6. Sample: 40,000 dpm. Detector: radioactivity monitor.

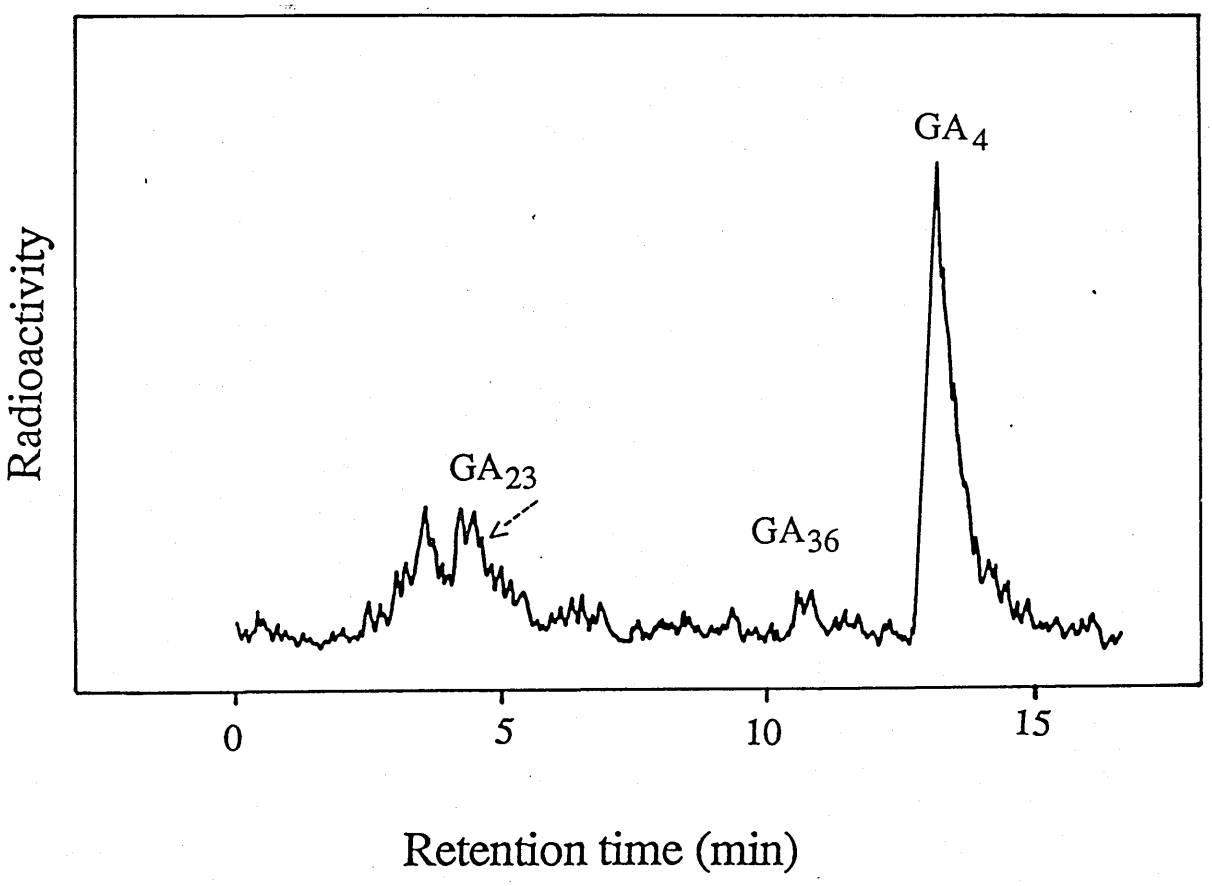


Figure 13 Metabolism of [^{3}H]GA_{36} by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Hammonds Dwarf Scarlet. Reversed-phase HPLC conditions as for Fig. 6. Sample: 30,000 dpm. Detector: radioactivity monitor.

Metabolism of [^{3}H]GA$_4$

Despite repeated incubations of [^{3}H]GA$_4$ with S-1 preparation from Prizewinner and Hammonds Dwarf Scarlet, no metabolism was observed.

Table 4. Metabolism of [^{3}H]GA$_{24}$ and [^{3}H]GA$_{36}$ by cell-free preparation from immature seeds of *Phaseolus coccineus* cv. Hammonds Dwarf Scarlet. HPLC-RC data of product based on co-chromatography with [^{3}H]-labelled GA standards.

Substrate	Putative product	% Recovered radioactivity	GA standard	HPLC R_t min R-P underiv.	N-P underiv.
[^{3}H]GA$_{24}$	[^{3}H]GA$_4$	23		12.9[a]	
			[^{3}H]GA$_4$	12.9[a]	
[^{3}H]GA$_{36}$	[^{3}H]GA$_4$	67		13.8[a]	11.3[b]
			[^{3}H]GA$_4$	13.8[a]	11.3[b]

Mobile phase: a) gradient 0 min - 50% MeOH, 15 min - 80% MeOH, 19 min - 100% MeOH, b) 20% ethyl acetate in hexane.

Metabolism of [^{2}H$_3$] GA$_{19}$

Unavailability of radio-labelled GA$_{44}$ standard hampered investigation of this section of the 13-hydroxylated route. Large-scale incubations of [^{14}C]GA$_{12}$-aldehyde were carried out in an attempt to accumulate this intermediate but conversions were low. Furthermore, with KOH treatment prior to incubation, problems similar to those encountered with [^{3}H]GA$_{15}$, meant that [^{14}C]GA$_{44}$ was unmetabolised on a routine basis.

Deuterated, [^{2}H$_3$]GA$_{19}$ was incubated with S-1 preparation from Prizewinner and the resultant extract fractionated by preparative HPLC to a number of zones. These were then analysed by combined GC-MIM as the TMS derivatives with Table 5 showing the identified products as [^{2}H$_3$]GA$_1$, [^{2}H$_3$]GA$_5$ and [^{2}H$_3$]GA$_{20}$. These conversions constitute loss of C-20, together with either 3β-hydroxylation or

introduction of a 2,3 double bond, respectively.

Table 5. GC-MIM data of metabolites from $[^2H_3]GA_{19}$ incubation with cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner.

Sample/ standard	m/z % abundance M$^+$	M$^+$+1	M$^+$+2	M$^+$+3	M$^+$+4	GC R_t	KI
$[^2H_3]GA_{19}$	*434(6)	435(38)	436(88)	437(100)	438(56)		2612
GA_1	506(100)	507(33)	508(11)	509(4)	510(1)	23.8[1]	2676
$[^2H_3]GA_1$	506(100)	507(51)	508(47)	509(32)	510(20)	23.7[2]	2676
GA_5	416(100)	417(33)	418(11)	419(4)	420(0)	18.3[1]	2504
$[^2H_3]GA_5$	416(100)	417(48)	418(35)	419(21)	420(15)	18.2[2]	2504
GA_{20}	418(100)	419(33)	420(11)	421(4)	422(1)	18.4[1]	2512
$[^2H_3]GA_{20}$	418(32)	419(42)	420(100)	421(91)	422(43)	18.3[2]	2512

*base peak, KI- Kovats Indices, [1]- R_t of M$^+$, [2]- R_t of M$^+$+3

Metabolism of $[^3H]GA_{20}$

$[^3H]GA_{20}$ (200,000 dpm, 20 ng) incubated with S-1 preparation from Prizewinner was metabolised to two products with the HPLC R_ts of $[^3H]GA_1$ and $[^3H]GA_5$ and in addition, to two peaks more polar than $[^3H]GA_8$ (Fig. 14A). The identities of the GA_1 and GA_5 peaks were confirmed by reversed- and normal-phase HPLC of the free acids and their methoxycoumaryl ester derivatives with the details shown in Table 6. The unidentified peaks were treated with cellulase enzyme but, as no free GAs were liberated, it was concluded that these metabolites were unlikely to be GA glucoside conjugates.

S-1 preparation from Hammonds Dwarf Scarlet metabolised $[^3H]GA_{20}$ (300,000 dpm, 31 ng) to GA_1- and GA_5-like peaks and to two polar peaks with similar HPLC R_ts to those obtained in incubations of Prizewinner preparation (Fig. 14B).

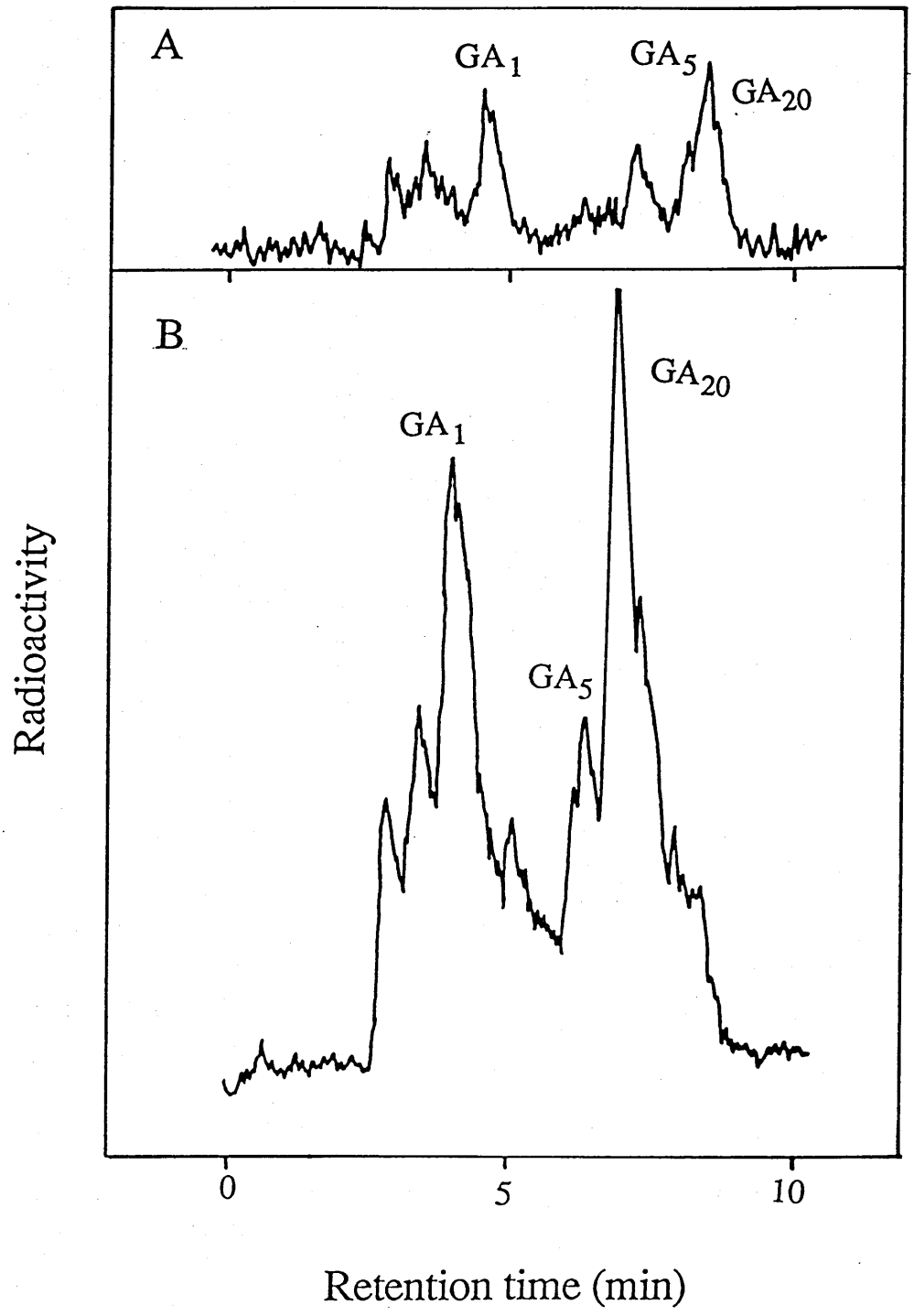


Figure 14 Metabolism of [^{3}H]GA_{20} by cell-free preparation from immature seed of *Phaseolus coccineus* A) cv. Prizewinner and B) cv. Hammonds Dwarf Scarlet. Reversed-phase HPLC conditions as for Fig. 6. Sample: A) 25,000 dpm and B) 90,000 dpm. Detector: radioactivity monitor.

Table 6. Metabolism of [^{3}H]GA_5 and [^{3}H]GA_{20} by cell-free preparation from immature seeds of *Phaseolus coccineus* cv. Prizewinner and Hammonds Dwarf Scarlet. HPLC data of product based on co-chromatography with [^{3}H]-labelled GA standards.

Cultivar	Substrate	Putative product	% Recovered radioactivity	GA standard	Isocratic HPLC R_t min R-P under.	R-P deriv.	N-P deriv.
Prizewinner	[^{3}H]GA_{20}	[^{3}H]GA_1	24		11.8[a]	10.3[c]	11.5[e]
				[^{3}H]GA_1	11.8[a]	10.3[c]	11.5[e]
		[^{3}H]GA_5	15		9.5[b]	6.7[d]	
				[^{3}H]GA_5	9.5[b]	6.7[d]	
	[^{3}H]GA_5	[^{3}H]GA_6	31		5.1[b]	9.1[d]	6.5[e]
				*GA_6	5.1[b]	9.1[d]	6.5[e]
Hammonds	[^{3}H]GA_{20}	[^{3}H]GA_1	25		9.7[a]		7.2[g]
				[^{3}H]GA_1	9.7[a]		7.2[g]
		[^{3}H]GA_5	14		7.5[b]		13.8[f]
				^{3}H]GA_5	7.5[b]		13.8[f]

Mobile phase: a) 35% MeOH in 1% acetic acid, b) 50% MeOH, c) 60% MeOH, d) 65% MeOH, e) 40% dichloromethane in hexane, f) 25% ethyl acetate in hexane, g) 45% ethyl acetate. * Detected by an absorbance monitor at 206 nm.

