AN INVESTIGATION OF THE GIBBERELLINS OF *PISUM SATIVUM* L.

By

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This thesis is dedicated to my parents, without whose support and encouragement it would not have been possible.

SUMMARY

An investigation was made of the endogenous GAs of young Pisum sativum seedlings, and of the metabolism of [^{3}H]GA_9, [^{3}H]GA_{12} aldehyde, [^{3}H]GA_{14} and [^{3}H]GA_{20} by Pisum seedlings. An extract of ten-day-old light-grown seedlings of the tall cultivar Alaska was purified and then separated by HPLC into seventy fractions, which were analysed using three bioassays and a GA_1 radioimmunoassay. Active fractions were subsequently analysed by GC-MS. Mass spectra of GA_{20} and GA_{29} were obtained. GC-MS failed to detect GA_1 in a HPLC fraction that had chromatographic and assay properties similar to those of GA_1. GC-MS did however detect the presence of a novel GA-like compound in this fraction. Although the identity of this component was not determined its mass spectrum contained ions characteristic of the presence of a 13-hydroxyl group.

Extracts of eight-day-old shoots from light- and dark-grown seedlings of the tall cultivar Alaska and the dwarf cultivars Meteor and Progress No.9 were each separated into 35 fractions by HPLC, and each fraction analysed for the presence of GA-like substances using a GA_1 radioimmunoassay. Light-grown shoots of all three cultivars appeared to contain similar GA-like substances, at least four being detected. The amount of GA_{20} in light grown shoots was estimated and varied from 70 to 330 pg seedling^{-1} (ca. 0.4 to 1.6 ng g.f.wt^{-1}.). These estimates are corrected for the recovery of internal standards in the extracts and for the cross-reactivity of GA_{20} in the GA_1 RIA. Dark-grown shoots contained

much lower amounts of all the GA-like substances detected, regardless of whether the amount was expressed on a per seedling or on a per unit weight basis.

Tritiated products from feeds of [^{3}H]GAs to seedlings were analysed by HPLC-RC. Some of the [^{3}H]GA_{14} metabolites were also analysed by GC-MS. [^{3}H]GA_9 was metabolised extensively by light-grown seedlings of the cultivars Alaska, Meteor and Progress No.9, and also by dark-grown seedlings of the cultivar Alaska. At least twenty seven metabolites were detected, but only GA_{20} and possibly GA_{29} were identified. The pattern of metabolism appeared identical in cv. Meteor and in cv. Progress No.9, and this was very similar to the spectrum of metabolites observed from light- and dark-grown seedlings of the cultivar Alaska.

Only low amounts of a single possible metabolite of [^{3}H]GA_{20} were detected. This putative metabolite was present in similar amounts in extracts of light-grown shoots of the cultivars Alaska, Meteor and Progress No.9. The metabolite was not identified but was not GA_1 or GA_8, and probably not GA_{29} or GA_{29} catabolite.

At least fourteen metabolites were observed from a feed of [^{3}H]GA_{14} to young light-grown seedlings of the cultivar Alaska. One product was identified by GC-MS as GA_{18}, but the other metabolites were not identified, although HPLC and GC-MS analysis showed some of them to be distinct from a range of GAs, including GA_1, GA_8, GA_{18}, GA_{23}, GA_{28}, GA_{38} and GA_{42}, which were identified by Durley et al. (1974a,b) as products of [^{3}H]GA_{14} fed to pea seedlings.

[^{3}H]GA_{12} aldehyde was converted to small amounts of a number of products, but these were not distinguished from breakdown products observed in control extractions.

ACKNOWLEDGEMENTS

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The [^{3}H]GA_{14} and [^{3}H]GA_{20} used were a gift from Professor R.P. Pharis (University of Calgary). Dr. V. Sponsel (University of Bristol) kindly provided a sample of GA_{29} catabolite while the remainder of the standards were generously supplied by Dr. T. Yokota (University of Tokyo).

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ABBREVIATIONS

AMO-1618	2'-Isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate.
B	Binding of radiotracer to antibody in presence of sample or standard GA.
B_o	Binding of radiotracer to antibody in the absence of sample or standard GA.
BuOH	Butanol
CCC	2-Chloroethyltrimethylammonium chloride
cpm	Counts per minute
CPP	Copalyl pyrophosphate
cps	Counts per second
CPS	Cyanopropylsilane
cv.	Cultivar
DCM	Dichloromethane
dpm	Disintegrations per minute
EtOAc	Ethyl acetate
EtOH	Ethanol
f.s.d.	Full scale deflection
f.wt.	Fresh weight
GA	Gibberellin
GA CE	Gibberellin-methoxycoumaryl ester
GA Me	Gibberellin methyl ester
GA MeTMSi	Gibberellin methyl ester trimethylsilyl ether
GC-MS	Combined gas chromatography-mass spectrometry
GC-RC	Combined gas chromatography-radioisotope counting
GA_1 RIA	GA_1 radioimmunoassay
GGPP	Geranygeranyl pyropnosphate

GC-SICM	Gas chromatography with selected ion current monitoring
HPLC	High performance liquid chromatography
HPLC-RC	Combined HPLC-radioisotope counting
LD	Long day
MeOH	Methanol
MVA	Mevalonic acid
m/z	Mass to Charge ratio
ODS	Octadecylsilane
PVP	Polyvinyl pyrrolidone
RIA	Radioimmunoassay
R_{10}	Percentage of MeOH giving a retention time of 10 minutes
R_t	Retention time
SD	Short days
THF	Tetrahydrofuran
TIC	Total ion current
TMSi	Trimethylsilyl derivative
v/v	Volume for volume
w/v	Weight for volume

CONTENTS

	Page No
Summary	i
Acknowledgements	iv
Abbreviations	v
Contents	vii
1. INTRODUCTION	
1.1. The Gibberellins	1
1.2. The Pea Plant	6
1.3. Gibberellins in Peas	
1.3.1. Endogenous gibberellins of peas	8
1.3.2. GA biosynthetic sites and GA translocation	13
1.3.3. Sub-cellular localisation of GAs	17
1.4. Biosynthesis of Gibberellins	20
1.4.1. (I) The formation of *ent*-kaurene	20
1.4.2. (II) *ent*-kaurene to GA_{12} aldehyde	26
1.4.3. (III) Further metabolism of GA_{12} aldehyde	26
1.4.4. Summary	35
1.5. Gibberellins and the Determination of Stem Length in *Pisum sativum*	36
1.5.1. Apical senescence	36
1.5.2. Stem elongation	38
1.5.3. Photo-inhibition of stem elongation	49
1.6. Summary and Objectives	56

Page No

2. MATERIALS AND METHODS

2.1. Plant Material 59

2.2. Application of [^{3}H]GAs, Extraction and Partitioning Procedures 59

2.3. DEAE-A25 Anion Exchange Chromatography 61

2.4. Gel Permeation Chromatography 62

2.5. Sep-Pak C_{18} Cartridges 63

2.6. Preparative High Performance Liquid Chromatography 63

2.7. Analytical High Performance Liquid Chromatography 64

2.8. Bioassays 66

2.9. GA_1 Radioimmunoassay 68

2.10. Liquid Scintillation Counting 69

2.11. Preparation of Derivatives 70

2.12. Combined Gas Chromatography-Mass Spectrometry 71

3. RESULTS

3.1. Growth of Experimental Plants 74

3.2. Reverse Phase HPLC of Gibberellins 76

3.3. GC-MS Analysis of the Endogenous GAs of cv. Alaska 79

3.4. Comparison of the Endogenous Gibberellins of Tall and Dwarf Cultivars by Immunoassay 88

Page No

3.5. [^{3}H]GA_{14} Feeds

3.5.1. [^{3}H]GA_{14} Feeds: Introduction 96

3.5.2. [^{3}H]GA_{14}: Time course feed 96

3.5.3. GC-MS analysis of [^{3}H]GA_{14} metabolites 107

3.5.4. [^{3}H]GA_{14} Feeds: Conclusion 118

3.6. [^{3}H]GA_{12} aldehyde Feeds

3.6.1. [^{3}H]GA_{12} aldehyde experiments: Introduction 119

3.6.2. Stability of [^{3}H]GA_{12} aldehyde 119

3.6.3. [^{3}H]GA_{12} aldehyde time course feeds 122

3.7. [^{3}H]GA_9 Feeds

3.7.1. [^{3}H]GA_9 Feeds: Introduction 125

3.7.2. [^{3}H]GA_9 Feeds: Preliminary experiments 125

3.7.3. [^{3}H]GA_9 Time Course Feeds: Initial analyses of individual EtOAc fractions 131

3.7.4. [^{3}H]GA_9 Time Course Feeds: Analyses of combined EtOAc extracts 149

3.7.5. [^{3}H]GA_9 Feeds: Summary 170

3.8. [^{3}H]GA_{20} Metabolism 171

4. DISCUSSION

4.1. [^{3}H]GA Feeds

4.1.1. Introduction 176

4.1.2. [^{3}H]GA_{12} aldehyde feeds 177

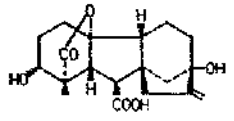
		Page No
4.1.3.	[^{3}H]GA_{14} feeds	178
4.1.4.	[^{3}H]GA_{20} feeds	179
4.1.5.	[^{3}H]GA_9 feeds	181
4.2.	Analysis of the Endogenous GAs of Pisum	184
4.3.	Conclusions	189
REFERENCES		191

INTRODUCTION

1.1. THE GIBBERELLINS

The gibberellins (GAs) are a group of diterpenoid acids which appear to function as endogenous regulators of plant growth. This role is generally accepted as GAs have been found in a wide variety of higher plants and because applications of small quantities of GAs to plants can induce a wide range of plant growth responses. Exogenous GAs can promote stem elongation, leaf enlargement, flowering, fruit set, parthenocarpic fruit development and germination. They can also break bud dormancy and retard senescence of stem apices and of leaves (see Krishnamoorthy, 1975 and Letham et al., 1978). Commercially GAs are sprayed onto vines to produce large berries in open clusters. They are also used to speed the malting of cereal grains, to improve fruit set and delay senescence of citrus crops, to prolong the harvest period of the globe artichoke and to force rhubarb in the absence of cold exposure. Commercial uses of GAs have been reviewed by Martin (1983) and by Weaver (1972).