Table 7 shows confirmation of these steps by reversed- and normal-phase HPLC analyses of the free acids and their methoxycoumaryl ester derivatives.

Metabolism of [^{3}H]GA_5

Incubation of [^{3}H]GA_5 (260,000 dpm, 7.5 ng) with Prizewinner S-1 preparation resulted in the production of a peak with the HPLC R_t of GA_6 (Fig. 15), confirmed by reversed- and normal-phase HPLC of the free acid and the corresponding methoxycoumaryl ester derivative (Table 6).

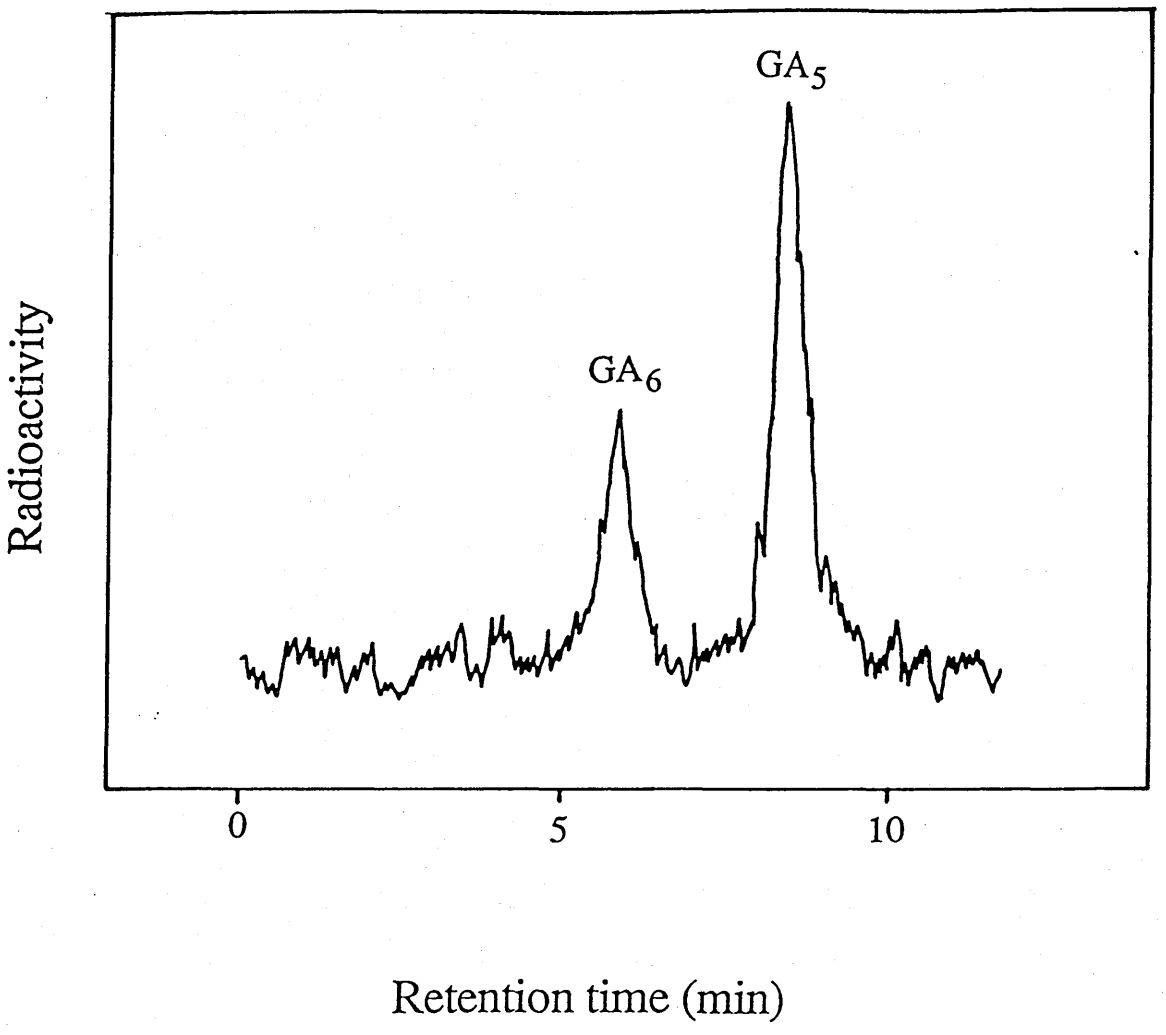


Figure 15 Metabolism of [^{3}H]GA_5 by cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. Reversed-phase HPLC conditions as for Fig. 6. Sample: 40,000 dpm. Detector: radioactivity monitor.

Metabolism of [^{3}H]GA$_1$

Despite repeated incubation of [^{3}H]GA$_1$ with S-1 preparation from Prizewinner and from Hammonds Dwarf Scarlet, no metabolism was observed.

Analysis of GA-metabolising enzymes.

Fast protein liquid chromatography and SDS-PAGE

Cell-free seed preparations from Prizewinner and Hammonds Dwarf Scarlet were subjected to FPLC in an attempt to isolate GA-metabolising activity from the total crude enzyme preparation. After gradient elution FPLC, the sample was collected as a number of successive fractions which were incubated with cofactors and radio-labelled GA substrate. Incubation products were examined by isocratic reversed-phase HPLC, revealing fractions in which GA metabolism was occurring. Fractions capable of a variety of metabolic conversions were compared and in some instances were subjected to SDS-PAGE to separate the constituent polypeptides. [^{3}H]GA$_{20}$ was used routinely as a substrate for the assay and only fractions in which 3β-hydroxylation occurred to produce [^{3}H]GA$_1$ were subsequently examined by SDS-PAGE.

Figures 16 and 17 show typical FPLC-UV traces obtained with gradient elution of 150 mg of cell-free seed preparation from Prizewinner and Hammonds Dwarf Scarlet. In order to check that enzymic activity was not lost as a consequence of 0.22 μm filtration, prior to FPLC separation, an aliquot of filtrate was incubated with cofactors and radio-labelled substrate. The results of this incubation could then be compared to the products of post-FPLC assays. Active fractions metabolised [^{3}H]GA$_{20}$ to [^{3}H]GA$_1$, with identifications being based on a comparison of isocratic reversed-phase HPLC R_t with that of authentic [^{3}H]GA$_1$ standard, (Table 7).

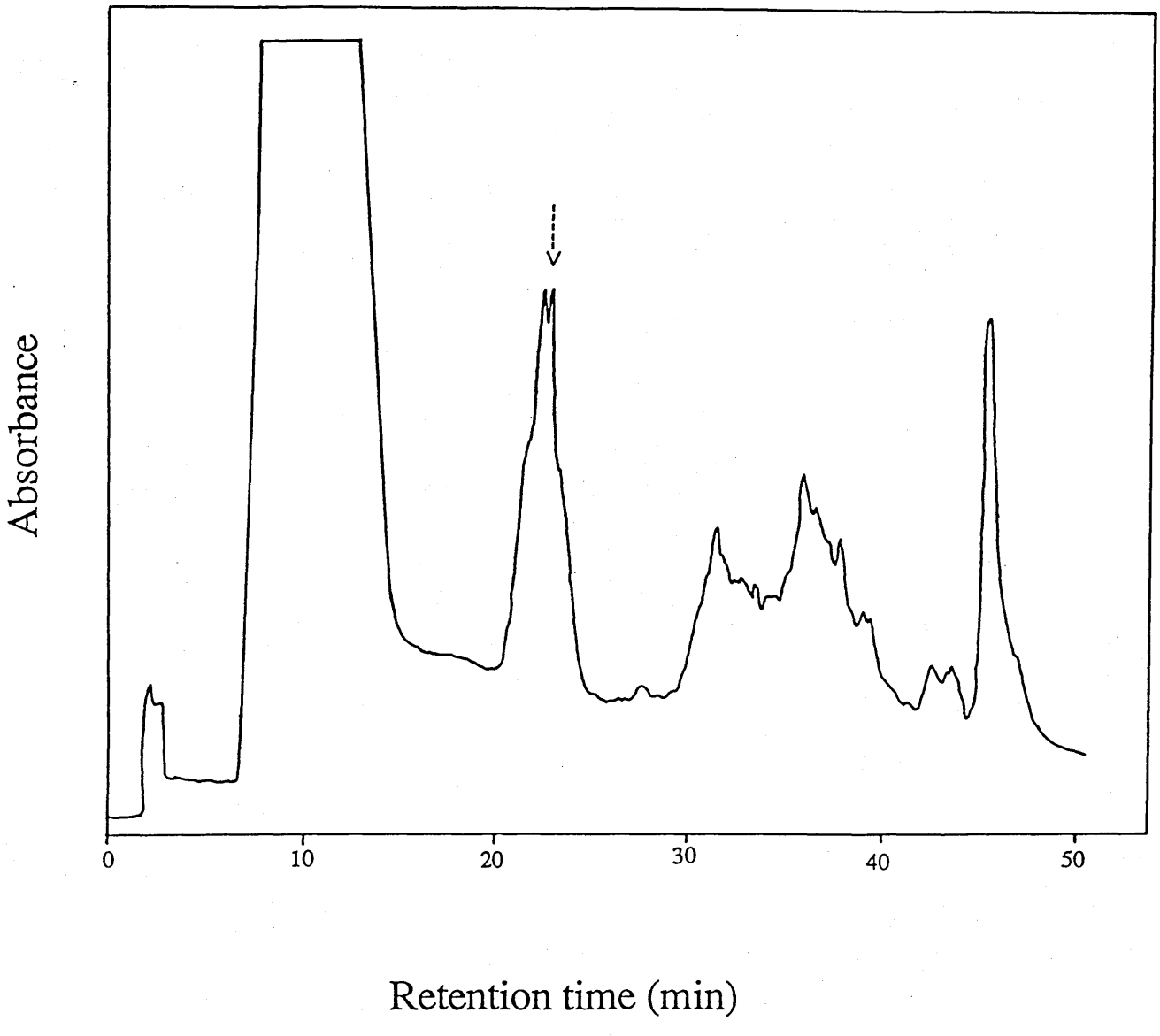


Figure 16 FPLC-UV trace of 150 mg cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner. FPLC: Mono Q column (50 mm x 5 mm i.d). Mobile phase: A) 0.02 M Tris HCl, pH 7.5; B) 0.02 M Tris HCl, pH 7.5, 1 M NaCl. A) and B) contained 0.4 mM dithiothreitol and 1.0 mM benzamidine. Programme: 0-22 min, 0% B; 22-24 min, 0-4.5% B; 24-26min, 4.5% B; 26-40 min, 4.5-35% B; 40-50 min, 35-100% B. Detector: UV monitor at 280 nm, 1.0 AU.

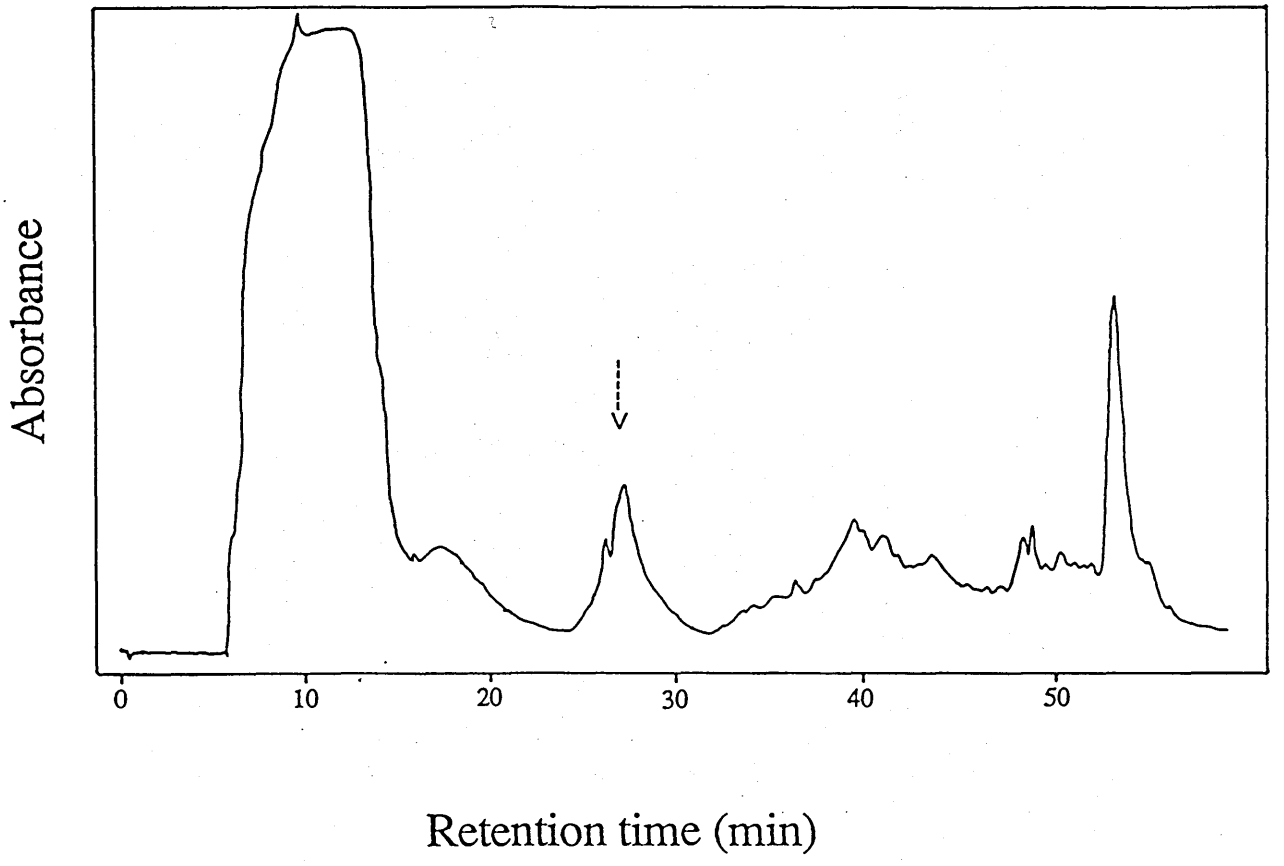


Figure 17 FPLC-UV trace of 150 mg cell-free preparation from immature seed of *Phaseolus coccineus* cv. Hammonds Dwarf Scarlet. FPLC conditions as for Fig. 17. Programme: 0-22 min, 0% B; 22-24 min, 0-4% B; 24-31 min, 4% B; 31-49 min, 4-35% B; 49-59 min, 35-100% B. Detector: UV monitor at 280 nm, 1.0 AU.