Despite the wide range of effects that exogenous GAs can cause, relatively little is known about the role of endogenous GAs and their mechanism of action. This is principally because GAs are present in tissues in very low amounts. Shoot and leaf tissues typically contain only a few ng per g f.wt. of a GA, although levels in seeds can be up to several µg per g f.wt. The low amounts present and the large number of structurally similar GAs make identification difficult, and it has not been until relatively recently that suitable techniques have been widely employed.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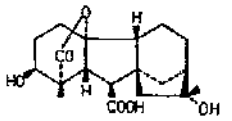
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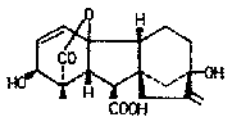
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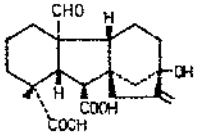
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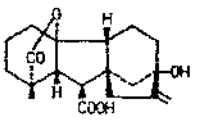
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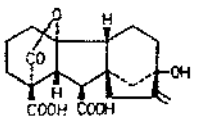
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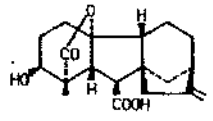
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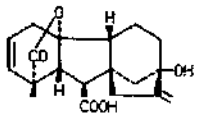
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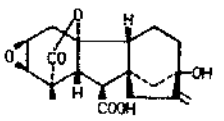
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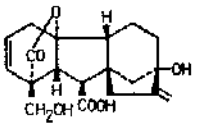
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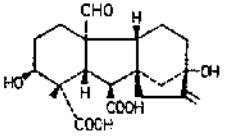
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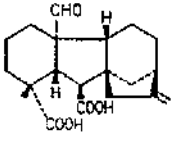
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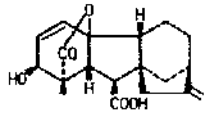
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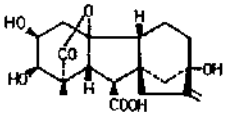
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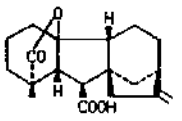
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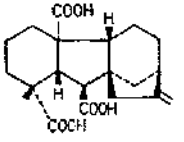
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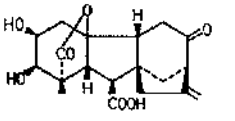
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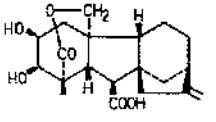
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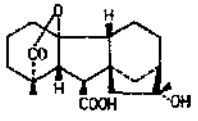
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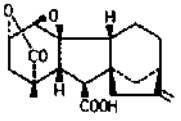
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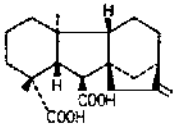
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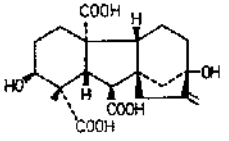
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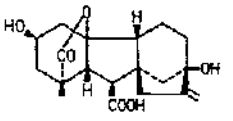
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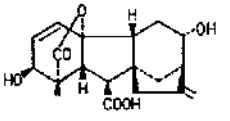
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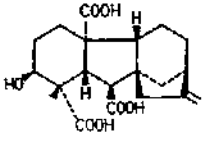
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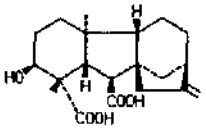
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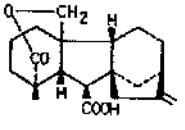
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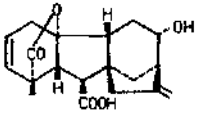
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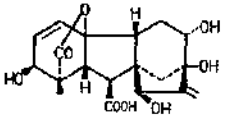
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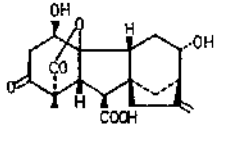
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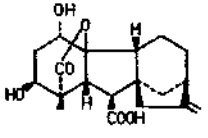
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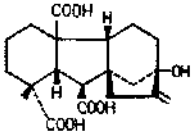
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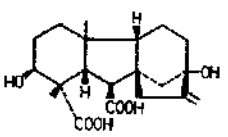
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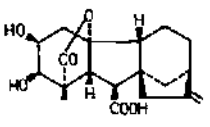
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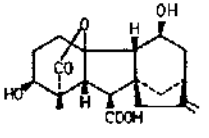
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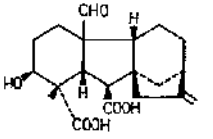
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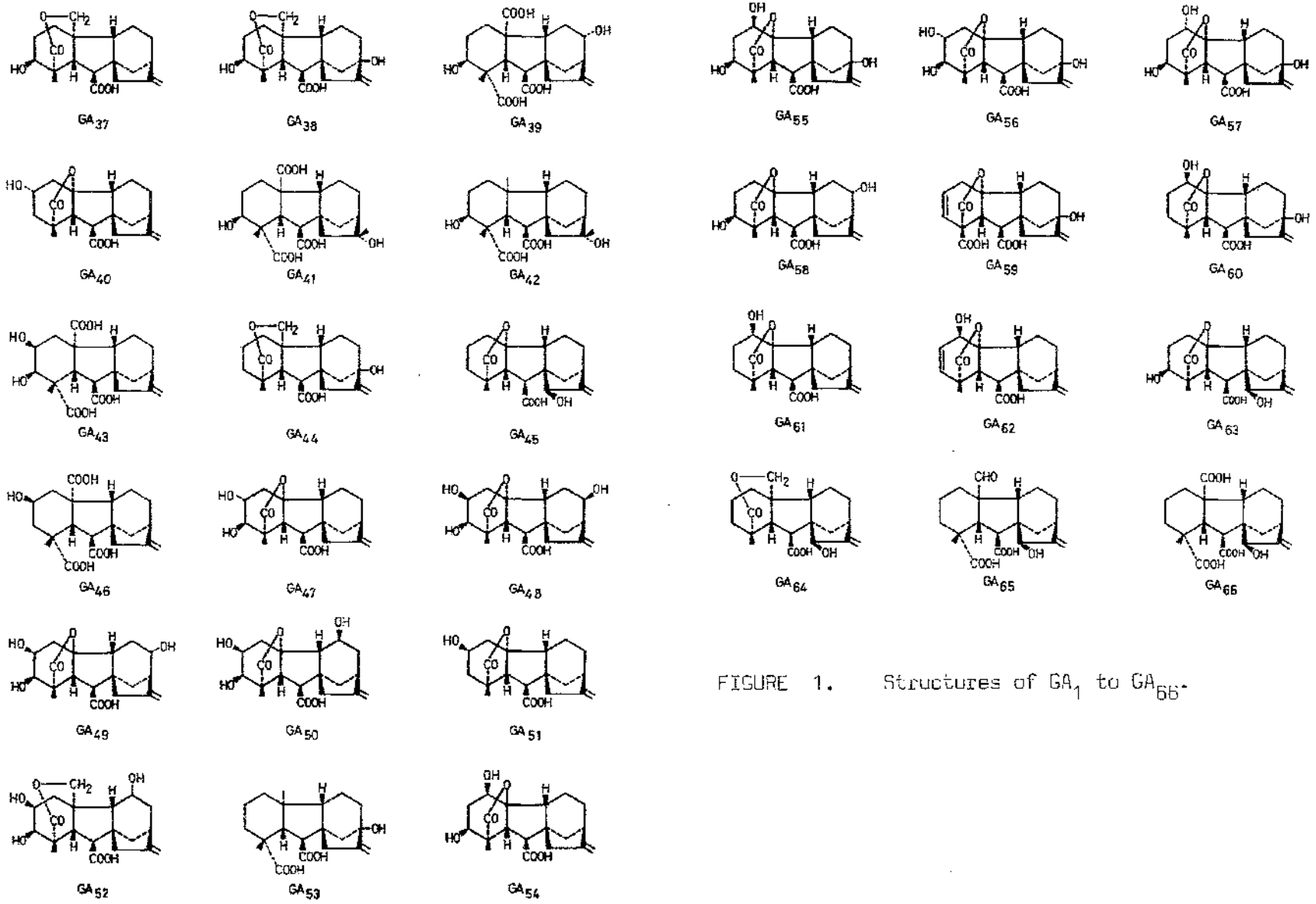
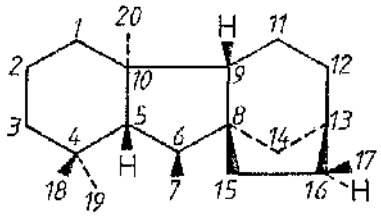


FIGURE 1. Structures of GA_1 to GA_{66}.

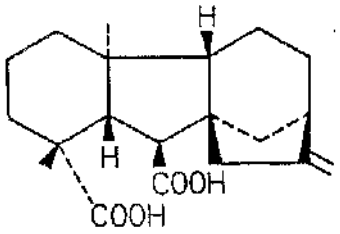
Sixty-six naturally occurring GAs have been reported (figure 1). Bearder (1980) comprehensively listed the species and the tissues from which GAs had been isolated at that date. Information on more recent identifications can be found in Crozier (1983). The systematic nomenclature of the GAs is based on ent-gibberellane (1), but as the systematic nomenclature is complex the GAs are reported by their A numbers, allocated in order of discovery.

Two major groups of GAs may be distinguished. C_{20}-GAs retain carbon-20, which can be present as either a $-CH_3$, $-CH_3OH$, -CHO or -COOH function (figure 2). All the C-20 alcohol GAs isolated have a δ-lactone ring (3), but this may be an artifact formed on extraction. C-20 aldehyde GAs exist in an equilibrium mixture of free acid (4) and δ-lactol (5) in aqueous solution (Graebe and Ropers, 1978). The C_{20}-GAs can be further divided on the basis of the number and location of substituent hydroxyl groups. C_{20}-GAs generally have low biological activity and appear, with the possible exception of the C-20 carboxyl GAs, to be intermediates in the formation of the second group of GAs, namely the C_{19}-GAs.

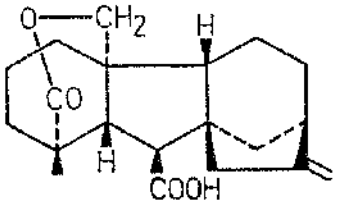
A 19→10 γ-lactone bridge is present in all C_{19}-GAs (7) except GA_{11}, which has a 19→2 γ-lactone. The remaining C_{19}-GAs are distinguished on the basis of different structural modifications of the ent-20-norgibberellane skeleton. The known structural modifications comprise: 2,3 and 1,10 epoxide groups; C-3 and C-12 keto groups; β-hydroxylation at C-1, C-2, C-3, C-11, C-12 and C-15; α-hydroxylation at C-1, C-2, C-12, C-13 and C-16; and the introduction of 1-2 or 2-3 double bonds.



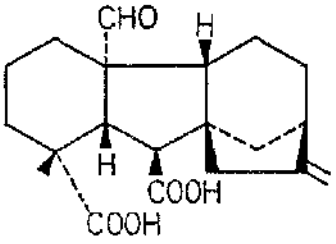
(1) *ent*-gibberellane



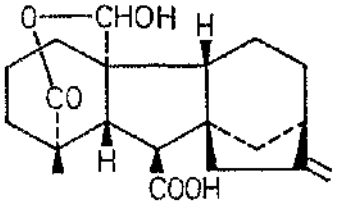
(2) C-20 methyl C_{20}-GA



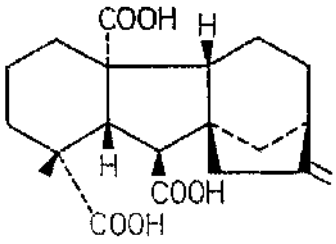
(3) δ-lactonic C_{20}-GA



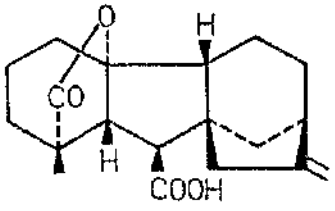
(4) C-20 aldehyde C_{20}-GA



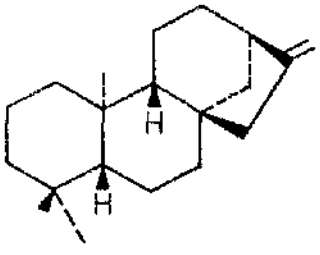
(5) δ-lactol C_{20}-GA



(6) C-20 carboxyl C_{20}-GA



(7) δ-lactonic C_{19}-GA



(8) *ent*-kaurene

FIGURE 2. Structures.

A number of conjugated GAs have been isolated (see Schneider, 1983). Most of these are either β-D-glucosyl ethers, in which the moiety is attached to the 2β-, 3β- or 11β-hydroxyl group, or β-D-glucosyl esters, in which the sugar is esterified to the C-7 carboxyl group. Besides these conjugates 3-O-acetyl GA_3 and 3-O-acetyl GA_1 have been isolated from Gibberella fujikuroi cultures, GA_3- and GA_1- n-propyl esters from Cucumis sativa, GA_9-methyl ester from Lygopodium japonicum and gibberethione (a sulphur containing derivative) from Pharbitis nil.

GAs are synthesised from mevalonic acid (MVA) via ent-kaurene (8). The systematic nomenclature of kaurenoid intermediates is based on the ent-kaurane skeleton. This can lead to confusion since the ent- prefix reverses the α- and β- designations of substituents at chiral centres. Thus 7β-hydroxykaurenoic acid is correctly termed ent-7α-hydroxykaurenoic acid, although the hydroxyl group at C-7 is above the plane of the ring.

1.2. THE PEA PLANT

The garden pea (Pisum sativum L.) is a member of the family Leguminosae, subfamily Papilionidae, tribe Viciae. Six Pisum species are recognised although they are differentiated with difficulty and their nomenclature is confused. P. sativum is not known in the wild state and its origin is undefined (Allen and Allen, 1981).

The advantages of the pea as an experimental plant have been reviewed by Went (1957). Peas are easily grown and are widely

available. The pea is self-fertilized and it is easy to obtain both inbred homozygous strains and a range of commercial cultivars. The genetics of the pea have been extensively studied, and a wide range of mutants are available for physiological investigation (see Blixt, 1972). For the above reasons peas have been widely used as experimental plants and a considerable amount of information is available about the species (see Sutcliffe and Pate, 1977). This is an additional advantage when considering using the pea as an experimental plant, since it allows new results to be directly related to previous ones in the same species, without the possible complications of effects due to species differences. Thus the depth of currently available knowledge makes it much easier to interpret the possible significance of new results.

The pea was chosen as an experimental plant in the current investigation for the above reasons, and because it has been widely used in investigations into the biochemistry and physiology of GAs (for example, see Sponsel, 1983; Hedden et al., 1978; and Graebe and Ropers, 1978). The following introduction reviews the available information on GA occurrence and metabolism in peas, referring to results from other species when pertinent. Subsequently the possible role of GAs in controlling stem elongation is discussed, principally with reference to peas although again drawing on the results from other species when these are relevant.

A range of different pea cultivars and lines have been used in previous work. There is evidence that the endogenous GAs of peas are influenced by certain genetic loci (see section 1.5.2.).

TABLE 1. Genotypes of commonly used pea types, with reference to some of the stem elongation genes.

Type	Phenotype	Genotype
Alaska	Tall	Na Le Cry (La or la)*
Progress No.9	Dwarf	Na le Cry* (La or la)
Meteor	Dwarf	Na le (Cry and La- one or both dominant)
G2	Dwarf	Na le " " " " " " "
Grosser Schnabel mit Gedrücktem Korn	Tall	Na Le " " " " " " "

* see McComb and McComb (1970)

The genotypes with reference to these loci, of some of the most commonly used types of peas, are listed in Table 1.

1.3.1. ENDOGENOUS GIBBERELLINS OF PEAS

The GA content of plants may differ both between species (see Sponsel, 1983) and between different genotypes of the same species (see Phinney, 1984). A single species may contain a variety of GAs. Early bioassay investigations of the endogenous gibberellins of peas suggested the presence of GA_1-like and GA_5-like compounds (see Lang, 1970), while more recent investigations have suggested the presence of at least 6 (Railton and Reid, 1974a). The GAs that have been conclusively identified from peas are detailed in Table 2.

The first GAs conclusively identified in peas were from seeds, as the levels of endogenous GAs in seeds can be much higher than those found in other tissues, for example Frydman et al. (1974)

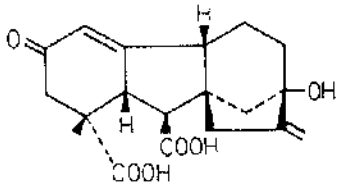
Type	Tissue	GAs	Reference
Progress No.9 (dwarf)	seeds	GA_9, GA_{17}, GA_{20}, GA_{29}, GA_{44}, GA_{51}, GA_{29}cat.	Frydman and MacMillan (1973) Frydman et al. (1974) Sponsel and MacMillan (1977, 1978, 1980), Kirkwood (1979)
	young etiolated seedlings	GA_{20}, GA_{29}, GA_{29}cat.	Sponsel and MacMillan (1978)
	young light grown epicotyls	GA_{20}, GA_{29}, GA_{29}cat.	Kirkwood and Sponsel (unpublished, cited by Hedden et al., 1978) Kirkwood (1979)
Alaska (tall)	seeds	GA_{20}	Eeuwens et al. (1973)
Greenshaft (dwarf)	seeds	GA_{20}	Ingram and Browning (1979)
G_2 (dwarf)	seeds	GA_9, GA_{17}, GA_{19}, GA_{20}, GA_{29}, GA_{44}, GA_{29}cat.	Ingram and Browning (1979) Davies et al. (1982)
	leaves and shoots	GA_{19}, GA_{20}, GA_{29}, GA_{29}cat.	Davies et al. (1982)
Various (dwarf)	seeds	GA_9, GA_{17}, GA_{19}, GA_{20}, GA_{29}, GA_{44}, GA_{51}, GA_{29}cat.	Ingram (1980)
	young seedlings	GA_{20}, GA_{29}, GA_{29}cat.	Ingram (1980)
Tall F_3, of line 77 (tall) and line 2 (dwarf) cross.	unexpanded apical tissue	GA_1, GA_{29}, GA_{29}cat.	Ingram et al. (1983)
line 88 (tall)	unexpanded apical tissue	GA_1, GA_8, GA_{17}, GA_{20}, GA_{29}, GA_{29}cat.	Ingram et al. (1983)

TABLE 2. Gibberellins conclusively identified from Pisum sativum. GA_{29}cat. = GA_{29}catabolite.

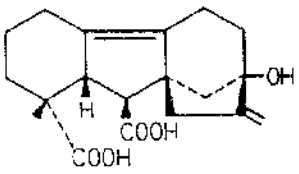
detected ca 5µg seed^{-1} GA_{20}. Levels in vegetative tissue are much lower (Kirkwood, 1979; Davies et al., 1982). The levels of GA_1, GA_8, GA_{20} and GA_{29} in stem tissue have recently been estimated by Ingram et al. (1984) to be several nanograms per plant.

Until recently it was thought that, despite the early bioassay evidence, GA_1 was not present in peas (see Sponsel, 1980a). However, studies by Ingram et al. (1983) have provided mass spectrometric evidence for the presence of GA_1 in pea seedlings. The GA_5-like peak detected in the early bioassay experiments is thought to be caused by GA_{20}, which has similar biological activity and chromatographic mobility. Possible reasons for the failure to identify GA_1 in earlier investigations have been suggested by Ingram et al. (1983). In addition to the identifications listed in Table 2 Keller and Coulter (1977, 1982) have claimed to identify and quantify GA_5 and GA_1 or GA_{29} from pea epicotyls. However, in view of the experimental procedure employed these identifications are unlikely to be accurate.

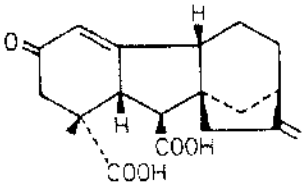
GA inactivation has been suggested to occur via two routes, conjugation and catabolism. GA conjugates have been detected in a range of plants and have been shown to accumulate in maturing seeds of some species (see Schneider, 1983). Sponsel (1980a) states that the levels of endogenous conjugates in pea seeds appear low. However, there is little published evidence on the subject .



(9) GA_{29}-catabolite



(10) GA_{20}-oper lactone



(11) GA_{51}-catabolite

FIGURE 2 (continued). Structures

A number of GA breakdown products, such as GA_{29}-catabolite (9), GA_{20}-open lactone (10) and GA_{51}-catabolite (11) have been detected in pea seeds (Sponsel, 1983). GA_{29}-catabolite is biologically inactive and may represent part of a deactivation mechanism for GA_{20} (see Sponsel, 1983).

There is evidence of temporal and spatial changes in GA levels within peas. Frydman et al. (1974) quantified the levels of GA_9, GA_{17}, GA_{20} and GA_{29} during the development of Pisum seeds. The levels detected may be underestimates as no internal standards were added to quantify losses during the extraction procedures. Later studies with internal standards suggested higher levels of

GA_{29} (Sponsel and MacMillan, 1978). Each GA reached a maximum amount and then declined as the seeds developed. It was found that the time at which the maxima were reached was dependent on seed fresh weight rather than seed age (Sponsel and MacMillan, 1977). There is also variation between seed batches (Sponsel and MacMillan, 1980). The experiments were later extended to include quantification of GA_{29}-catabolite (Sponsel and MacMillan, 1980). Ingram and Drowning (1979) and Durley et al. (1971) also reported considerable quantitative changes, during maturation, in the seed GAs of Pisum sativum and Phaseolus species respectively.

Sponsel (unpublished, cited by MacMillan, 1984) has shown that GA_{20} and GA_{29} are located almost exclusively in the cotyledons of pea seeds while GA_{29}-catabolite is located predominantly in the testa. It was shown that the conversion of GA_{20} to GA_{29} occurs in the cotyledons, while that of GA_{29} to GA_{29}-catabolite occurs in the testa. Similarly in Phaseolus coccineus GA_1 is found mainly in the cotyledons of maturing seeds while GA_8 is only found in the testa. Albone et al. (unpublished, cited by MacMillan, 1984) have shown results of a similar nature in a cucurbit, "cho-cho" (probably Sechium edule).

The results of Potts et al. (1982a) and Potts and Reid (1983) indicate that differences in the content of endogenous GAs may occur between seed and vegetative tissue, and between young and mature vegetative tissue. These differences appear to be qualitative and quantitative. Kaufman et al. (1976) using bioassay techniques

demonstrated different GA levels in different parts of wheat plants, the inflorescence and nodes having high levels. Roots had lower levels of GAs (expressed either on a per plant or a per unit weight basis). There appeared to be temporal changes in the p-1 internode as it developed. Metzger and Zeevaart (1980a) identified GAs from shoots and roots of Spinacia oleracea. The results suggested that there may be quantitative and qualitative differences between the GAs of roots and shoots. On the basis of bioassay evidence it was estimated that the GA pool in the roots was ca. 3 times less than the pool in the shoots (on a per unit weight basis). Kirkwood (1979) failed to detect GAs from radicles of 8-day-old pea seedlings, cv. Progress No.9, although GAs were identified from epicotyls. Sebanek et al. (1978) claimed to show GA-like activity in crude extracts of pea roots from 3- and 6-day-old seedlings. However, the small amounts of tissue extracted and methods employed make the results of questionable value.