Table 7. Incubation of immature seed cell-free preparations from *Phaseolus coccineus* cv. Prizewinner, with [^{3}H]GA_{12}, [^{3}H]GA_{14} and [^{3}H]GA_{20}, and cv. Hammonds Dwarf Scarlet with [^{3}H]GA_{20}, after FPLC. HPLC-RC data based on co-chromatography of incubation products and [^{3}H]-labelled GA standards.

Cultivar	Substrate	Putative product	GA standard	R_t (min)	% MeOH
Prizewinner	[^{3}H]GA_{20}	[^{3}H]GA_1		6.5	50
			[^{3}H]GA_1	6.5	50
Hammonds	[^{3}H]GA_{20}	[^{3}H]GA_1		6.5	50
			[^{3}H]GA_1	6.5	50
Prizewinner	[^{3}H]GA_{12}	[^{3}H]GA_{37}		7.8	60
			[^{3}H]GA_{37}	7.8	60
		[^{3}H]GA_{24}		15.1	60
			[^{3}H]GA_{24}	15.1	60
		[^{3}H]GA_4		9.1	60
			[^{3}H]GA_4	9.1	60
	[^{3}H]GA_{14}	[^{3}H]GA_{37}		7.4	65
			[^{3}H]GA_{37}	7.4	65

Generally, post-filter, pre-column incubations also contained several peaks more polar than GA_8. Two of these peaks typically occurred in post-FPLC assays of Prizewinner preparation but Hammonds Dwarf Scarlet fractions synthesised one only. It is possible that these unidentified incubation products correspond to those observed when crude S-1 preparation from Prizewinner and Hammonds Dwarf Scarlet is incubated with [^{3}H]GA_{20}. All enzyme activity corresponded in FPLC R_t to the UV peaks on Figs. 16 and 17 indicated by arrows. Figure18 represents the distribution and relative percentages of the various metabolites of [^{3}H]GA_{20} incubation with Prizewinner and Hammonds Dwarf Scarlet and shows that 3β-hydroxylation was not eluted separately from the other activities

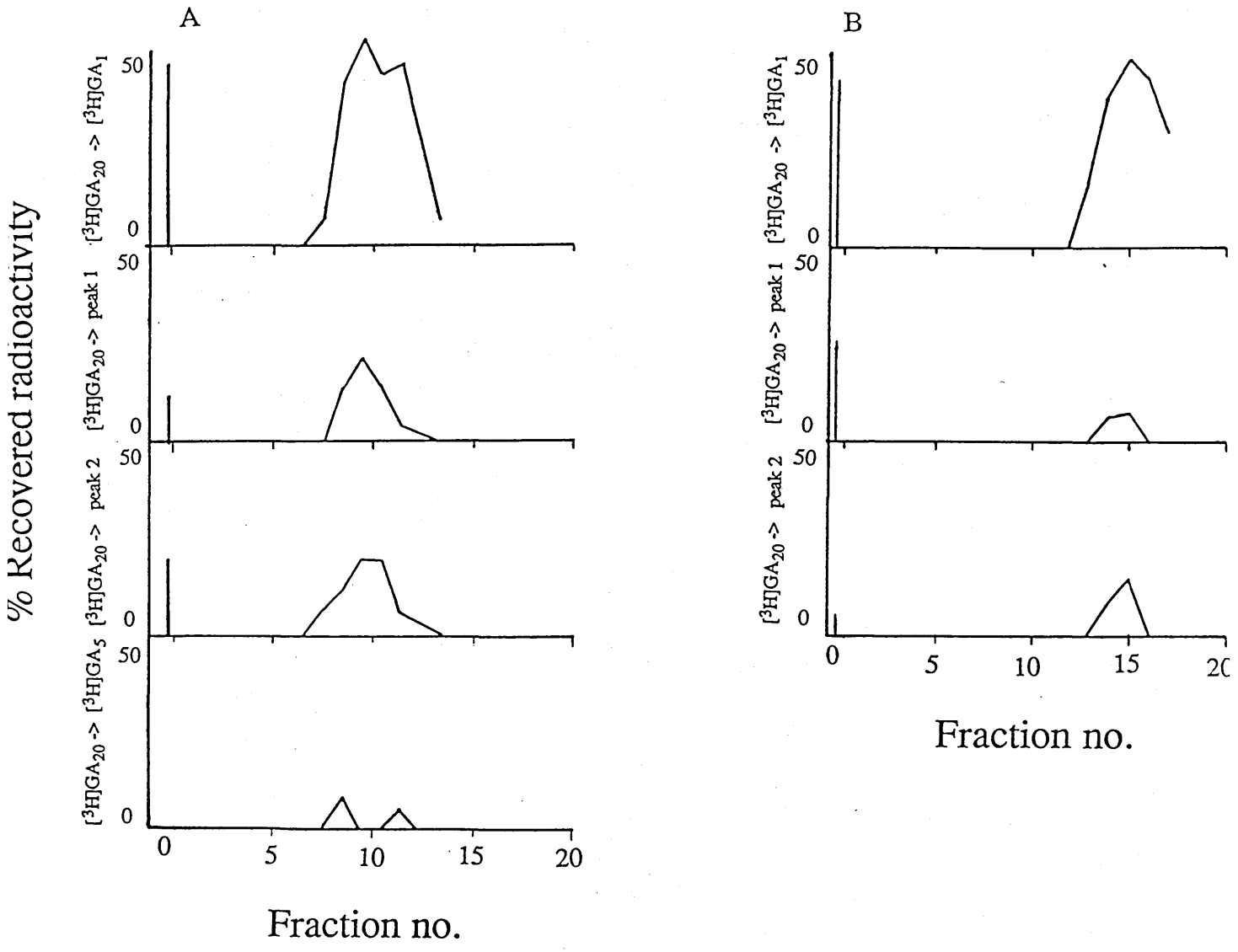


Figure 18 Post FPLC incubation of cell-free preparation from immature seed of *Phaseolus coccineus* A) cv. Prizewinner and B) cv. Hammonds Dwarf Scarlet with $[^3H]GA_{20}$. All fractions were assayed, but only the enzymic conversions and their distributions in the active region are shown. Fraction 0 represents incubation of an aliquot of total enzyme preparation after filtration, but prior to FPLC.

with the FPLC conditions used.

A preliminary analysis of the active fractions from the FPLC runs shown in Figs. 16 and17 was by SDS-PAGE. Aliquots of active zones and standard mixture proteins were loaded onto an 11% polyacrylamide slab gel, subjected to electrophoresis and subsequently stained and destained prior to examination. Figure 19 shows the constituent polypeptide bands, after FPLC, of the active fractions from cell-free seed preparations of Prizewinner and Hammonds Dwarf Scarlet.

In Fig. 19A, positions 1 and 2, and on Fig. 19B position 1, represent(s) standard mixture proteins of known molecular weights, with which to compare sample bands. In descending order, the standard mixture proteins are BSA (68 k daltons), alcohol dehydrogenase (41 k daltons), myoglobin (17.2 k daltons) and cytochrome C (12.1 k daltons). Table 8 summarises the sample band data obtained from this experiment. SDS gels of preparations from Prizewinner and Hammonds Dwarf Scarlet have bands in common at 36.6 k daltons, ~30 k daltons and the Prizewinner sample has a single strongly indicated band at 49.4 k daltons which may correspond with any of the separated bands from 47.0 to 54.6 k daltons in the Hammonds Dwarf Scarlet sample.

After FPLC, Prizewinner S-1 cell-free preparation was also incubated with [^{3}H]GA_{12} and [^{3}H]GA_{14}. In active zones, the former substrate was metabolised to peaks with the reversed-phase HPLC R_ts of [^{3}H]GA_4, [^{3}H]GA_{24} and [^{3}H]GA_{37} (Table 7). Figure 20A shows the distribution of each enzyme activity in the fractions, with production of [^{3}H]GA_4 and [^{3}H]GA_{37} being relatively restricted while that of [^{3}H]GA_{24} is more widespread. The [^{3}H]GA_4 that accumulated could have originated from either [^{3}H]GA_{24} or [^{3}H]GA_{37}, but it would seem more probable that [^{3}H]GA_{24} is the precursor, as the peak in the [^{3}H]GA_4 production corresponds to a trough in the level of

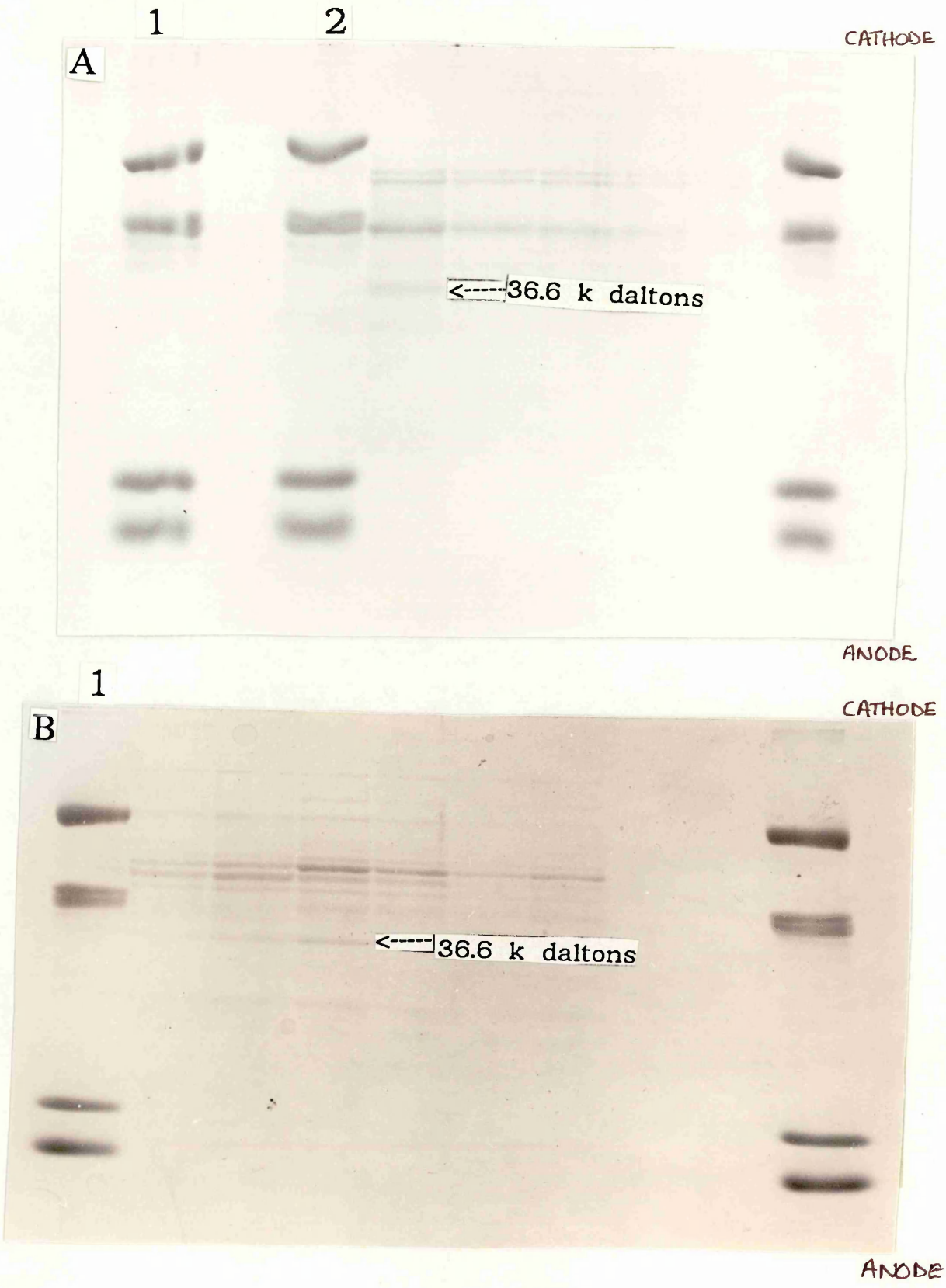


Figure 19 SDS-PAGE of cell-free preparations from immature seed of *Phaseolus coccineus*, A) cv. Prizewinner and B) Hammonds Dwarf Scarlet, after FPLC separation. FPLC fractions analysed metabolised [^{3}H]GA_{20} to [^{3}H]GA_1 .