1.3.2. GA BIOSYNTHETIC SITES AND GA TRANSLOCATION

The evidence regarding sites of GA biosynthesis has been reviewed by Graebe and Ropers (1978) and by Stoddart (1983). There is strong evidence that GA biosynthesis occurs in pea seeds. The evidence is (i) the occurrence of GAs in pea seeds, (ii) the demonstration of GA biosynthesis in pea seed cell-free systems and (iii) the reduction in endogenous GA-like substances in pea seeds caused by AMO-1618 (Baldev et al., 1965). All stages of GA biosynthesis from MVA to C_{19}-GAs have been demonstrated in cell free systems from pea seeds (Coolbaugh and Moore, 1971a; Ropers et al.,

1978; Kamiya and Graebe, 1983). Coobaugh and Moore (1971a), using cell-free systems, found that the capacity for ent-kaurene biosynthesis was located mainly in the cotyledons rather than in the embryo or seed coat.

The results indicating that GA biosynthesis occurs in pea seeds are consistent with data obtained from other species. GA_1 has been identified in suspensors of Phaseolus coccineus (Alpi et al., 1979) and all the main biosynthetic stages from MVA to GA_8 have been demonstrated in cell-free systems of this tissue (Ceccarelli et al., 1979, 1981a, 1981b). Synthesis of GAs from MVA has also been demonstrated in endospermic cell-free systems from Marah macrocarpus and from Cucurbita maxima (see Hedden, 1983 and Coolbaugh, 1983). There is also strong circumstantial evidence implicating the embryo of barley grains as a site of GA synthesis (see Graebe and Ropers, 1978).

Suppositions about the sites of synthesis of GAs in the vegetative tissues of peas are largely speculative. It is possible that the GAs of young seedlings could be to some degree derived from the seed. In peas the levels of GAs in mature seeds are low (Frydman et al., 1974). However, the first internode of nana peas (which appear to be able to synthesise GAs in the seed but not in the shoot, see section 1.5.1.) elongates to a greater extent than the later internodes, which may indicate that seed derived GAs stimulate early shoot development. Sponsel (1980a) discussed the possible role, during germination and seedling growth, of GAs derived during seed maturation. Use of a growth retardant,

known to inhibit GA biosynthesis in Gibberella fujikuroi and Marah macrocarpus did not affect germination, but did inhibit growth of 5-6 day-old seedlings, suggesting to Sponsel that de novo synthesis is occurring at this stage.

The possible sites of GA biosynthesis in Helianthus seedlings were studied by Jones and Phillips (1966) using an agar diffusion technique. They concluded that the young leaves of the apical bud were a major source of GA. Root tips were also thought to be able to synthesise GAs. Other evidence frequently cited as supporting the hypothesis that roots are biosynthetic organs is the detection of GA-like substances in the exudates from the stock of excised shoots (see Graebe and Ropers, 1978).

Investigations into the sources of GAs in pea seedlings have generally involved excising an organ or tissue and investigating whether exogenous GA could restore growth. Such results are difficult to interpret as excision of tissues may cause a variety of changes. Lockhart (1957) found that the reduction in stem height caused by excising apices could be partially counteracted by GA_3, and concluded that the apex was a source of GA. However, Kuraishi and Muir (1964) found that exogenous GA_3 would not counteract the effects of apical excision. Shininger (1972) showed that GA could partly replace excised cotyledons, and concluded that these were a source of GAs in pea seedlings. However, AMO-1618 inhibited growth of seedlings with excised cotyledons, suggesting that GA biosynthesis was also occurring in the seedling. Conversely, Lockhart (1957) and Moore

(1967), also using excision experiments, concluded that GA was unlikely to be supplied to young shoots from the cotyledons.

GA biosynthesis from MVA has not been demonstrated in cell-free systems from vegetative tissues of any species, although ent-kaurene biosynthesis has been shown to occur in a number of pea tissues (Coolbaugh, 1982). The conversion of C-20-methyl to C_{19}-GAs has been demonstrated in intact plants by Durley et al. (1974a, 1974b) and by Gianfagna et al. (1983). Ent-kaurene biosynthesis has not been demonstrated in pea roots (Coolbaugh, 1982). However, Froneberg (unpublished, cited by Graebe, 1982) has observed the conversion of ent-kaurene to ent-7α-hydroxy-kaurenoic acid, and of GA_{12} aldehyde to GA_{12}, 12α-hydroxy $-GA_{12}$ aldehyde and 12β-hydroxy-GA_{12} aldehyde in a cell-free system from Cucurbita maxima roots.

After reviewing the literature on GA biosynthesis in vegetative tissues Graebe and Ropers (1978) concluded that although the evidence was weak it was likely that GAs were biosynthesised in shoot and root tips and young leaves, although they did not exclude other tissues as having some GA biosynthesising ability. Stoddart (1983) arrived at similar conclusions.

There is evidence that GAs are translocated in plants. This evidence comes from: (i) the detection of GA-like substances in xylem and phloem sap (see Graebe and Ropers, 1978), and (ii) the movement of applied label from radioactive feeds (e.g. Zweig et al., 1961; Nash and Crozier, 1975). However, the quantities, directions and types of GAs translocated in vivo are not known.

In peas McComb (1964) demonstrated, using autoradiography,

that [^{14}C] was translocated from [^{14}C]GA_3 treated leaves mainly to young leaves and internodes. By applying GA_3 at different nodes, and timing the period until stem elongation increased, he estimated the rate of movement of GA_3 along the stem at 5cm hr^{-1}. Grafting studies have suggested the *in vivo* translocation of GAs in peas (Reid *et al.*, 1983) and maize (Katsumi *et al.*, 1983). Jones and Lang (1968) found that the GA_1-like component of pea apices would diffuse into agar, but that the GA_5-like component would not, suggesting that only some of the endogenous GAs are mobile.

1.3.3. SUB-CELLULAR LOCALISATION OF GAs

There is some evidence to suggest that there is differential sub-cellular compartmentation of GAs and possibly differential sub-cellular metabolism. Several reviews have covered the subject, including Hedden *et al.* (1978), Rappaport and Adams (1978), Graebe and Ropers (1978) and Crozier (1981). The endoplasmic reticulum, mitochondria, plastids and vacuoles are all potential sites of localisation. Evidence from cell-free systems (see Hedden, 1983) suggests that the series of biosynthetic stages from *ent*-kaurene to oxidation of GA_{12} aldehyde are located in the endoplasmic reticulum. Hilton and Smith (1980) found no GA-like activity from purified mitochondrial fractions, and Simcox *et al.* (1975) concluded that little or no *ent*-kaurene synthetase was present in mitochondria. Thus there is no evidence at the moment for a direct role of mitochondria in GA metabolism.

There have been a number of reports of GA-like substances being isolated from plastids (Cooke and Saunders, 1975; Cooke et al., 1975; Cooke and Kendrick, 1976; Evans and Smith, 1976; Hilton and Smith, 1980). However, the only conclusive identification of GAs from plastids, reported by Browning and Saunders (1977) has not been repeatable (Saunders, unpublished, cited by Sembdner et al., 1980). Little work seems to have been reported on the identification of endogenous GAs from vacuoles, and evidence for their role has come from tracer experiments.

It has been suggested in a number of reports that GA release from etioplasts may be a phytochrome mediated response, promoted by red light. However this hypothesis must be regarded as tentative as (i) it has not been demonstrated that phytochrome is definitely associated with etioplasts in vivo (Hilton and Smith,1980) and (ii) many of the results show a very small difference in GA-levels after red light treatment. Graebe and Ropers (1978) and Stoddart (1983) have questioned the significance of some of the differences detected.

Hedden et al. (1978) have reviewed the evidence that suggests that plastids are capable of the early stages of GA biosynthesis. The only studies of GA metabolism by chloroplasts have been reported by Railton and co-workers (Railton, 1977a,b; Railton and Rechav, 1979; Railton and Reid, 1974b). These results are inconclusive as the chloroplast preparations used are likely to have been impure and the separation and identification of products was usually inadequate.

Evidence for a possible role of vacuoles in GA metabolism has come from metabolism studies with [^{3}H]GA_1 in barley (Hordeum vulgare) and cowpea (Vigna sinensis) (see Garcia-Martinez et al., 1981; Keith et al., 1982; Ohlrogge et al., 1980). Rappaport and Adams (1978) suggested that different patterns of GA metabolism could occur at different sites within the cell, citing the results of Hartmann et al. (1977) as evidence. Hartmann et al. showed that alkylation of the triterpene, cycloartenol, is mediated by a microsomal enzyme, whereas glucosylation is mediated by a plasmalemma-associated enzyme.

1.4. BIOSYNTHESIS OF GIBBERELLINS

It is convenient to discuss the pathway of GA biosynthesis in three stages: (I) the formation of ent-kaurene, (II) the conversion of ent-kaurene to GA_{12} aldehyde, and (III) the conversion of GA_{12} aldehyde to GAs (Graebe et al., 1980). The pathway has been reviewed in Crozier (1983), by Hedden et al. (1978) and by Graebe and Ropers (1978). Metabolic studies in Gibberella fujikuroi have provided the background for higher plant studies and have been comprehensively reviewed by Bearder (1983). In higher plants the pathway to GA_{12} aldehyde has been studied almost exclusively in cell-free systems. The main systems have been prepared from Marah macrocarpus (previously identified as Echinocystis macrocarpus), Cucurbita maxima (previously identified as Cucurbita pepo) and Pisum sativum, although other species have also been used. Coolbaugh (1983) and Hedden (1983) have recently reviewed the early stages of GA biosynthesis. The available evidence suggests that GAs in all species are synthesised by a common path as far as GA_{12} aldehyde. The pathways subsequent to GA_{12} aldehyde depend on the tissue being studied. The outline of biosynthesis given here refers mainly to Pisum sativum, although comments regarding other species are made where pertinent. Crozier (1983) should be consulted for a broader coverage.