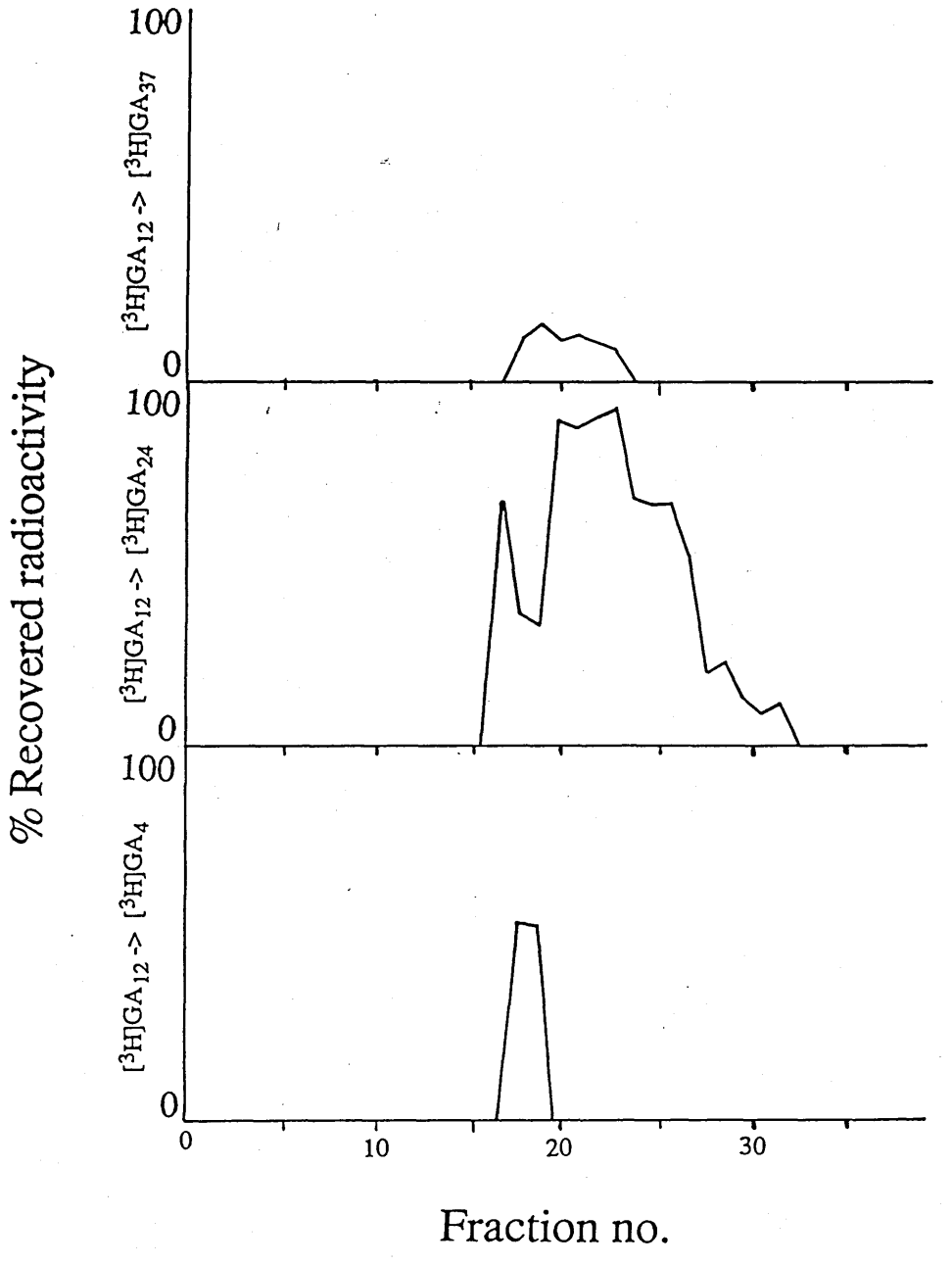


Figure 20A Post FPLC incubation of cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner with [^{3}H]GA_{12}. All fractions were assayed, but only the enzymic conversions and their distributions in the active region are shown.

[^{3}H]GA_{24}. No similar relationship was found between [^{3}H]GA_{37} and [^{3}H]GA_4.

Table 8. Post-FPLC analysis of immature seed cell-free preparation derived from Prizewinner and Hammonds Dwarf Scarlet. Fractions which metabolised [^{3}H]GA_{20} to [^{3}H]GA_1 were subjected to SDS-PAGE and the MW of the bands estimated by comparison with standards.

Prizewinner			Hammonds Dwarf Scarlet		
Sample/ standard	Distance travelled	MW (k daltons)	Sample/ standard	Distance travelled	MW (k daltons)
BSA	13 mm	68.0	BSA	13 mm	68.0
alcohol dehydrog.	20 mm	41.0	alcohol dehydrog.	22 mm	41.0
myoglobin	47 mm	17.2	myoglobin	44 mm	17.2
cytochrome C	52 mm	12.1	cyt. C	49 mm	12.1
			Band 1	7 mm	85.6
			Band 2	9 mm	77.5
Band 1	13.5 mm	63.4	Band 3	11 mm	70.1
Band 2	14.5 mm	60.3	Band 4	16 mm	57.4
Band 3	20 mm	49.4	Band 5	17 mm	54.6
			Band 6	18 mm	51.9
			Band 7	20 mm	47.0
Band 4	26 mm	36.6	Band 8	25 mm	36.6
Band 5	30 mm	31.5	Band 9	32 mm	27.1

The absence of [^{3}H]GA_{36} concurs with the results of cell-free incubations of total enzyme preparations where conversion of GA_{36} to GA_4 occurred readily, with no substrate remaining at the end of the incubation period. It would seem unlikely that the enzyme activities responsible for the observed metabolism could be separated by alteration of the FPLC conditions, as the [^{3}H]GA_{24} zone starts marginally before the others and ends after them.

Post FPLC incubation of [^{3}H]GA_{14} produced one peak with the reversed-phase HPLC R_t of [^{3}H]GA_{37} which further supports the above view that [^{3}H]GA_4 production was via [^{3}H]GA_{24}, (Table 7, Fig. 20B).

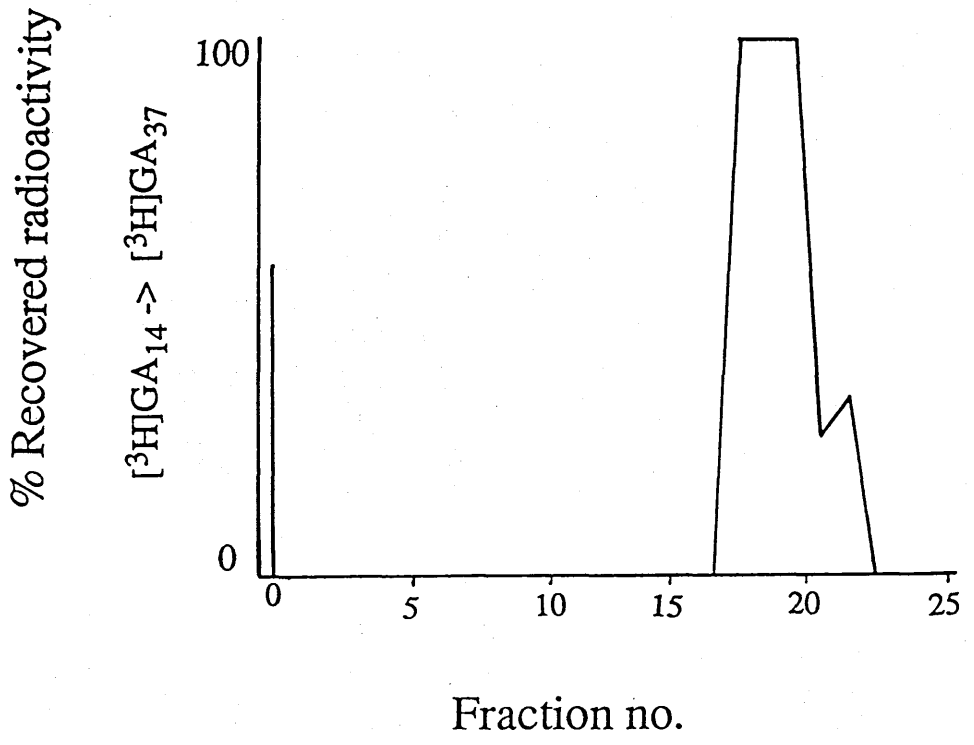


Figure 20B Post FPLC incubation of cell-free preparation from immature seed of *Phaseolus coccineus* cv. Prizewinner with $[^3H]GA_{14}$. All fractions were assayed, but only the enzymic conversions and their distributions in the active region are shown. Fraction 0 represents incubation of an aliquot of total enzyme preparation after filtration, but prior to FPLC.

Estimation of endogenous GA_{20} content of seedlings of Prizewinner and Hammonds Dwarf Scarlet.

Three batches of Prizewinner and Hammonds Dwarf Scarlet, comprising 100 seeds per batch, were separately grown, extracted and analysed for endogenous GA_{20} content. Plants were grown as described in the Materials and Methods section, harvested and deuterated GA_{20} in methanol added, prior to homogenisation. Following extraction and purification by Sephadex DEAE-25, samples were fractionated by gradient elution reversed-phase HPLC (programme: 0-15 min, 50-80% MeOH; 15-19 min, 80-100% MeOH) and the GA_{20} region (8.5 - 10 min), retained and dried *in vacuo* prior to analysis by combined GC-MS.

In order to estimate the amount of deuterated GA_{20} added to each sample, known amounts of GA_3 standard were subjected to isocratic elution reversed-phase HPLC with an absorbance monitor operating at 208 nm. A standard curve plot of peak height against ng was constructed. Aliquots of deuterated GA_{20} dissolved in methanol, were similarly analysed and the amount μl^{-1} was deduced from the GA_3 standard curve. It was then possible to calculate the weight of deuterated GA_{20} added g^{-1} tissue and to relate this to the endogenous GA_{20} content, via the ratio of M^+: M^++2 obtained by GC-MS analysis.

Due to the small quantity of available sample, it was not feasible to compare ions other than the M^+ and the M^++2. Combined GC-MS of TMS derivatives was performed in the multiple ion monitoring (MIM) mode and as the GA of interest was GA_{20}, m/z 418 and 420 were scanned.

Table 9. Estimation of endogenous GA_{20} content in seedlings of Prizewinner and Hammonds Dwarf Scarlet by GC-MIM using $[^2H_2]GA_{20}$ as an internal standard.

Sample	m/z 418/420 %	$[^2H_2]GA_{20}$ added ($ng\,g^{-1}$)	Endog. GA_{20} ($ng\,g^{-1}$)	Mean
Prizewinner A	44	1.78	0.78	
Prizewinner B	44	3.25	1.43	1.14
Prizewinner C	44	2.76	1.21	
Hammonds A	25.5	2.53	0.64	
Hammonds B	34	3.88	1.32	0.95
Hammonds C	26	3.46	0.95	

$[^2H_2]GA_{20}$ m/z 420 (100%), m/z 418 (1.2%)

Table 9 shows the m/z 418 (endogenous) expressed as a % of m/z 420 (deuterated) and from the weight of deuterated GA_{20} added g^{-1} tissue, an estimate of the endogenous GA_{20} was calculated. There is no obvious difference between estimates of endogenous GA_{20} in Prizewinner and Hammonds Dwarf Scarlet. Although it would have been more useful to investigate GA_1 and GA_4 contents in dwarf and tall varieties, sufficiently pure deuterated standards were not available.

GA metabolism in seedlings.

Seven days old seedlings of Prizewinner (tall) and Hammonds Dwarf Scarlet (dwarf) were injected with radio-labelled GA at the base of the epicotyl. After 5 h, the plant tissue was homogenised in methanol prior to petroleum ether/aqueous (pH 8.0), then ethyl acetate/aqueous (pH 2.5) partitioning. In each experiment, all but negligible radioactivity remained in the ethyl acetate fraction and the aqueous extract was then discarded. After further purification, the nature of the metabolites was investigated by HPLC.

Metabolism of [^{3}H]GA$_4$

When fed with [^{3}H]GA$_4$ (730,000 dpm seedling^{-1}, 10 seedlings, 135 ng seedling^{-1}), extracts from both Prizewinner and Hammonds Dwarf Scarlet yielded a metabolite with the HPLC R_t of [^{3}H]GA$_1$ and a [^{3}H]GA$_8$-like peak (Figs. 21 and 22). These identities were confirmed by co-chromatography of the metabolites with [^{3}H]-labelled GA standards by reversed-phase HPLC (Table 10).

Table 10. HPLC-RC data on products of [^{3}H]GA$_4$ application to seedlings of *Phaseolus coccineus* cv. Prizewinner and cv. Hammonds Dwarf Scarlet.

Cultivar	Applied GA	Putative product	% Recovered radioactivity	Standard R_t (min)	Product R_t(min)	% MeOH
Prizewinner	[^{3}H]GA$_4$	[^{3}H]GA$_1$	43	12.3	12.3	35
		[^{3}H]GA$_8$	19	5.6	5.6	35
Hammonds	[^{3}H]GA$_4$	[^{3}H]GA$_1$	58	12.5	12.5	35
		[^{3}H]GA$_8$	16	5.8	5.8	35

A number of other unidentified products were obtained, one of which had the same R_t as GA$_4$ glucosyl ester. The possibility that GA$_{34}$ is among the products was not investigated but as Turnbull *et al.* (1988) observed [^{3}H]GA$_{34}$ when [^{3}H]GA$_4$ was fed to 24 h germinated seeds of *Phaseolus coccineus*, this cannot be ruled out. It is interesting to note that while seedlings readily metabolise [^{3}H]GA$_4$ to [^{3}H]GA$_1$, no metabolism of this substrate was observed on incubation with seed cell-free preparation from either cultivar.