1.4.1. (I) THE FORMATION OF ENT-KAURENE

This part of the pathway is catalysed by soluble enzymes and is detailed in figure 3. The conversion of MVA to ent-kaurene in peas has been reported frequently (e.g. Anderson and Moore, 1967;

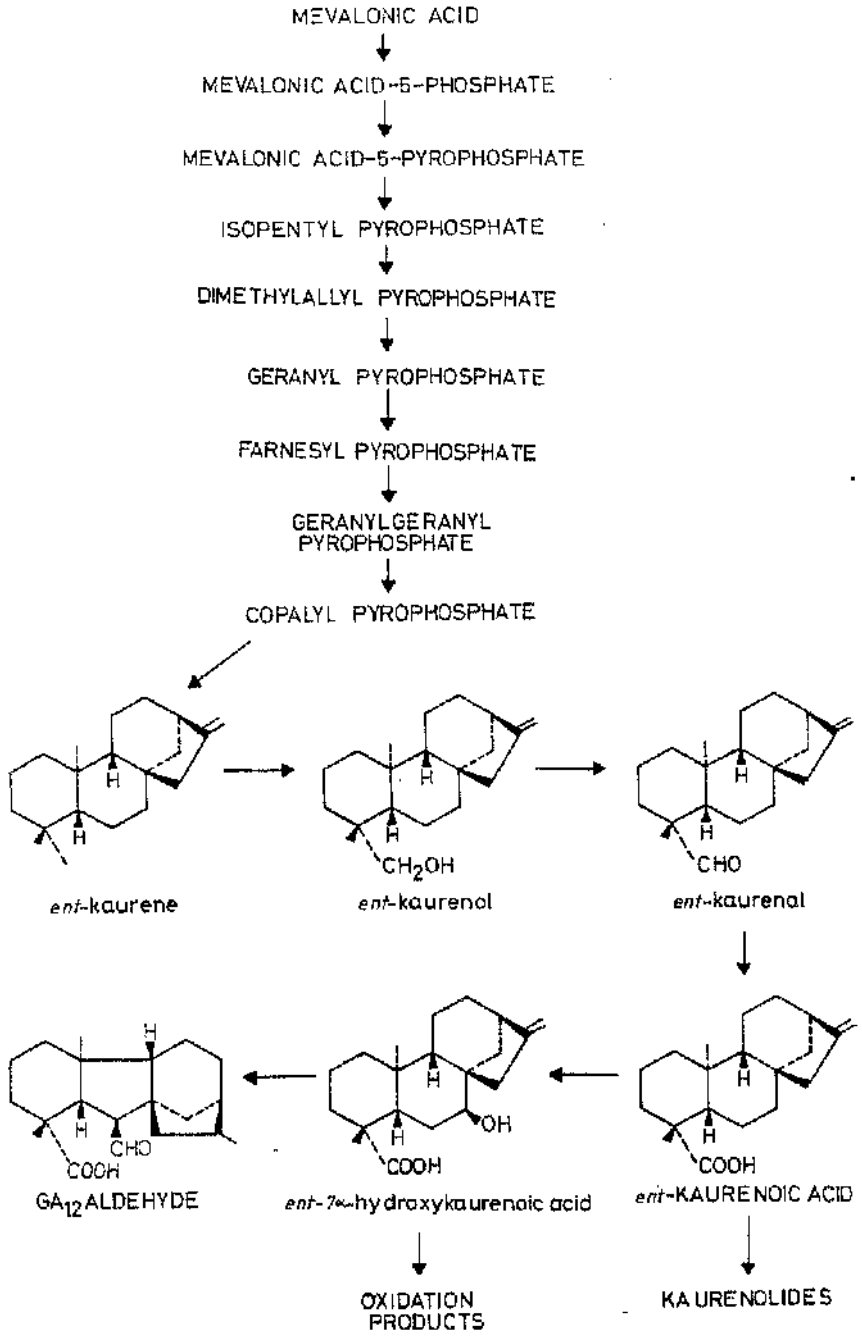


FIGURE 3. GA biosynthetic pathway from mevalonic acid to GA_{12} aldehyde.

Graebe, 1968; Ecklund and Moore, 1974). Synthesis has been demonstrated in young fruits (Graebe, 1968), in seeds (Ecklund and Moore, 1974; Coolbaugh and Moore, 1971a), in shoot tips (Coolbaugh *et al.*, 1973; Ecklund and Moore, 1974) and in leaves and internodes (Coolbaugh, 1982). Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) have been confirmed as intermediates by Moore and Coolbaugh (1976). Shoot tip systems have been found to synthesise approximately 150 times less *ent*-kaurene than seed systems (Coolbaugh *et al.*, 1973). A root tip system was almost inactive (Coolbaugh, 1982). The synthesising ability of seeds seems to be located mainly in the cotyledons (Coolbaugh and Moore, 1971a).

Cyclisation of GGPP to *ent*-kaurene is catalysed by *ent*-kaurene synthetase. The reaction proceeds in two stages, *ent*-kaurene synthetase A catalysing the conversion of GGPP to copalyl pyrophosphate (CPP), and *ent*-kaurene synthetase B that of CPP to *ent*-kaurene. The cyclisation of GGPP to *ent*-kaurene is an important stage in the biosynthetic pathway as it commits the molecule to biosynthesis of either GAs or a number of other classes of polycyclic diterpenoid metabolites of more limited distribution. It is therefore a possible stage for the control of GA biosynthesis. West *et al.* (1982) have reviewed the evidence suggesting a control mechanism at this stage. Another possible mechanism controlling GA biosynthesis was suggested by Rappaport and Adams (1978). They pointed out that in *in vitro* *Marah macrocarpus* systems different concentrations of different ions are necessary for different biosynthetic stages, and that ion flux could provide a control

mechanism.

A number of inhibitors of GA biosynthesis are known (see Sembdner et al., 1980). It is known that 2-chloroethyltrimethylammonium chloride,(CCC) and 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate,(AMO-1618) inhibit ent-kaurene synthesis (see Graebe and Ropers, 1978). In cell-free systems from peas CCC is much less effective than AMO-1618 in inhibiting the incorporation of MVA into ent-kaurene (Anderson and Moore, 1967). CCC also appears less effective in inhibiting pea stem elongation (Choinski and Moore, 1980). It is clear that AMO-1618 also affects a range of other cellular processes including the incorporation of MVA into sterols (Douglas and Paleg, 1972; Douglas et al., 1981). Phosfon D, which is similar structurally to AMO-1618, uncouples photosynthetic electron transport from ATP synthesis (Lendzian et al., 1978). There are also reports of AMO-1618 and CCC causing increases in endogenous GA levels (e.g. Reid and Crozier, 1970a,b; Halevy and Shilo, 1970). The results of experiments in which AMO-1618 and CCC are used to reduce GA biosynthesis therefore need to be interpreted with some caution.

There is some evidence that plastids have the ability to catalyse a number of the stages of ent-kaurene biosynthesis (see Hedden et al., 1978). Evidence for the localisation of the CDP to ent-kaurene stage in plastids of peas has been presented by Simcox et al. (1975). Evidence is also presented by Moore and Coolbaugh (1976), but this is based on very low counts and is unconvincing.

There have been several attempts to relate developmental phenomena in peas to differences in the ability of cell-free systems to synthesise ent-kaurene. However, equating the ent-kaurene synthesising ability of cell-free systems with the synthetic ability of the intact tissue requires caution as homogenisation breaks down inter- and intra-cellular compartmentation. This leads to unusual mixing of enzymes, cofactors and inhibitors which may alter the ent-kaurene synthesising capacity.

Coolbaugh and Moore (1969) followed the ent-kaurene synthesising capacity of peas during seed development. Synthetic ability was maximal at about half-maximum fresh weight and then declined. Graebe (1980) reported similar results. Ecklund and Moore (1974) studied the development of ent-kaurene synthesising ability in shoot tips of cv. Alaska. Synthesis was first detected in 3-day-old seedlings and by day 9 had increased to a maximum level which was sustained until day 24.

Ecklund and Moore (1974) also reported that shoot tips from the tall cultivar Alaska could synthesise more ent-kaurene than shoot tips from the dwarf cultivar Progress No.9. Synthesis was less in cell-free systems from dark-grown tissue of both species, and increased when dark-grown plants were transferred to the light. However, experiments with late flowering cultivars yielded inconclusive results.

Although most of the investigations into the conversion of MVA into ent-kaurene have been done using cell-free systems there are also a few reports of the conversion being observed in intact plants (See Graebe and Ropers, 1978, and Hedden et al., 1978).

1.4.2. (II) ENT-KAURENE TO GA_{12} ALDEHYDE

The intermediates between ent-kaurene and GA_{12} aldehyde are shown in figure 3. The pathway has been confirmed in Marah macrocarpus, Cucurbita maxima, Pisum sativum, Phaseolus coccineus and Gibberella fujikuroi. The enzymes catalysing the pathway seem to be microsomal mixed function oxidases, requiring molecular oxygen and reduced pyridine nucleotides. These stages of the GA biosynthetic pathway have been reviewed by Heddon (1983).

Coolbaugh and Moore (1971b) identified [^{14}C] ent-kaurenol and obtained tentative identifications of [^{14}C] ent-kaurenal and [^{14}C] ent-kaurenoic acid, originating from [^{14}C] MVA fed to cotyledon preparations from Pisum sativum, cv. Alaska. Ropers et al., (1978) identified similar radiolabelled products, along with [^{14}C]-labelled ent-7α-hydroxykaurenoic acid and GA_{12} aldehyde following the incubation of [^{14}C] ent-kaurene with a preparation from immature seeds of the cultivar "Grosser Schnabel mit Gedrücktem Korn". The wide distribution of ent-kaurenoid compounds as secondary plant products indicates that the pathway as far as ent-7α-hydroxy-kaurenoic acid is not exclusive to GA biosynthesis. Two main groups of metabolites, the kaurenolides and the oxidation products of ent-6α,7α-hydroxykaurenoic acid, have been found together with GAs in seeds of higher plants. Their formation appears to be closely related to GA biosynthesis (see Graebe et al., 1980, and Hedden, 1983). Neither group has been reported as metabolites in cell-free systems from peas.

1.4.3. (III) FURTHER METABOLISM OF [^{3}H]GA_{12} ALDEHYDE

The pathway from GA_{12} aldehyde to C_{19}-GAs has been demonstrated in G. fujikuroi and in cell-free systems from C. maxima and P. sativum. The conversion of ent-kaurene to C_{19}-GAs has also been observed in a cell-free system from P. coccineus suspensors (Ceccarelli et al., 1981a,b). The in vivo conversion of exogenous G20-methyl GAs to C_{19}-GAs has been reported in two species. Durley et al. (1974a,b) observed conversion of [^{3}H]GA_{14} to GA_1 in P. sativum, while Gianfagna et al. (1983) observed conversion of [^{2}H]GA_{53} to [^{2}H]GA_{20} in Spinacea oleracea.

In both C. maxima and P. sativum, the only two systems from which data are available, the C-20 carbon atom seems to be lost from the C-20-aldehyde GA (Graebe et al, 1980; Kamiya and Graebe, 1983), with oxidation to the acid representing a side branch. In both these systems the open ring form of the C-20-alcohol GAs seems to be the substrate for oxidation to the C-20-aldehyde. The corresponding δ-lactone C-20-alcohols are not further oxidised. It is not clear at which stage the C-20 carbon atom is lost from G. fujikuroi (see Bearder, 1983).

The identification of twenty six C_{20}-GAs showing ten different hydroxylation patterns, and the large number of structurally different C_{19}-GAs suggest that there may be several pathways from GA_{12} aldehyde to C_{19}-GAs. Microsomal enzymes appear to be involved in the initial oxidation of GA_{12} aldehyde, but later reactions appear to be catalysed by soluble enzymes (see Hedden, 1983).

Two parallel paths from GA_{12} aldehyde to C_{19}-GAs, a non-

hydroxylated path and an early 13-hydroxylated path (figure 4), have been demonstrated in a cell-free system from the cultivar "Grosser Schnabel mit Gedrücktem Korn" (Kamiya and Graebe, 1983). These paths are consistent with the GAs known to be endogenous in peas (Table 2). Pea microsomal enzymes are able to oxidise GA_{12} and GA_{12} aldehyde at C-7 and at C-13 (Ropers *et al.*, 1978; Kamiya and Graebe, 1983). It is not known whether GA_{12} or GA_{12} aldehyde is the endogenous substrate for 13-hydroxylation. *In vitro* GA_{12} seems to be preferred (Kamiya and Graebe, 1983), but low amounts of GA_{53} aldehyde have been identified as a product from GA_{12} aldehyde (MacMillan, 1978).

Microsomal oxidation of GA_{12} aldehyde has also been studied in cell-free systems from *M. macrocarpus* and from *C. maxima*. In the former, GA_{12} was tentatively identified as a product from *ent*-kaurenoic acid (West, 1973). *C. maxima* microsomes are able to oxidise GA_{12} aldehyde at C-7 and C-12, and GA_{12} at C-13. 13-hydroxy GAs are not known to be endogenous to *C. maxima* (see Hedden, 1983). In *G. fujikuroi* GA_{12} aldehyde is preferred to GA_{12} for 3β-hydroxylation (see Hedden, 1983).

Soluble enzymes in the pea are able to metabolise GA_{12} aldehyde, but the product is GA_{12} aldehyde glucosyl ester. This compound has not been reported endogenously so the significance of this conversion is unknown (Kamiya and Graebe, 1983). GA_{12} is metabolised by the soluble enzymes to GA_9 and GA_{51}, both of which are found endogenously in peas. The soluble enzymes are also able to oxidise GA_{53} to GA_{20} and GA_{29}, but they have not been demonstrated to be able to 13-hydroxylate GAs. The microsomal

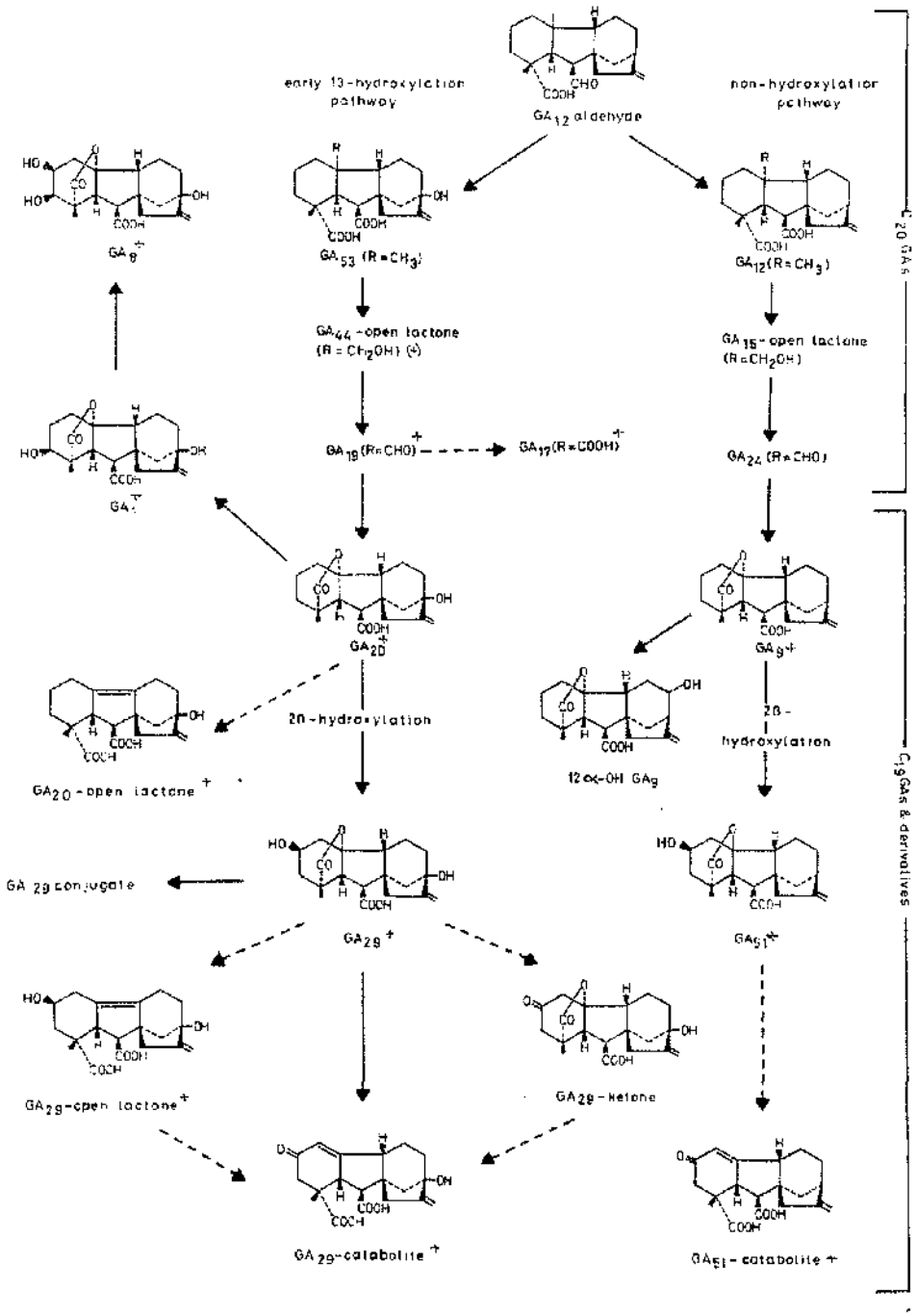


FIGURE 4. GA metabolic pathways in Pisum sativum.

→ metabolic conversion established from feeding studies

- - - → speculative metabolic conversion

\+ GA known to be endogenous to Pisum

(+) the lactone (GA_{44}) is present in plant extracts.

(Adapted from Sponsel, 1983)

13-hydroxylase is active on a range of GAs, but GA_{12} was converted with highest efficiency, suggesting that it may be the normal *in vivo* substrate (Kamiya and Graebe, 1983).

The soluble enzymes of *C. maxima* are able to oxidise GA_{12} aldehyde to GA_{12}, but show less substrate specificity than, and have different co-factor requirements to, the microsomal enzyme. (The soluble enzymes are also able to oxidise GA_{14} aldehyde and 12α-hydroxy GA_{12} aldehyde at C-7, while the microsomal enzyme acts only on GA_{12} aldehyde). The soluble enzymes are able to convert GA_{12} aldehyde to GA_4 and GA_{43}, the conversions involving C-20 loss, and oxidations at C-2, C-3 and C-7. Recent evidence suggests that parallel pathways may operate for 12α-hydroxy GA_{12} aldehyde (see Hedden, 1983).

Work on cell-free systems has therefore suggested that initial oxidation of GA_{12} aldehyde is by microsomal enzymes, but that subsequent metabolism and C-20 loss are mediated by soluble enzymes. In peas 13-hydroxylation seems to be an early event catalysed by microsomal enzymes, with subsequent metabolism being catalysed by soluble enzymes. Little is known about these enzymes, but a 2β-hydroxylase isolated from pea seeds seemed to have low substrate specificity, unlike a similar enzyme isolated from *Phaseolus vulgaris* (Hoad *et al.*, 1982). The activity of the *Pisum* enzyme was studied at stages during seed maturation and was maximum at a time when the endogenous levels of GA_{29} (the presumed *in vivo* product) peaked.

The *in vivo* ability of pea seedlings, cv. Meteor to oxidise $[^3H]GA_{14}$ to C_{19}-GAs was shown by Durley *et al.* (1974a,b). GA_1, GA_8,

GA_{18}, GA_{23}, GA_{28} and GA_{38} were identified as products. Solely on the basis of percentage incorporations after 20 and 40h the authors suggested a $GA_{14} \rightarrow GA_{18} \rightarrow GA_{38} \rightarrow GA_{23} \rightarrow GA_1 \rightarrow GA_8$ pathway. GA_{14} has not been found *in vivo* but the early 13-hydroxylation observed may reflect a natural *in vivo* step.

Further information on the pathways of GA metabolism in the pea comes from *in vivo* and *in vitro* feeds of various C-19 GAs. Each substrate will be considered individually and only feeds to peas are discussed.

[^{3}H]GA_9: Sponsel and MacMillan (1977), and Frydman and MacMillan (1975) reported the *in vitro* metabolism of [15,17-^{3}H]GA_9 in pea seeds. Two sizes of seeds were used, *ca.* 0.25g f.wt. and *ca.* 0.75g f.wt. (endogenous GA_9 levels estimated at *ca.* 10ng seed^{-1} and *ca.* 100ng seed^{-1} respectively by Frydman *et al.*, 1974). The seeds had a high capacity to metabolise exogenous [^{3}H]GA_9. The larger seeds could take up and completely metabolise *ca.* 8µg per seed in 2 days, while the younger seeds could metabolise *ca.* 1µg per seed over the same period. Metabolism appeared to be independant of dose and was similar *in vivo* and *in vitro*.

The main product from seeds of both sizes was a conjugate of 12α-hydroxy GA_9, found in the acidic ethyl acetate (EtOAc) and butanol (BuOH) partitions, which accounted for up to *ca.* 60% of absorbed radioactivity. (All percentage conversions refer to percentages of absorbed radioactivity). A small amount of GA_{51}-conjugate (*ca.* 15%) was a product from one feed. Free GAs were produced in relatively small amounts. Small seeds produced GA_{20}

and 12α-OH GA_9 (each 1-10%). GA_{51} was the major product (2-28%) from the larger seeds, together with smaller amounts of 12α-OH GA_9 (0-6%). No other products were identified and a maximum of ca. 65% of absorbed label was accounted for. As $[^3H]GA_{20}$ was only observed as a product from small seeds and as the amount detected was small the authors postulated that GA_9 was not the major source of GA_{20} in vivo.

Railton et al. (1974a,b) fed 5µg $[17\text{-}^3H]GA_9$ to 5-day-old dark grown seedlings of the cultivar Meteor. The recovery of radioactivity in the acidic EtOAc fractions was low, but since no details were given of the amount of radioactivity removed by pre-extraction washes of the plant surface, the amount of absorbed radioactivity cannot be calculated. GA_{20} (up to 4%), 12α-OH GA_9 (up to 6%) and GA_{10} were identified by GC-MS as products (percentages are of applied radioactivity). GA_{10} was thought to be a non-metabolic product. Railton and co-workers reported further studies on the metabolism of $[^3H]GA_9$ (Railton, 1974, 1977 b; Railton and Reid, 1974b). It was concluded that metabolism of $[^3H]GA_9$ was faster in the dark than in the light, and that chloroplasts metabolise $[^3H]GA_9$. However, the data presented do not justify these conclusions. The analysis of the products of $[^3H]GA_9$ was inadequate and the chloroplast preparations used were likely to have been impure.

$[^3H]GA_9$ metabolism has also been studied in relation to the photoperiodic control of apical senesence in peas (Proebsting et al., 1978; Proebsting and Heftmann, 1980). $[^3H]GA_9$ was metabolised to

at least five products, but none was identified. Metabolism may have been faster in mature than in young leaves, and in short days (SD) than in long days (LD). Proebsting et al. (1978) concluded that a unique metabolite (GA_E) was produced by the line G2 in SD, but this may be incorrect since the chromatographic resolution of the system used to analyse the products was low, and inadequate to distinguish GA_E from other products (GA_D and GA_F).