Metabolism of [^{3}H]GA$_{20}$

Extracts derived from 15 Prizewinner seedlings fed with [^{3}H]GA$_{20}$

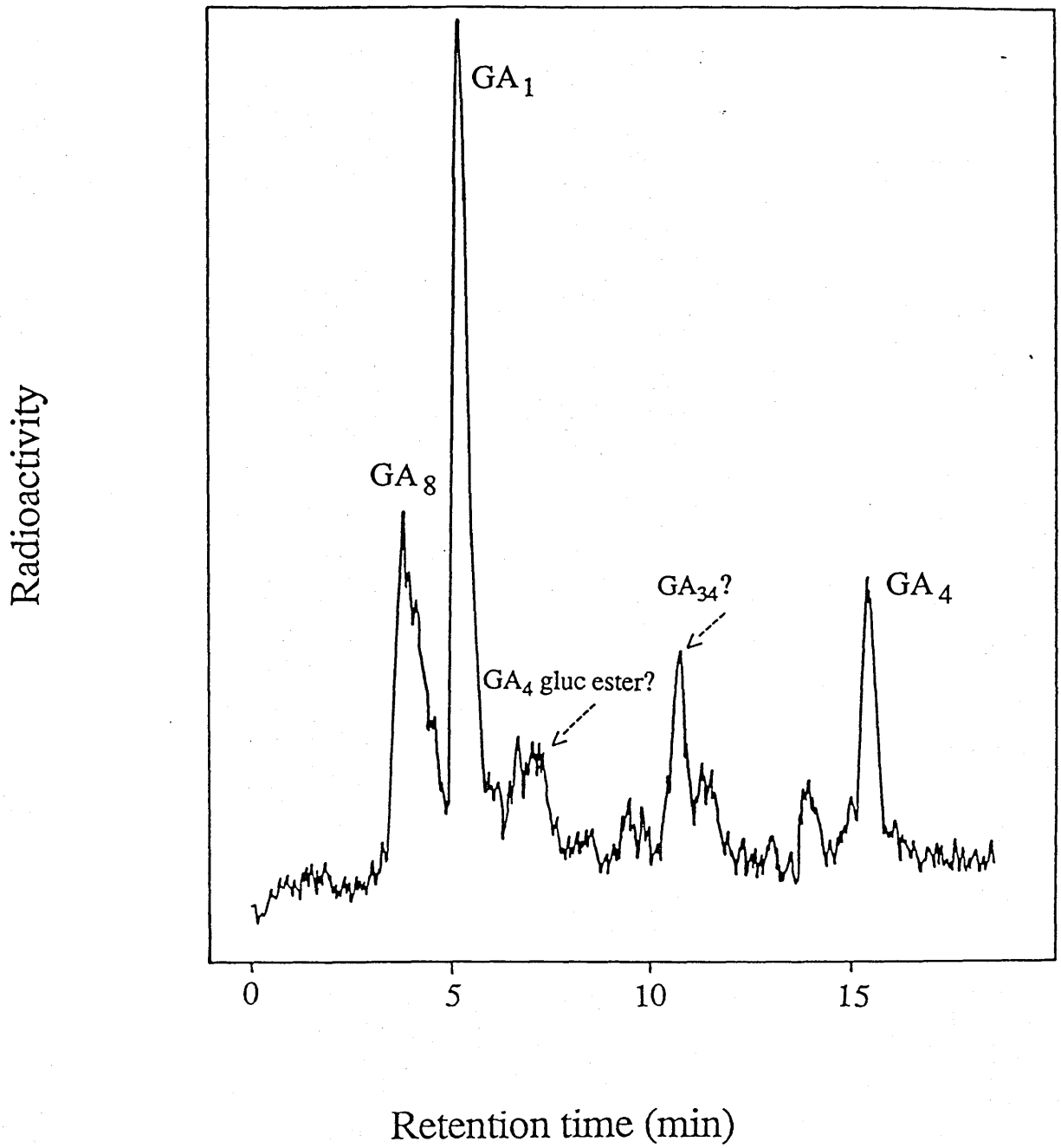


Figure 21 Metabolism of [^{3}H]GA_4 by seedlings of *Phaseolus coccineus* cv. Prizewinner. HPLC conditions as for Fig. 6. Sample: 100,000 dpm. Detector: radioactivity monitor.

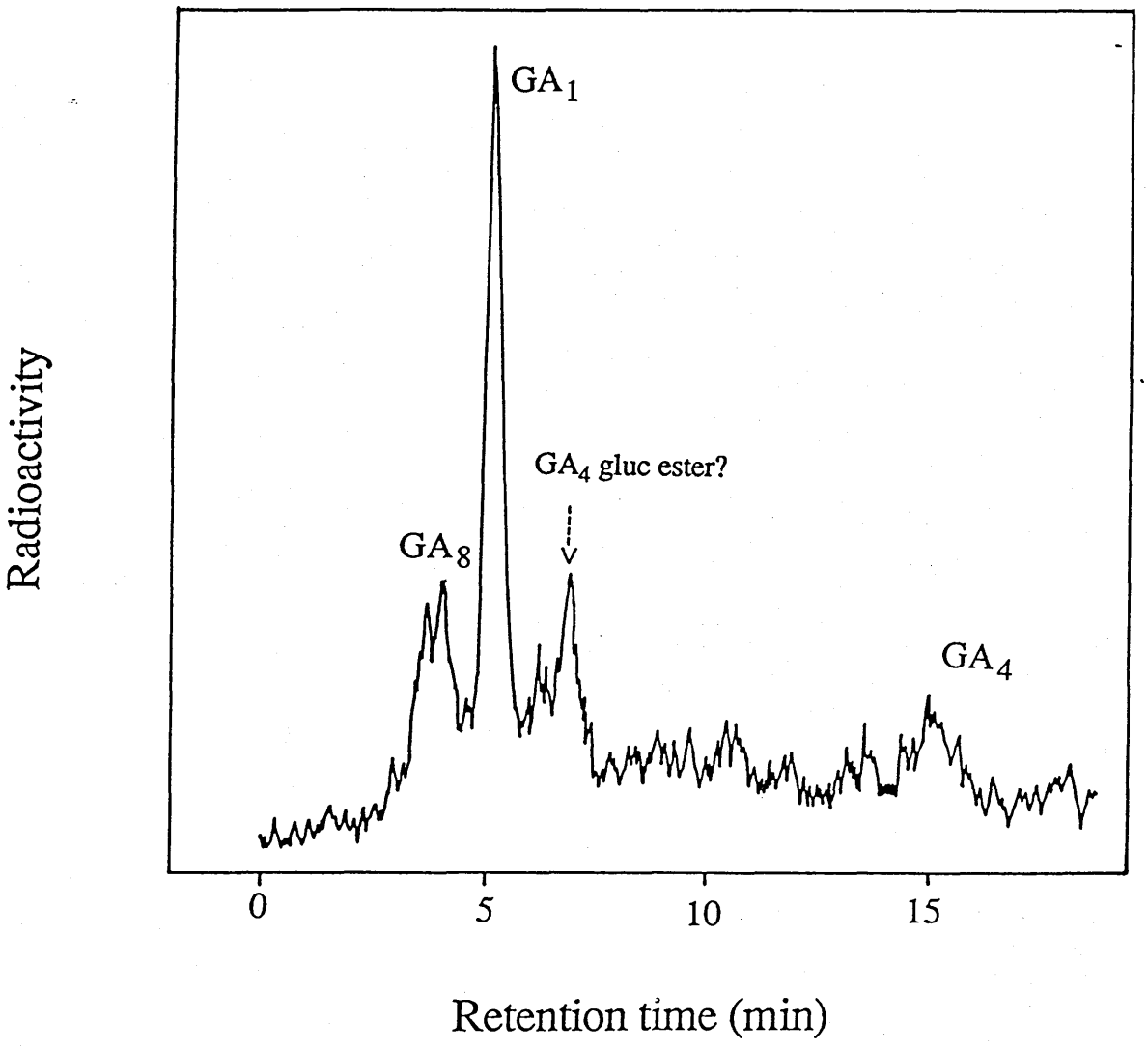


Figure 22 Metabolism of [^{3}H]GA$_4$ by seedlings of *Phaseolus coccineus* cv. Hammonds Dwarf Scarlet. HPLC conditions as for Fig. 6. Sample: 80,000 dpm. Detector: radioactivity monitor.

(430,000 dpm, 45 ng seedling^{-1}) yielded a GA_5-like peak and a minor product with the HPLC R_t of GA_1, in addition to several unidentified metabolites (Fig. 23A). A similar experiment with Hammonds Dwarf Scarlet seedlings also produced HPLC peaks with the R_t of GA_1 and GA_5 (Fig 23B).

Table 11. HPLC-RC data on products of [^{3}H]GA_{20} application to seedlings of *Phaseolus coccineus* cv. Prizewinner and cv. Hammonds Dwarf Scarlet.

Cultivar	Applied GA	Putative product	% Recovered radioactivity	Standard R_t (min)	Product R_t (min)	% MeOH
Prizewinner	[^{3}H]GA_{20}	[^{3}H]GA_1	31	11.8	11.8	35
		[^{3}H]GA_5	7	11.3	11.3	50
Hammonds	[^{3}H]GA_{20}	[^{3}H]GA_1	43	11.8	11.8	35

These identities were confirmed by co-chromatography of the metabolites with [^{3}H]-labelled GA standards by reversed-phase HPLC (Table 11). None of the unidentified metabolites corresponded in R_t with any of the available GA_1, GA_5, GA_8 or GA_{20} conjugates.

Metabolism of [^{3}H]GA_5

When fed to seedlings of Prizewinner and Hammonds Dwarf Scarlet (250,000 dpm, 7.2 ng seedling^{-1}, 10 seedlings), [^{3}H]GA_5 was not further metabolised. Nash (1976) fed [^{3}H]GA_5 to *Phaseolus coccineus* seedlings and obtained only minor quantities of a product tentatively identified as [^{3}H]GA_8 after enzymic hydrolysis of the acidic butanol-soluble fraction. These results are in contrast to those obtained when [^{3}H]GA_5 is incubated with seed cell-free preparation from Prizewinner, to produce [^{3}H]GA_6 by epoxide formation between C-2 and C-3.

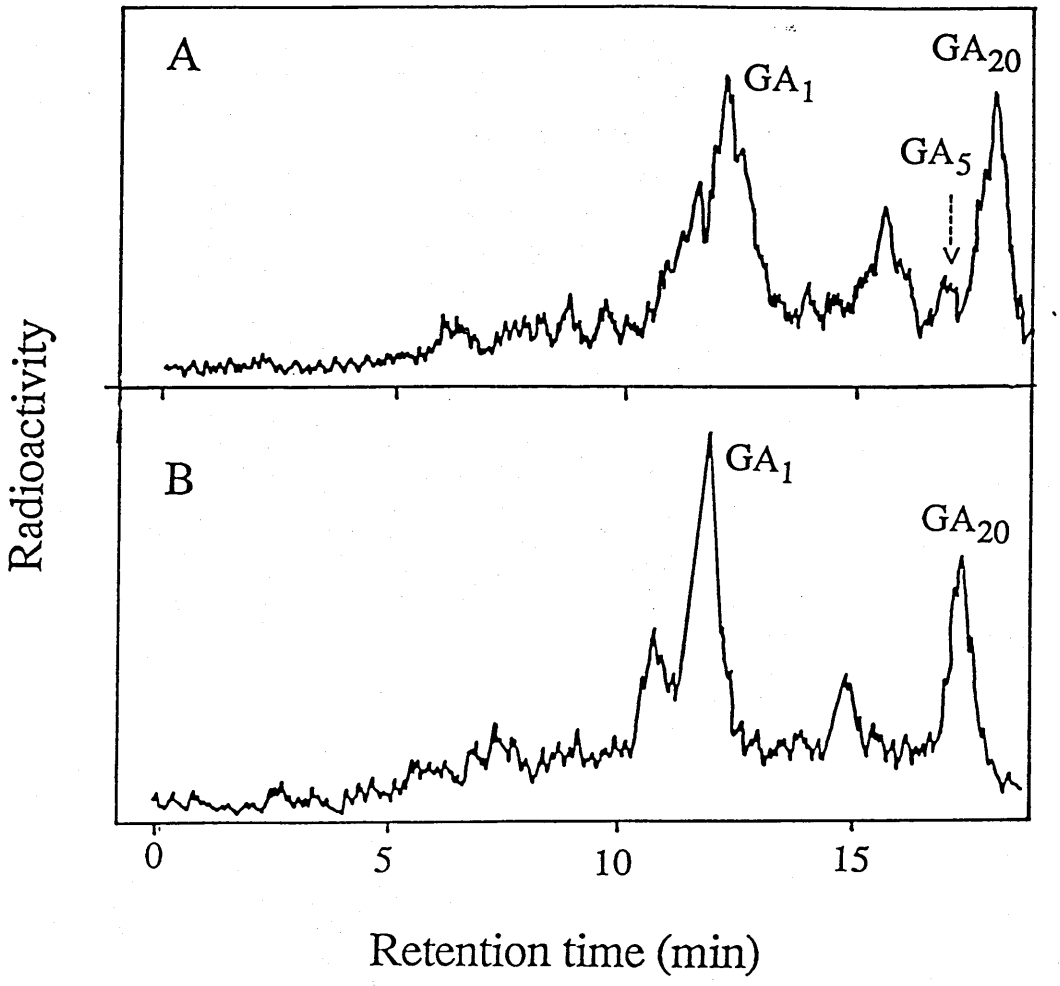


Figure 23 Metabolism of [^{3}H]GA_{20} by seedlings of *Phaseolus coccineus* A) cv. Prizewinner and B) cv. Hammonds Dwarf Scarlet. HPLC conditions as for Fig. 6. Programme: 0-15 min, 35-65% B; 15-18 min, 65-100% B; 18-22 min, 100% B. Sample: 40,000 dpm. Detector: radioactivity monitor.

Metabolism of [^{3}H]GA$_1$

[^{3}H]GA$_1$ (430,000 dpm, 2.3 ng seedling^{-1}, 10 seedlings) was fed to seedlings of Prizewinner and Hammonds Dwarf Scarlet. After extraction, both samples contained a peak with the HPLC R_t of [^{3}H]GA$_8$ (Fig. 24). This conversion was confirmed by analysis of the metabolites by reversed-phase HPLC (Table 12). In contrast, no conversion of [^{3}H]GA$_1$ was observed when this GA was used as a substrate with S-1 seed preparation from Prizewinner or Hammonds Dwarf Scarlet.

Table 12. HPLC-RC data on products of [^{3}H]GA$_1$ application to seedlings of *Phaseolus coccineus* cv. Prizewinner and cv. Hammonds Dwarf Scarlet.

Cultivar	Applied GA	Putative product	% Recovered radioactivity	Standard R_t (min)	Product R_t (min)	% MeOH
Prizewinner	[^{3}H]GA$_1$	[^{3}H]GA$_8$	55	5.4	5.4	35
Hammonds	[^{3}H]GA$_1$	[^{3}H]GA$_8$	36	5.6	5.6	35

Further investigation of [^{3}H]GA$_4$ metabolism

Having established that the main metabolic route to GA$_1$, in seedlings, proceeded via GA$_4$, it was then decided to investigate whether any root/shoot differences in GA metabolism existed.