[^{3}H]GA_{20}: Frydman and MacMillan (1975) and Sponsel and MacMillan (1977, 1978) have reported the metabolism of labelled GA_{20} in pea seeds. [14, 15, 17-^{3}H]GA_{20} and [1β, 3α-$^2H_2$] [1β, 3α-$^3H_2$]GA_{20} were very efficiently converted to GA_{29}, which was the only detected metabolite. Conversion was highest when the time of feeding coincided with the maximum endogenous levels of GA_{20} in the seed. Durley et al. (1979) and Railton et al. (1974c) observed similar patterns of metabolism, also in seeds.

[^{3}H]GA_{20} fed to the apices of dark grown seedlings of the cultivar Meteor was metabolised to [^{3}H]GA_{29}, although the conversion was only 3.4% of the applied radioactivity after 20h. [^{3}H]GA_{29} was the only radioactive compound detected in the EtOAc phase after partitioning (Railton et al., 1974c). Railton (1974) reported an inconclusive investigation into the effect of light on GA_{20} metabolism. Railton and Reid (1974b) suggested that chloroplast preparations could metabolise [^{3}H]GA_{20}. This work is subject to similar criticisms as their studies of [^{3}H]GA_9 metabolism by chloroplasts.

Ingram et al. (1984) have recently reported the metabolism of [17-^{13}C, $^{3}H_2$]GA_{20} by the young expanding shoot tissue of several types of peas, differing at the Le locus. In Le plants the products identified were GA_{29}, GA_1 and GA_8 while GA_{29} and GA_{29}-catabolite were identified in extracts from le plants. The percentage conversions reported were low.

[^{3}H]GA_{29}: [^{3}H]GA_{29} has been fed only to seeds of peas. Initially no metabolism of [^{3}H]GA_{29} was detected from feeds of [14, 15, 17-^{3}H] GA_{29} or from [^{3}H]GA_{29} synthesised in situ from applied [14, 15, 17-^{3}H]GA_{20} (Frydman and MacMillan, 1975). Further studies using a double isotope labelling technique showed exogenous [2α-^{2}H]GA_{29} to be metabolised more slowly than the endogenous GA_{29} pool, which was thought likely to be due to a primary isotope effect (Sponsel and MacMillan, 1978) although lack of penetration to the active site was also recognised as a possibility. [1β, 3α-$^{2}H_2$] [1β, 3α-$^{3}H_2$]GA_{29} produced in situ from labelled GA_{20} was metabolised at the same rate as the endogenous GA_{29}, but some radioactivity seemed to be lost from the extracts during work-up.

The above results led the authors to suggest that GA_{29} was being further metabolised at the C-2 position. A specific search led to the finding of a 2-keto compound, GA_{29}-catabolite, which had also been identified from [^{3}H]GA_{20} feeds by Durley et al. (1979). Feeds of [17-$^{13}C_1$]GA_{29} confirmed the formation of this product from GA_{29} (Sponsel and MacMillan, 1980). The catabolite can occur in very large amounts (up to 24μg seed^{-1}, Sponsel, 1983) and there is evidence that it is further metabolised. Sponsel (1980b) attributed

the original failure to observe metabolism of [14, 15, 17-^{3}H]GA_{29} to the inadequate duration of the feed reported by Frydman and MacMillan (1975).

[^{3}H]GA_5: Durley et al. (1973) fed ca. 2.7µg [^{3}H]GA_5 to the apices of dark-grown seedlings, cv. Meteor. GA_3 and a compound very similar to GA_3 were detected by gas chromatography-radiolabel counting (GC-RC). Each product represented ca. 1% of the applied label. A large proportion of the radioactivity was recovered in the acidic butanol phase, suggesting metabolism to more polar compounds.

Musgrave and Kende (1970) fed [^{3}H]GA_5 to seedlings and germinating seeds of the cultivar Progress No.9. The [^{3}H]GA_5 was metabolised to more polar compounds, one of which was chromatographically-similar to GA_1.

[^{3}H]GA_1: Durley et al. (1974b) fed [^{3}H]GA_1 to dark-grown seedlings, cv. Meteor. After extraction and partitioning between 70 and 80% of the absorbed radioactivity was present in the acidic EtOAc phase. Chromatography showed at least three compounds, one of which was identified by GC-RC as [^{3}H]GA_8. The other two products were more polar than [^{3}H]GA_8.

Stoddart et al. (1974) observed conversion of [^{3}H]GA_1 to [^{3}H]GA_8 (tentative identification) in excised pea epicotyls. Conversion was extremely rapid, ca. 50% of the absorbed label being present as [^{3}H]GA_8 within 1h of the feed commencing. Kende (1967) and Barendse et al. (1968) also reported [^{3}H]GA_1 metabolism in

peas, but no products were identified.

1.4.4. SUMMARY

The pathways of GA biosynthesis in the pea appear similar to those that have been found in other species. In all of the species studied GAs have been found to be synthesised from GA_{12} aldehyde, which is itself synthesised from MVA. The pathways in the different species diverge subsequent to GA_{12} aldehyde. In peas two pathways from GA_{12} aldehyde to C_{19}-GAs have been found, a non-hydroxylated path and a 13—hydroxylated path (figure 4). The recent identification of GA_1 from peas raises the possibility that 3β-hydroxylated and 3β, 13—hydroxylated paths may also be present.

13—Hydroxylated GAs are present in pea seeds in higher amounts than non-hydroxylated GAs. Only the former have been found in seedlings. 13—Hydroxylated GAs are the predominant products formed from GA_{12} in cell-free systems when the microsomes are present (Kamiya and Graebe, 1983). Only small amounts of $[^3H]GA_{20}$ have been detected as a product from $[^3H]GA_9$ feeds, and GA_{12} was the preferred substrate for 13—hydroxylation *in vitro*. These observations suggest that the 13—hydroxylation pathway is more important than the non-hydroxylated pathway, but estimates of GA turnover are required to substantiate this.

GA-conjugates have not been found endogenously in peas, although conjugated products have been detected from $[^3H]GA$ feeds. Conversion to 2-keto compounds may represent an important GA

inactivation pathway in peas, and also in some other species.

1.5. GIBBERELLINS AND THE DETERMINATION OF STEM LENGTH IN PISUM SATIVUM

Stem length in P. sativum is determined by the length and the number of internodes produced before apical senescence occurs. There is evidence that GAs are involved in the control of apical senescence in the pea and this is considered in section 1.5.1.

GAs also have a more direct effect on stem length by increasing the rate of elongation and this is discussed in section 1.5.2. The rate of stem elongation is affected by environmental factors, especially light, and there is evidence to suggest that GAs may be involved in the photo-inhibition of stem elongation, which is discussed in section 1.5.3.

1.5.1. APICAL SENESCENCE

There is evidence, from the application of exogenous GAs and of the growth retardant AMO-1618, that GAs are involved in the control of shoot senescence in the pea (Lockhart and Gottschall, 1961; Ecklund and Moore, 1968). Shoot senescence is normally associated with flowering and the development of fruit. Recent investigations of the role of GAs in shoot senescence in peas have used lines of peas in which the genotype, with respect to the major flowering genes, is known.

Four major genes are known to control flowering in peas, one of which, Sn, has a pleiotropic effect on stem elongation (see Murfet, 1977; Ingram, 1980). Recent investigations into

senescence in the pea have mainly used the line G2 (lf E Sn Hr). This genotype flowers and fruits in both long days (LD) and short days (SD) but apical senescence only occurs in LD. Using G2 enables senescence to be studied independently of floral and fruit development (Proebsting et al., 1976), although Ingram and Browning (1980) reported that seed development is slightly retarded in SD. Proebsting et al. (1976) showed that senescence was delayed by removal of fruits in LD. Grafting studies suggested that vegetative G2 stocks were able to produce a senescence inhibiting factor, although this seemed to require the presence of Hr in the scion to be active (Proebsting et al., 1977).

It was found that GA_3 and GA_{20} could delay apical senescence when applied to G2 plants grown in LD, but GA_9 was ineffective. (Proebsting et al., 1978). This led to an investigation of the metabolism of $[^3H]GA_9$ in SD and in LD by G2, and also in the photoperiodically-insensitive I line (Proebsting et al., 1978). The authors concluded that a unique product, GA_E, was produced by G2 in SD (i.e. when senescence is retarded). The authors went on to investigate, by bioassay, the endogenous GA content of mature leaves and stipules of G2 and I plants grown in SD, and of G2 plants grown in LD. At least 3 GAs were present, and the levels of the most polar (designated E) appeared highest in G2 in SD, which led the authors to suggest that this was the senescence retarding factor in G2. They suggested that this GA might be GA_1.

Ingram and Browning (1979) and Davies et al. (1982) have

reported analyses, using GC-MS, of the endogenous GAs of G2, from both fruits and vegetative tissue. GA_1 was not found and it was suggested that the biological activity designated E, reported by Proebsting et al. (1978) may have been caused by GA_{19}. This seems unlikely if the earlier observations were correct, since GA_{19} has low biological activity and could not represent a metabolite of $[^3H]GA_9$. Ingram (1980) investigated the endogenous GAs of the seeds of a number of lines of peas, differing in their flowering genes, but could find no correlation between GA content, genotype and photoperiod.

The possible role of GAs in reproductive development and senescence in peas therefore remains unresolved. It would be of value to re-investigate the results of Proebsting et al. (1978) to confirm that GA_E (a metabolite from $[^3H]GA_9$) is produced only by G2 in SD, and to confirm the identity of the GA responsible for the peak of biological activity, designated E, which appeared to be present in larger amounts in G2 in SD.

1.5.2. STEM ELONGATION

There is strong evidence that GAs are involved in controlling stem elongation in the pea. Initial evidence for the involvement of GAs came from investigations into the effects of the application of exogenous GAs, which demonstrated that GAs could cause dramatic stem elongation in Pisum sativum. The results of these experiments have shown that:

(i) All internodes appear capable of responding to exogenous GA_3,

providing that they are either growing or embryonic at the time of treatment (Arney and Mancinelli, 1966; Giles and Myers, 1966; Spiker et al., 1976). Moore (1967) has suggested that the internodes of cv. Alaska have different sensitivities to applied GA_3. He suggested that the sensitivity of successive internodes decreases, until, as the plants enter the linear growth phase there is little further enhancement of growth by GA_3. To explain the results of Lockhart and Gottschall (1961) he also suggested that the linear phase of growth was followed by a period of increasing sensitivity to GA_3. This later phase of sensitivity seems to be partly due to GA_3 delaying senescence, rather than entirely due to a direct stimulatory effect on stem elongation. Spiker et al. (1976), showed that internodes 6-18 of the cv. Little Marvel (a dwarf) were all highly responsive to GA_3.

(ii) The time for which the increased growth persists is proportional to the dose of GA_3 applied (Brian and Hemming, 1955). Repeated applications of GA_3 sustain the high growth rate (Spiker et al., 1976).

(iii) Both tall and dwarf cultivars will respond to GA_3 application when light grown, but the response of the dwarfs is often greater (e.g. Sale and Vince, 1960; Brian and Hemming, 1955). Dwarf and tall cultivars given saturating doses of GA_3 in the light can reach the same height (e.g. Vince, 1967).