[^{3}H]GA$_4$ (300,000 dpm, 55 ng seedling^{-1}, 20 seedlings) was injected at the base of the epicotyl as before, but prior to extraction, root and shoot tissues were separated. HPLC analysis of root extracts from Prizewinner and Hammonds Dwarf Scarlet with 25% and 19% of the recovered radioactivity respectively, each contained a GA$_1$-like peak and a number of other unidentified products and the Prizewinner extract also yielded a GA$_8$-like product (Fig. 25). These

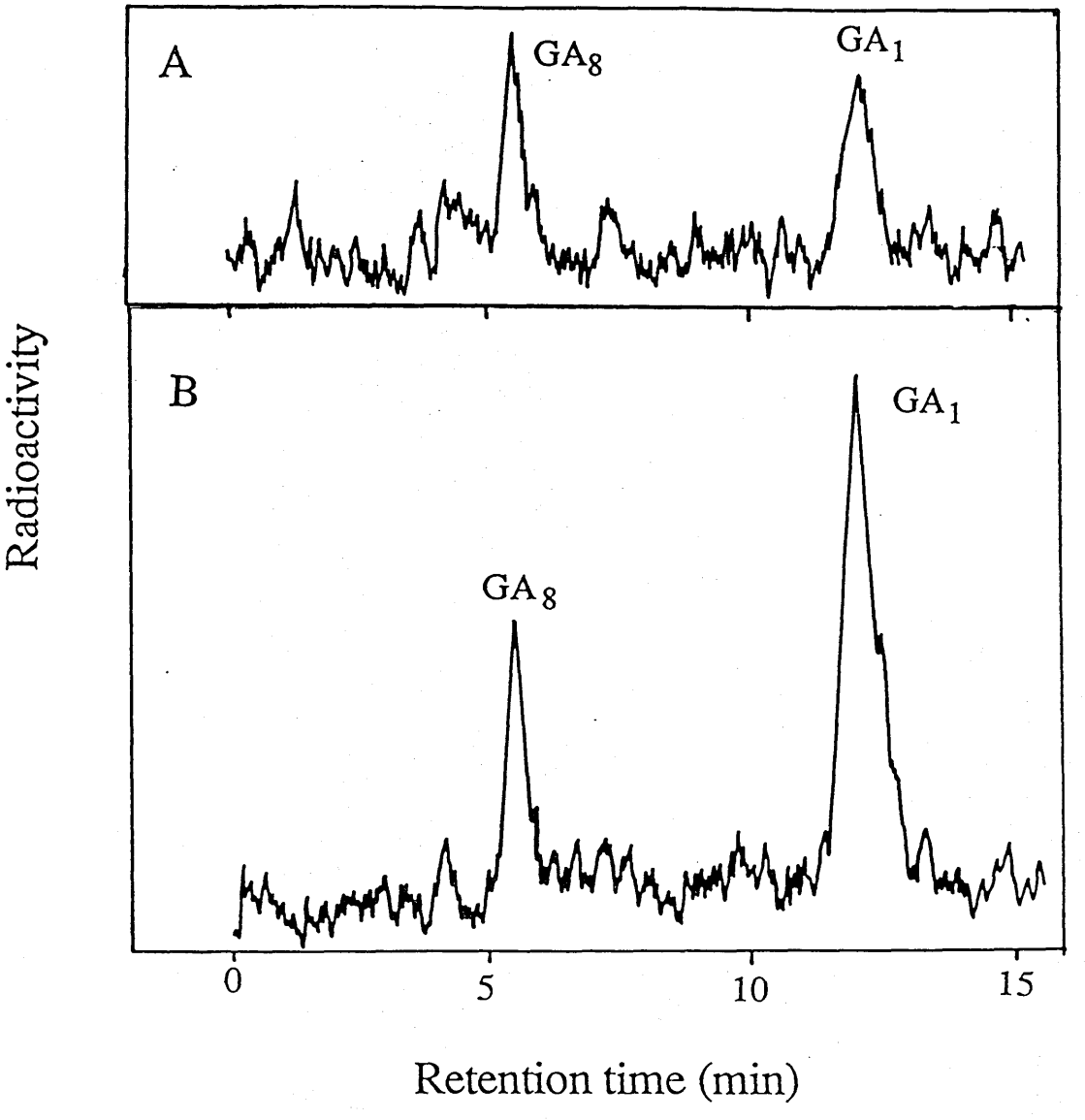


Figure 24 Metabolism of [^{3}H]GA$_1$ by seedlings of *Phaseolus coccineus* A) cv. Prizewinner and B) cv. Hammonds Dwarf Scarlet. Isocratic reversed-phase HPLC 35% B. Sample: A) 35,000 dpm B) 45,000 dpm. Detector: radioactivity monitor.

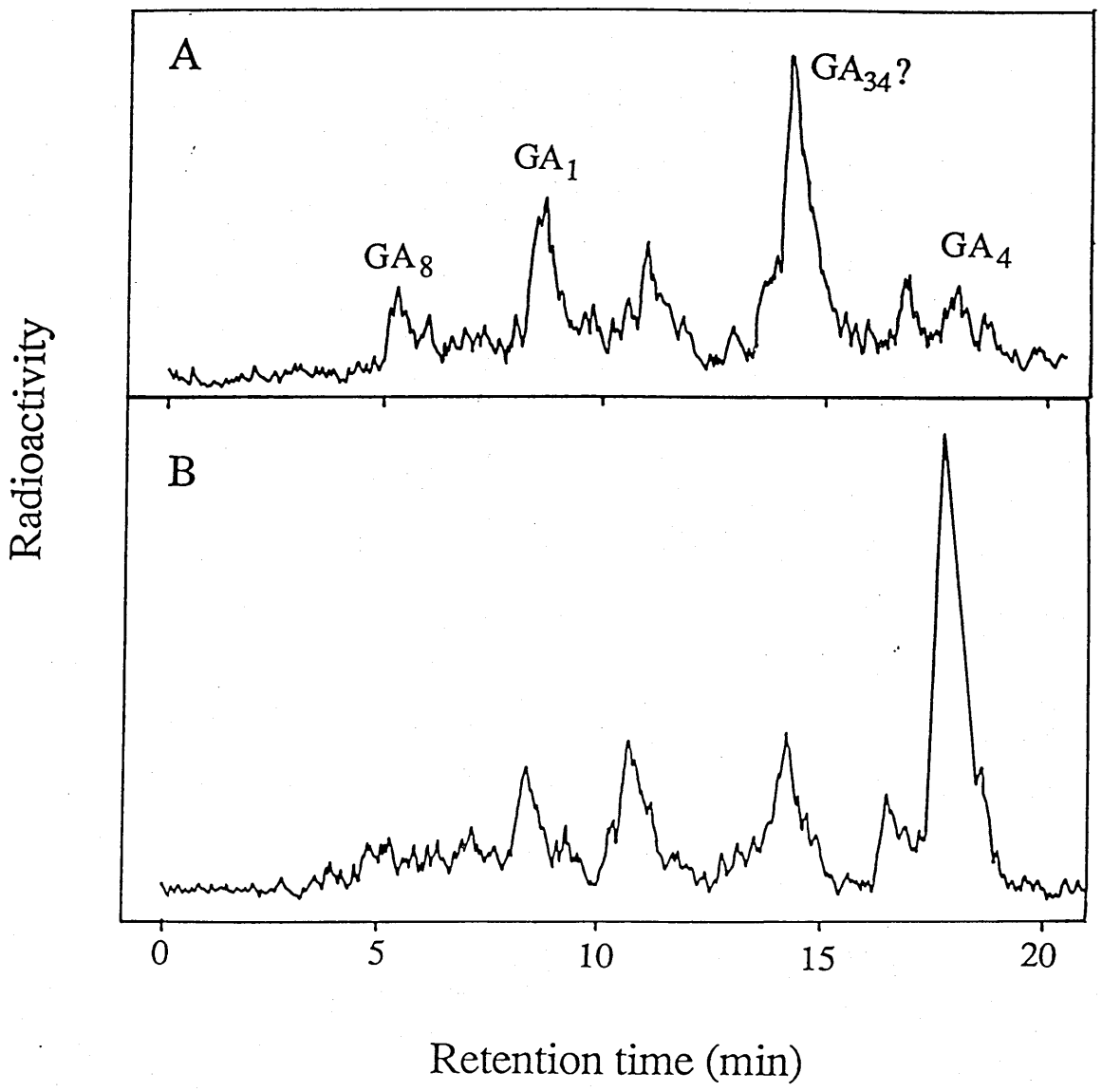


Figure 25 Metabolism of [^{3}H]GA_4 in roots of seedlings of *Phaseolus coccineus* A) cv. Prizewinner and B) cv. Hammonds Dwarf Scarlet. Reversed-phase HPLC conditions as for Fig. 6 Programme: 0-15 min, 40-80% B; 15-18 min, 80-100% B;18-22 min, 100% B. Sample: A) 35,000 dpm and B) 50,000 dpm. Detector: radioactivity monitor.

tentative identifications were confirmed by co-chromatography of sample peaks with the appropriate [^{3}H]-labelled GA standards by reversed-phase HPLC (Table 13).

Table 13. HPLC-RC data on products of [^{3}H]GA_4 metabolism accumulating in roots of *Phaseolus coccineus* cvs. Prizewinner and Hammonds Dwarf Scarlet.

Cultivar	Standard/ substrate	Putative product	% Recovered radioactivity	Standard R_t(min)	Product R_t(min)
Prizewinner	[^{3}H]GA_4	[^{3}H]GA_1	20	8.4	8.4
		[^{3}H]GA_8	10	4.9	4.9
Hammonds	[^{3}H]GA_4	[^{3}H]GA_1	12	8.4	8.4

Mobile phase: gradient 0 min - 40% MeOH in 1% acetic acid, 15 min - 80% MeOH, 18 min - 100% MeOH.

Turnbull *et al.* (1986b) fed [^{3}H]GA_4 to 5 days old *Phaseolus coccineus* seedlings and obtained GA_4-3-*O*-glucoside, GA_4-glucosyl ester, GA_8-2-*O*-glucoside and a GA_{34}-*O*-glucoside, in addition to GA_1 and GA_8. When analysed by HPLC, extracts from Prizewinner and Hammonds Dwarf Scarlet shoot tissue, containing 75% and 81% of the recovered radioactivity respectively, showed the same overall profile of metabolites, both to each other and to those observed with root extracts, including the presence of GA_1- and GA_8-like peaks (Fig. 26).

Table 14. HPLC-RC data on products of [^{3}H]GA_4 metabolism in shoots of *Phaseolus coccineus* cvs. Prizewinner and Hammonds Dwarf Scarlet.

Cultivar	Applied GA	Putative product	% Recovered radioactivity	Standard R_t (min)	Product R_t (min)
Prizewinner	[^{3}H]GA_4	[^{3}H]GA_1	19	8.3	8.3
		[^{3}H]GA_8	13	5.0	5.0
Hammonds	[^{3}H]GA_4	[^{3}H]GA_1	20	8.5	8.5
		[^{3}H]GA_8	14	5.2	5.2

Mobile phase : gradient 0 min - 40% MeOH in 1% acetic acid, 15 min -80% MeOH, 18 min - 100% MeOH.

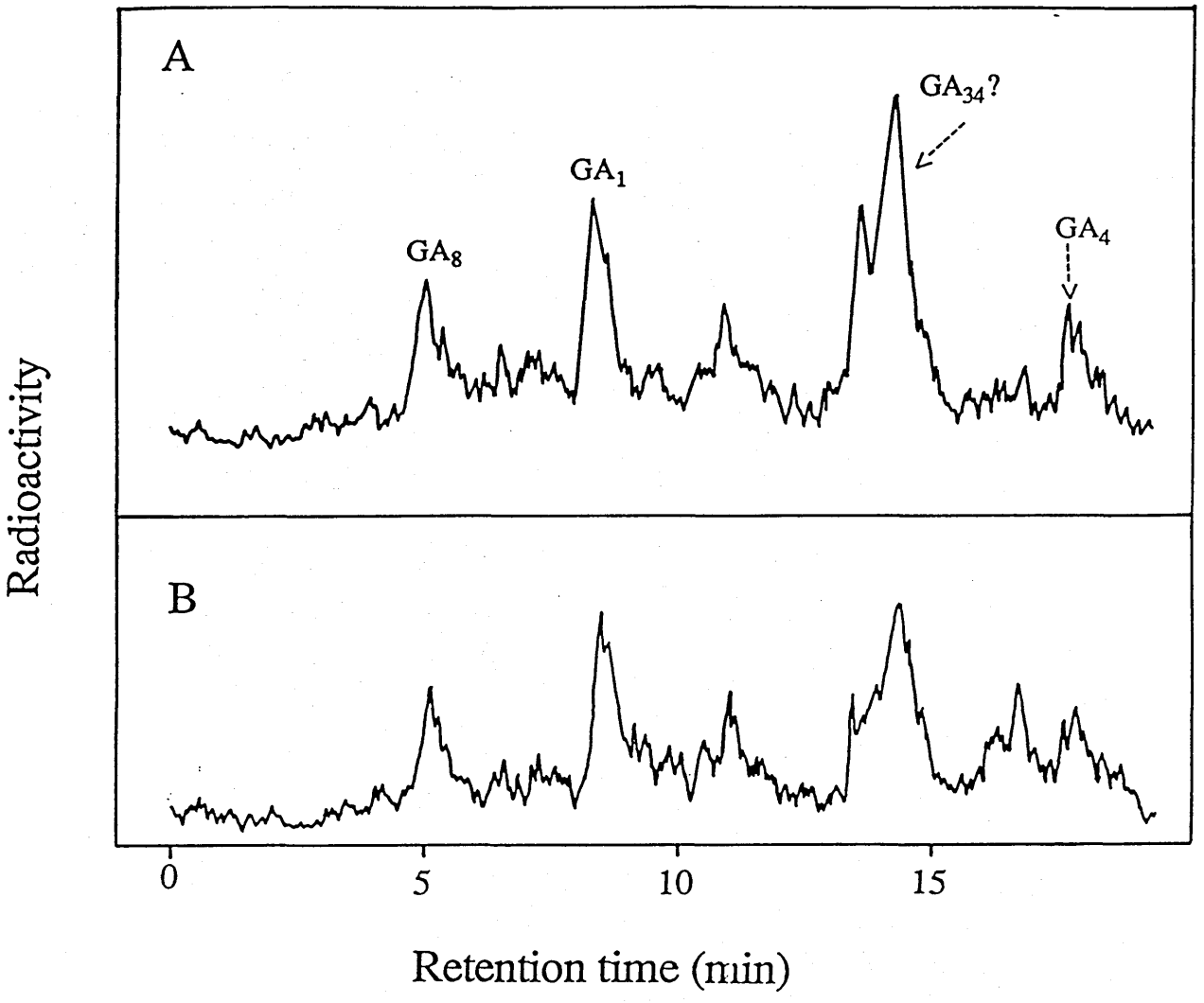


Figure 26 Metabolism of [^{3}H]GA_4 in shoots of seedlings of *Phaseolus coccineus* A) cv. Prizewinner and B) cv. Hammonds Dwarf Scarlet. Reversed-phase HPLC conditions as for Fig.6. Sample: A) 50,000 dpm and B) 35,000 dpm. Detector: radioactivity monitor.

The identities of these metabolites were also confirmed by co-chromatography with [^{3}H]-labelled GA standards by reversed-phase HPLC (Table 14).

Although roots and shoots fed with [^{3}H]GA_4 were examined separately in this study, the seedlings were intact at the time of [^{3}H]-labelled GA application. It cannot therefore be argued that both roots and shoots metabolise [^{3}H]GA_4 but it can be concluded that if conversion is occurring in only one of the two regions, then transport of the full range of products to the other region takes place.

DISCUSSION

Before discussing the *in vitro* GA interconversion sequence in seeds of *Phaseolus coccineus*, the general metabolic trends and physiological implications will be considered briefly. Seeds are known to contain relatively large endogenous pools of a variety of GAs (see Sponsel, 1983), whose levels vary with the stage of seed development attained. For example, Durley *et al.* (1971) examined changes in the endogenous levels of GA_1, $GA_{5/20}$, GA_6 and GA_8 in seeds of *Phaseolus coccineus* during maturation. They found that the concentrations of GA_1 and $GA_{5/20}$ were low throughout development but dropped almost to zero at maturation. In contrast, the GA_6 level was high at the immature seed stage, declining to zero at maturation, while GA_8 showed high accumulation at both extremes of the study period, with a reduction in the middle.

The incidences of 2β-hydroxylated GAs (Sponsel, 1987) and conjugated GAs (Schneider, 1983) are reported to increase towards the end of seed maturation. This view is supported by the observation of 2β-hydroxylation of both $[^3H]GA_1$ and $[^3H]GA_4$ in cell-free preparations from mature, imbibed seeds of *Phaseolus coccineus* (Turnbull and Crozier, 1988). However, Garcia-Martinez *et al.* (1987) found GA_1, GA_8, GA_{20} and GA_{29} at very early developmental stages in pea seeds, but only GA_{20} and GA_{29} as maturation progressed. Regarding a role for these GAs, pod growth was correlated with GA_1 content early in development, but it was concluded that the endogenous GA_{20} and GA_{29} observed in older seeds did not affect maturation or subsequent germination.

The possibility that GAs from suspensor tissue influence early embryo development by their transportation and subsequent regulation of protein synthesis was discussed by Quatrano (1987).

Ceccarelli *et al.* (1979;1981b) demonstrated with *Phaseolus coccineus* that embryos from which the suspensors were removed did not survive and suspensor tissue both synthesised and metabolised radio-labelled GA precursors to C_{19}-GAs (Ceccarelli *et al.*, 1979; 1981b).

The work of Zeevaart (1966) on *Pharbitis nil* seeds suggests that GAs exert little or no influence on growth, at least during the later stages of development. If CCC, an inhibitor of *ent*-kaurene synthetase (see Coolbaugh, 1983) is applied before flower opening, the resultant seeds show a dramatic reduction in extractable GA-like activity and ultimately abort. Treatment of seeds with inhibitor three weeks after anthesis also resulted in depleted GA levels but there was no difference in the final fresh/dry weight of treated and control seeds. The resultant seedlings from retardant-treated seeds were dwarfed. However, it cannot be concluded that this is a consequence of a lack of GAs during seed maturation, as the plants contained residual CCC which could limit the rate of shoot growth by inhibiting *de novo* GA biosynthesis.

In summary, no function has been established for GAs during the later stages of seed maturation. Although no clear picture emerges as to seed GA function compared to those in seedlings, seed systems provide a useful starting point for determining the GA metabolic potential of a species.

A study of GA metabolism by cell-free preparations from immature seed of Prizewinner (tall) established that many of the steps in the hypothetical scheme shown in Fig. 5 were occurring. [^{14}C]GA_{12}-aldehyde was found to be oxidised at C-7, C-20 and hydroxylated at C-3 and/or C-13 to produce a range of GAs whose metabolism proceeds along two parallel routes to GA_1. The 13-hydroxylated branch comprises GA_{53}, GA_{44}, GA_{19}, GA_{20} and GA_1 while the non-13-hydroxylated pathway is from GA_{15} to GA_{37}, GA_{24}, GA_{36} and GA_4.

Incubation of intermediates on the non-13-hydroxylated route, namely [^{3}H]GA_{14}, [^{3}H]GA_{24} and [^{3}H]GA_{36}, yielded [^{3}H]GA_4 but not [^{3}H]GA_1 (Tables 2 and 3). Incubation of [^{3}H]GA_4 itself, revealed that the cell-free preparation derived from immature seeds of *Phaseolus coccineus* could not 13-hydroxylate [^{3}H]GA_4 to [^{3}H]GA_1.

In contrast, immature seed cell-free preparation from Prizewinner metabolised [^{3}H]GA_{20} to [^{3}H]GA_1, [^{3}H]GA_5 and a number of other unidentified products (Fig. 14, Table 6). This led to the conclusion that the 13-hydroxylated branch (Fig. 5) is the main metabolic route to GA_1 in this tissue. However, when [^{14}C]GA_{12}-aldehyde is incubated with Prizewinner cell-free preparation, comparison of [^{14}C]GA_4 and [^{14}C]GA_1 accumulation reveals [^{14}C]GA_4 to be the more abundant product, (Fig. 6).

The probable GA metabolism pathway from GA_{12}-aldehyde through to GA_1, GA_4 and GA_6 in *in vitro* seed preparations from *Phaseolus coccineus*, cv. Prizewinner is summarised in Fig. 27. Without a hypothesised physiological role for GAs in seeds, it is difficult to discuss the significance of specific observed steps and the absence of others. However, considering the generally greater biological activity of C_{19}- than C_{20}-GAs in bioassay systems (Crozier, 1981), it will be assumed that if any seed GAs perform a regulatory function, it will be the C_{19} compounds. Although the importance of GA_1 for development has been demonstrated for seedlings, (Phinney and Spray, 1982), the situation in seeds is not known and the possibility remains that GA_4 *per se* controls some aspect of development.

Alternatively, the greater accumulation of [^{14}C]GA_4 than [^{14}C]GA_1 when [^{14}C]GA_{12}-aldehyde is incubated, may be a consequence of the *in vitro* nature of these enzymatic steps. The disruption caused to the system may be sufficient to result in "abnormal" ratios of each metabolic branch. The possibility remains that the lack of [^{3}H]GA_4

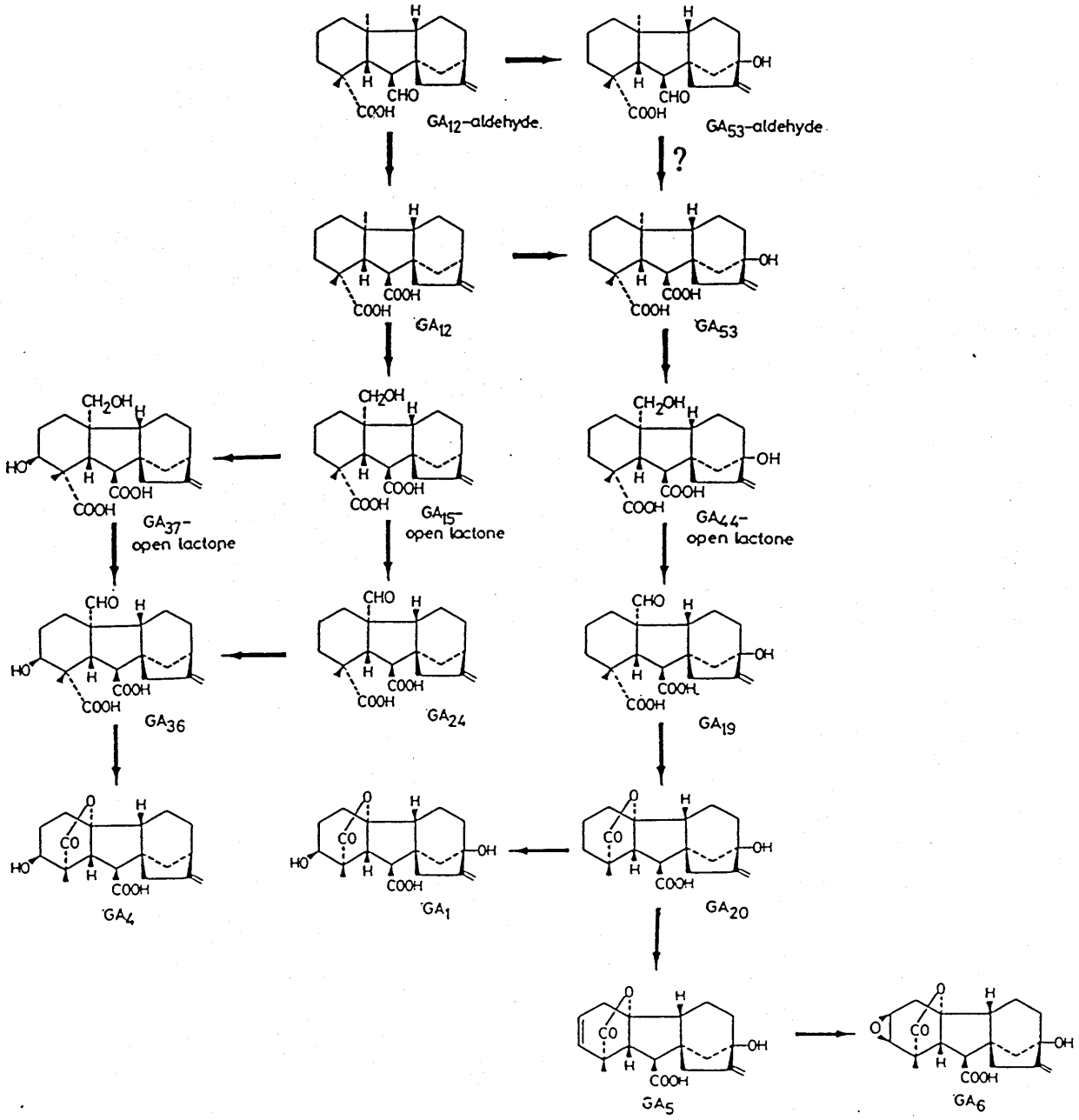


Figure 27 Summary of the probable GA metabolic steps occurring in *Phaseolus coccineus* seeds, cv. Prizewinner.

conversion can be similarly explained.

A third explanation for the observed results is that GA_1 synthesis is important throughout the life cycle of *Phaseolus coccineus* but that the metabolic route leading to its formation can be altered. That a cell-free preparation produced from the cotyledons of mature, 24 h imbibed Prizewinner seeds did not metabolise [^{3}H]GA_{20} but did metabolise [^{3}H]GA_4 (Turnbull and Crozier, 1988), would suggest that between the immature seed stage and the onset of germination, a "switch" in the method of GA_1 production occurs.

2β-Hydroxylation, regarded as a deactivation step, has not been observed with the *Phaseolus* seed cell-free system. This may reflect the removal of the testas prior to enzyme preparation, as GA_8 was found to accumulate in the testas of *Phaseolus coccineus* seeds (Albone *et al.*, 1984). Alternatively, as already discussed, the absence of GA_8 in the immature seed may be due to the stage of development of the seed under study, as *in vitro* preparations from mature, imbibed *Phaseolus coccineus* seeds 2β-hydroxylate both [^{3}H]GA_1 and [^{3}H]GA_4 to[^{3}H]GA_8 and[^{3}H]GA_{34} respectively, (Turnbull and Crozier, 1988).

Incubations of radio-labelled GA substrates with S-1 preparation from Hammonds Dwarf Scarlet were performed to investigate possible differences from Prizewinner and especially, the capacity for 3β-hydroxylation of GA_{20}. Overall, GA metabolism by the dwarf preparation was found to be very similar to that of Prizewinner with GA_1 synthesis from GA_4 absent, but 3β-hydroxylation of GA_{20} occurring readily. If specific GA biosynthetic routes can be genetically switched off and on at various developmental stages, then GA_1 synthesis with the dwarf seed preparation does not guarantee its presence in seedlings.

In summary, the range of GA conversions observed with a cell-free preparation from immature seeds of the tall cultivar

Prizewinner followed closely those predicted from a knowledge of the endogenous compounds of this tissue, except that GA_4 is not 13-hydroxylated to produce GA_1. No qualitative differences in GA metabolism were found when experiments with preparations from the dwarf variety were carried out. The cell-free preparations from tall and dwarf immature seeds provided a useful and experimentally versatile starting point for the elucidation of GA metabolism in *Phaseolus coccineus*, and also made possible an investigation of the nature of the enzymes involved and prompted *in vivo* metabolic studies with seedlings.

As the 3β-hydroxylation of GA_{20} is the final step leading to the production of GA_1 in the *in vitro* preparations from *Phaseolus coccineus* seeds, a preliminary investigation of the nature of this enzyme was undertaken. Aliquots of seed cell-free preparation were subjected to FPLC and all the resultant fractions assayed for conversion of $[^3H]GA_{20}$ to $[^3H]GA_1$. The fractions in which enzyme activity was situated were then analysed by SDS-PAGE to examine the constituent polypeptides. Gels of Prizewinner and Hammonds Dwarf Scarlet seed cell-free samples were found to have similar band patterns with subunits in common at approximately 30 k daltons, 36.6 k daltons and 50 k daltons. At this stage of experimentation, it is not possible to conclude which, if any, subunit(s) is responsible for the observed 3β-hydroxylation of GA_{20}, or for any of the other observed conversions which co-elute with this enzymic activity. Further experimentation might include attempts to separate the various enzymic activities with a view to further purification and characterisation. This might be achieved by sample elution from the Mono Q column by a more shallow gradient, followed by purification by gel filtration on a Superose 12 column. Again, assays would be performed to ascertain the position and nature of enzyme activity. SDS-PAGE of the active fractions could then be used to examine

whether bands obtained after FPLC on Mono Q only, were lost after an additional purification step. Any such bands could then be eliminated as regards their contribution to the observed enzyme activity.

For comparison with this study, results obtained by Smith and MacMillan (1984), showed that a 2β-hydroxylating enzyme from mature seed of *Phaseolus vulgaris*, purified by DEAE-cellulose chromatography, CM-cellulose chromatography and gel filtration, had a molecular weight of 36 k daltons and the characteristics of a dioxygenase.

On the basis of incubations of seed cell-free preparation with radio-labelled GAs and these preliminary FPLC/SDS-PAGE data, it is concluded that at the immature seed stage of the life cycle, there are no discernible differences between GA metabolism in tall and dwarf cultivars of the runner bean. Although it should be noted that these constitute *in vitro* results, most of the GAs involved are known endogenous components of this tissue (Sponsel,1983; Albone *et al.*,1984).

In addition to [^{3}H]GA_{20}, [^{3}H]GA_{12} and [^{3}H]GA_{14} were used as post-FPLC assay substrates. Incubation of Prizewinner cell-free preparation with [^{3}H]GA_{12}, after FPLC, yielded [^{3}H]GA_4, [^{3}H]GA_{24} and [^{3}H]GA_{37} in some fractions. As mentioned in the Results section, GA_4 can arise from GA_{24} or GA_{37} but the complete absence of metabolism beyond GA_{37}, when [^{3}H]GA_{14} is incubated after FPLC, suggests that GA_{24}, rather than GA_{37}, is converted to GA_4. This is supported by the fact that an increase in GA_4 production corresponds with a reduced accumulation of GA_{24} (see Fig 20). The lack of GA_{37} oxidation to GA_{36} (Fig. 20), is interesting when it is remembered that GA_{24} formation from GA_{15} occurs readily and by the same C-20 oxidation step. This suggests that either one enzyme can perform both oxidations, but GA_{24} is much more successful at

competing for active sites, or that two separate oxidation enzymes exist and the GA_{37}-specific protein is separated after FPLC from the fractions in which GA_{37} production occurs. The former idea seems unlikely as it is improbable that one substrate would be exclusively "favoured" over the other. The further possibility that GA_{37} oxidation requires highly specific cofactors is also unlikely as the parallel oxidation of GA_{15} occurs readily over a wide range of fractions. The occurrence of GA_4 in only a narrow band of fractions may reflect the limited distribution of the enzyme catalysing the synthesis of GA_{36} rather than of that converting GA_{36} to GA_4. If restriction of GA_4 synthesis from GA_{36} were occurring, one would expect to observe GA_{36} accumulation outwith the region of GA_4 production and within the zone of GA_{24} synthesis. No GA_{36} accumulation was noted and it was therefore concluded that the GA_{24} to GA_{36} conversion was the rate limiting step.

When $[^{14}C]GA_{12}$ is incubated with total enzyme preparation, both 13- and non-13-hydroxylated pathways occur, yielding $[^{14}C]GA_{15}$, $[^{14}C]GA_{44}$ and $[^{14}C]GA_{53}$ (Fig. 7A). After FPLC, $[^{3}H]GA_{12}$ incubation results only in non-13-hydroxylation to $[^{3}H]GA_4$, $[^{3}H]GA_{24}$ and $[^{3}H]GA_{37}$ (Fig. 20A). It is likely that the 13-hydroxylase, which is particulate in peas (Kamiya and Graebe, 1983), is removed in the filtration step prior to FPLC, hence the GA_{12} to GA_{53} conversion cannot take place.

It is interesting to note that cell-free incubation of total enzyme preparation from Prizewinner with $[^{3}H]GA_{14}$ produced a range of products including $[^{3}H]GA_4$, $[^{3}H]GA_{24}$ and $[^{3}H]GA_{37}$. After FPLC, however, incubation of $[^{3}H]GA_{14}$ with Prizewinner cell-free preparation yielded only $[^{3}H]GA_{37}$ which suggests, as with the $[^{3}H]GA_{12}$ incubation results, that the oxidase which could further metabolise this compound is separated from the fractions in which $[^{3}H]GA_{37}$ is produced.

The evidence, both for and against the hypothesis that GA_1 is the only GA active in the control of stem elongation, was presented in the Introduction. Having established the general metabolic scheme with a cell-free system from immature seed of *Phaseolus coccineus*, a comparison of *in vivo* GA metabolism in seedlings was carried out. If the simplest form of the current hypothesis (Phinney and Spray, 1982), holds true for this species, then seedlings of the dwarf cultivar, Hammonds Dwarf Scarlet, would not be expected to synthesise GA_1.

A number of radio-labelled GAs were fed to tall and dwarf seedlings and the resultant extracts were subjected to reversed-phase HPLC. Both [^{3}H]GA_4 and [^{3}H]GA_{20} were converted by tall and dwarf seedlings to [^{3}H]GA_1 and other products, with the [^{3}H]GA_4 route appearing to predominate. This is in contrast to the results of feeds to the immature seed cell-free preparation, when radio-labelled GA_4 was not metabolised. It can therefore be concluded that in contrast to the *dwarf*-1 mutant of *Zea*, seedlings of Hammonds Dwarf Scarlet have a functional 3β-hydroxylating enzyme and can synthesise GA_1 from GA_{20}. From these experiments alone, the possibility that Hammonds Dwarf Scarlet has an early metabolic block, similar to the *dwarf*-2, *dwarf*-3 and *dwarf*-5 of maize, cannot be dismissed. However, the presence of endogenous GA_{20} in these seedlings, would imply that at least the 13-hydroxylated branch is operational, hence GA_1 production is possible.

No comparison can be made between the *Phaseolus* dwarfing gene and the *le* allele of pea, which is reported only to be expressed in the light, as Hammonds Dwarf Scarlet seedlings were not grown in darkness.

The possibility that Hammonds Dwarf Scarlet seedlings are similar to the GA-deficient *na* pea mutant can be dismissed for the same reason as the *d*2, *d*3 and *d*5 of maize. That is, that the bean

seedlings contain endogenous GA_{20} and can therefore synthesise GAs that occur at the start of the metabolic sequence. However, as previously discussed, the *na* allele of pea is reported only to be expressed in the shoots, with roots of these seedlings possessing endogenous GA pools and the capacity for GA_1 synthesis (Ingram *et al.*,1985). In order to investigate root/shoot GA transport, the epicotyls of seedlings of Prizewinner and Hammonds Dwarf Scarlet were fed with [^{3}H]GA_4 and the root and shoot portions subsequently extracted and analysed separately. The metabolites obtained included [^{3}H]GA_1 and [^{3}H]GA_8 and were similar both for tall and dwarf and between tissues. Clearly, there is no restriction on shoot to root transport but it cannot be concluded from this experiment whether the applied GA is transported prior to metabolism, whether the metabolites from the shoot are exported or whether a combination of the two is occurring. This experiment does not permit any conclusions to be made about root to shoot transport of GAs.

Feeding [^{3}H]GA_5 to tall and dwarf *Phaseolus coccineus* seedlings produced no metabolites, whereas with the immature seed cell-free system, [^{3}H]GA_6 was synthesised.

[^{3}H]GA_8 is a product of both [^{3}H]GA_4 and [^{3}H]GA_{20} feeds, via 2β-hydroxylation of [^{3}H]GA_1. The capacity for 2β-hydroxylation is another feature of seedling GA metabolism which is in contrast to that of immature seeds. This conversion is generally regarded as a deactivation step and, if endogenous GA_1 is indeed implicated in the control of stem elongation, the capacity for 2β-hydroxylation may provide a means of regulating the GA_1 pool size. That the 2β-hydroxylating function appears to be absent from immature seed cell-free preparations from *Phaseolus coccineus* may reflect the physiological insignificance of GA_1 at this stage. If GA_1 had a precise role to play, then presumably some control of its concentration

would be required. The unidentified products of [^{3}H]GA_4 and [^{3}H]GA_{20} metabolism may be GA conjugates whose formation may represent another means of regulating the pool size of biologically active GA (see Schneider, 1983).

From the above study of *in vivo* GA metabolism, it is concluded that the dwarf growth habit of *Phaseolus coccineus* cv. Hammonds Dwarf Scarlet, is not due to an inability to synthesise GA_1. There are several other explanations, including the possibility that although the full complement of GAs is present in the dwarf, the levels may be depressed to such an extent, relative to the tall, that growth is reduced. In an attempt to investigate this, seedlings of Prizewinner and Hammonds Dwarf Scarlet were analysed for endogenous GA_1, GA_4 and GA_{20} content. Unfortunately in the cases of GA_1 and GA_4, the deuterated internal standards added were of insufficient purity to allow realistic estimation of the endogenous contents, so data were obtained for GA_{20} alone. The ng GA_{20} g^{-1} fresh weight figures for tall and dwarf extracts were similar (Table 9), but no real conclusion on this approach to the dwarfism problem can be drawn without estimates of endogenous GA_1 and GA_4 content.

Another theoretically feasible explanation for the Hammonds Dwarf Scarlet growth habit is that the mutation is expressed as an altered binding site for GA and/or a reduction in the magnitude of the ultimate growth response in the dwarf. The *slender* mutant of pea can have undetectably low GA concentrations but exhibits elongated internode length (Jolly *et al.*,1987), suggesting that either the binding sites are apparently GA-saturated, or that the eventual growth response is "switched on" in an exaggerated manner by very small amounts of endogenous GA. The opposite is possible for Hammonds Dwarf Scarlet seedlings, in that, for a given amount of plant growth substance, perception is reduced due to fewer total or fewer functional binding sites, or that a smaller growth response is

initiated, relative to that in Prizewinner seedlings. In order to investigate this, experiments require to be carried out where increase in fresh weight per unit of GA applied, is estimated for seedlings of both tall and dwarf varieties.

The role of GA_1 and possibly other GAs is unclear and is currently a matter for conjecture. Regarding the apparent switching on or off of various enzymic functions and particularly, the change with development of GA_1 synthesis from GA_{20} to GA_4 observed during this work, the following possibilities can be considered.

It is possible that the metabolic branch leading to GA_1 is unimportant and that there is a certain biochemical randomness involved in synthesis, making the apparent switch of the GA_1 precursor from GA_{20} to GA_4 a "red herring".

Alternatively, if the route of GA_1 synthesis in some way affects the role this GA plays in developmental regulation, a genetic switching on of one biosynthetic route and switching off of another is conceivable. The situation with *Phaseolus coccineus* is complex as immature seed cell-free preparations do not metabolise $[^3H]GA_4$, imbibed, mature seed cell-free preparations do not metabolise $[^3H]GA_{20}$, but intact seedlings metabolise both. From this it can be argued that some degree of GA_{20}-metabolising capacity is a redundant function from the seed or that GA_4 and GA_{20} both contribute to the same physiological response by their GA_1 production or that GA_1 derived from different precursors affects different responses. If the last suggestion is true, it implies that the identity of the GA_1 precursor and the subsequent side-chain addition determine the sub-cellular geography of GA_1 production. In the case of GA_4, 13-hydroxylation to produce GA_1 is membrane-associated in peas (Kamiya and Graebe, 1983) while the 3β-hydroxylation of GA_{20} is soluble. Thus, the same end product may be synthesised in areas of the intact cell distant from each other and, conceivably, the same

mode of perception may initiate a different response, depending upon where binding occurs. Until something is known about sub-cellular location of these metabolic conversions and about the nature of plant growth perception, conclusions will be restricted to comments on metabolic anomalies. Bearing this limitation in mind, the results of this work have shown that a change in the metabolic route to GA_1 in tall and dwarf *Phaseolus coccineus* varieties occurs with age of tissue and that dwarfism in the variety Hammonds Dwarf Scarlet is seemingly not simply a consequence of a metabolic block leading to GA_1 production.

Although a useful guide to the metabolic potential of tissues and the concentrations of endogenous plant growth substances present, traditional plant physiology-biochemistry research, such as that carried out during this project, has not as yet led to an understanding of the fundamental processes involved. It seems likely that one of the more direct routes to this knowledge will be by the characterisation of the genes responsible for all the events from plant growth substance production to completion of the physiological response. Work to find the gene(s) in *Zea mays* which influences stem elongation has begun (see Phinney and Spray, 1985) by inserting a transposable element, Robertson's mutator, into the genome and sequencing the DNA surrounding the inserted element after selection of plants with a dwarf characteristic (Barker *et al.*, 1984). The combination of experiments using such techniques but interpreted from a plant physiological stand point may prove very enlightening.

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