The main effect of GA_3 on stem height is due to an increase in the length of the internodes (e.g. Brian and Hemming, 1955),

although there may also be an increase in internode number (Arney and Mancinelli, 1966; Spiker et al., 1976; Vince, 1967). The latter effect is small and has not been noticed by all workers (e.g. Brian and Hemming, 1955). Exogenous GA seems to have little effect on stem or epicotyl width (Nakamura et al., 1970).

A number of reports have been published of the effects of GA at the histological level (e.g. Arney and Mancinelli, 1966; Nakamura et al., 1970; Vince, 1967). Exogenous GA_3 can increase both the number and length of cells in expanding stem tissue. The relative importance of these two effects has shown some variation. Nakamura et al. (1970) found that GA_3 had a dramatic effect on cell length in cv. Alaska epicotyls, but little effect on cell number. Arney and Mancinelli (1966) found that cell division and cell elongation contributed equally to GA_3 induced expansion of the 6th internode in the cv. Meteor. Vince (1967) found the major effect of GA_3 on the epidermis was through an effect on cell elongation.

The above observations suggest that GAs may have a role in the in vivo control of stem elongation, and to investigate this possibility a number of comparisons were made of the endogenous GAs of tall and dwarf cultivars (Kohler and Lang, 1963; Kende and Lang, 1964; Jones and Lang, 1968; Kohler, 1965a, 1970). The results generally failed to show any differences in the GA content of tall and dwarf tissue. Although Kohler (1965a) reported that the normal cultivar, Schnabel, contained more GA than the dwarf cv. Kleine Rheinlanderin later results contradicted this

(Kohler, 1970). All of the above comparisons used very crude purification procedures with detection by bioassay, and it is possible that the techniques used were inadequate to detect any differences in the endogenous GAs of the cultivars.

Lockhard and Grunwald (1970) and McComb and McComb (1970) used grafting studies to investigate whether a graft transmissible inhibitor was responsible for the difference in internode lengths between the tall cv. Alaska and the dwarf cv. Progress No.9. They confirmed that the difference was due to the Le locus, but found by grafting that the factor(s) responsible were not transmissible from roots or mature tissue to young shoots.

Thus, although the evidence from exogenous applications of GAs suggested a role for GAs in controlling internode elongation in the pea, investigations of the endogenous GA content of tall and dwarf peas failed to support this view. This led to some doubt as to whether differences in GA content controlled stem growth (Trewavas, 1981). Recently, however, Reid and co-workers have re-investigated the role of GAs in pea internode elongation using genotypes differing at a single allele. This elegant approach, used initially by Phinney and co-workers to study dwarfism in maize, has provided good evidence that stem growth in peas is at least partly controlled by the endogenous pools of certain GAs. Reid and co-workers have made investigations into the effects of five loci . The effects of these loci will now be discussed.

The Le Locus

Two alleles of this locus are known, Le plants being tall

and le plants dwarf. Dwarfism in most of the cultivars used in the earlier investigations into the role of GAs in the pea was caused by the homozygous presence of the le gene (Table 1), although other genetic differences could also have contributed to the differences in stem lengths observed between these cultivars. The le gene causes a reduction in the length of the upper internodes by 40-60% and a zig-zag appearance of the stem (Blixt, 1972). le has a lesser effect on the length of the earlier internodes, with virtually no effect on the first internode. The Le locus also seems to have a minor pleiotropic effect on flowering (see Reid et al., 1983).

Initial experiments (Potts et al., 1982a) suggested that the levels of biologically active GAs were lower in mature tissue than in young apical tissue, and that young dwarf and tall apical tissue differed in the presence of a specific polar GA. This GA was present in the two tall (Le) lines examined, but absent from all fourteen dwarf (le) lines examined. The GA had a similar retention time to GA_1 when chromatographed on a silica gel partition column, and was active in the Waito-C dwarf rice bioassay. Ingram et al. (1983) reported that application of AMO-1618 converted tall plants to the dwarf phenotype, and dwarf plants to the nana phenotype, providing evidence that the differences between these phenotypes may be due to differences in the endogenous GAs. Other experiments (Potts and Reid, 1983) suggested that maturing seeds of both dwarf and tall plants contained similar GAs, including the polar peak only found in the apical tissue of tall peas.

Reid et al. (1983) compared the number and sizes of cells in the 3rd and 7th internodes of tall and dwarf plants. There was no significant difference in the length of the 3rd internode of the two types, because although both epidermal and cortical cells were longer in Le plants both types of cells were more numerous in the le plants. The length of the 7th internode of the Le plants was ca. four times that of the le plants. In the epidermis ca. 60% of the increase was due to an increase in cell number. GA_3, applied to dwarf peas, has been found to increase both the lengths, and the numbers, of cells in internodes (Arney and Mancinelli, 1966). Reid et al. (1983) also confirmed earlier results (McComb and McComb, 1970; Lockhard and Grunwald, 1970) that the difference between tall and dwarf phenotypes is not transmissible between grafts of tall scions and dwarf stocks, or between the reciprocal grafts.

Support for the idea that the polar GA present only in tall tissue was GA_1 came from the GC-MS identification of this GA in purified extracts of the apical tissue of tall peas (Ingram et al., 1983). The presence of GA_{17}, GA_{20} and GA_8 was also suggested by selected ion current monitoring. GA_1 had not previously been detected in pea extracts (Table 2).

Ingram et al. (1983) showed that Le plants responded much better to exogenous GA_9 and GA_{20} than le plants. The response of the dwarf peas was consistent with the known structure-activity relationships of GAs in the dwarf pea bioassay (see Crozier, 1981; Hoad et al., 1976). Subsequently it was shown that [17-^{13}C ^{3}H]GA_{20}

was converted to GA_1 and GA_8 in Le plants, but not in le plants (Ingram et al., 1984). It was suggested that the observed path from exogenous GA_{20} to GA_1 and GA_8 reflected the endogenous path, and that the Le gene allows 3β-hydroxylation of GAs. It was also suggested that dwarfism in le plants results from a failure to produce enough GA_1, and that this is the major endogenous GA promoting stem elongation in peas.

The La and Cry loci

Two alleles of the La locus (La and la) and three of the Cry locus (Cry, cry^C, cry^S) are known, although additional alleles of the latter locus may exist. La and Cry are polymeric dwarfing factors. The combination la cry^S leads to slender plants in the presence of Le or le. The combination la cry^C gives the phenotype cryptotall if Le is present, and cryptodwarf if le is present. The combinations Le La Cry, Le la Cry, Le La cry^S and Le La cry^C are all phenotypically tall. Slender plants are characterised by rapid germination, long thin spindly stems, pale foliage, reduced branching, long peduncles, malformed and abortive flowers, reduced seed set and the production of parthenocarpic pods. Crypto- plants show rapid elongation of early internodes, but this rapid elongation ceases before the development of later internodes. These two loci react in a complex way with the flowering genes (Reid et al., 1983).

Reid et al. (1983) concluded that the difference between dwarf and cryptodwarf phenotypes was not graft transmissible, although the dwarf scion was grafted onto the 5th node of the

cryptodwarf stock, by which time the rapid elongation of the stock is less prominent.

Reid et al. (1983) found that le la Cry dwarfs had slightly longer internodes than le La Cry and le La cry^s plants, suggesting that La inhibits stem elongation to a larger extent than Cry. There is evidence that the internode length of slender plants is less affected by AMO-1618 than that of tall and dwarf phenotypes, and that the addition of the na gene has little effect on growth. This has led to the suggestion that some processes beyond GA perception are influenced by the La and Cry loci (Potts et al., 1982b).

The Na and Lm loci

The na allele is epistatic to the Le locus. na plants exhibit the nana phenotype which is typified by a major reduction in internode length and a darkening of foliage colour, although other characters (e.g. leaf size, stem diameter) are not reduced to the same extent as by the lm allele. The lm allele causes a reduction in all the dimensions of the plant, including the internode length of tall, dwarf, cryptodwarf and slender plants, leading to the micro-phenotype. It is not epistatic to le (see Reid et al., 1983).

The na allele causes a dramatic reduction of both cell number and cell length in both the epidermal and cortical tissue of internode 7. The effect of Na is transmissible into na scions from the roots and cotyledons, and from mature leaves and stem tissue (Reid et al., 1983). The nana phenotype is almost unaffected by AMO-1618 (Ingram et al., 1983), and is very responsive to exogenous GA_3. However the nana line L81 (le La cry^s na lm) is much less

responsive to GA_{20} than the nana line 1776 (Le La cry^{s} na Lm), which may be due to the inability of le plants to 3β-hydroxylate GA_{20} (Potts and Reid, 1983; Ingram et al., 1984).

It appears that nana phenotypes lack biologically active GAs in shoot tissue, although they are present in seeds (Potts and Reid, 1982a). Internode 1 is not dwarfed as dramatically as the others (e.g. Ingram et al., 1983). Ingram et al. (1984), using GC-MS failed to find evidence of GA_{20}, GA_{29}, GA_{1} or GA_{8} in nana dwarfs. It therefore seems that GA production is blocked at an early stage in na plants and that this results in the nana phenotype.

The work of Reid and co-workers has demonstrated that GAs are required for normal elongation of shoots in peas, and suggested that only one GA, probably GA_{1}, is highly active in promoting elongation. It is unclear whether the stem growth shown by le plants is due to some GA_{1} being formed or due to the activity per se of GAs earlier in the pathway.

Evidence also indicates that GAs are required for normal stem elongation in other species. This is best established for maize and rice (see Phinney, 1984) but Goodwin (1978) cites a number of other examples of species in which genetic dwarfs have been found, by bioassay, to have low levels of endogneous GAs (e.g. Pharbitis nil, Phaseolus vulgaris, Lycopersicon esculentum). It has been shown in a large number of species that GA application can cause considerable stem elongation (see Paleg, 1965).

Phinney and co-workers have studied GA metabolism in maize dwarfs, and have suggested that GA_{1} is the only GA promoting stem

elongation in maize (see Phinney and Spray, 1982, and Phinney, 1984). In the plants used dwarfism is expressed from the seedling to the mature plant. The dwarfs respond to GA application by normal growth, and do not respond to other hormones. A number of GAs have been isolated from the tassels of normal-type maize and the structures suggest the presence of an early 13-hydroxylation pathway. Some of the conversions in this hypothetical pathway have been confirmed by radioactive feeds (see Phinney, 1984).

GA biosynthesis in the d_5 mutant appears to be blocked between CPP and ent-kaurene. This conclusion is supported by (1) the observed biological activities of exogenous GAs applied to d_5, (2) bioassay studies, which have failed to detect endogenous GAs from d_5, and (3) studies of ent-kaurene biosynthesis in cell-free systems. The latter studies have shown that the d_5 mutant produces only $^1/_5$ of the ent-kaur-16-ene of the normal type, but produces large amounts of the isomer ent-kaur-15-ene.

In the d_1 mutant biosynthesis appears to be blocked at the 3β-hydroxylation stage. This conclusion is based on the response of the mutant to exogenous GAs, but is consistent with the results of (1) bioassay analysis indicating the presence of endogenous GAs in d_1, (2) grafting studies and (3) the bioactivity of 3β-chloro GA_{20}. GA biosynthesis in the d_2 and d_3 mutants appears to be blocked at the C-7 oxidation and C-13 hydroxylation steps respectively, although this conclusion is based only on the relative biological activities of different GAs applied to these dwarfs.

As a result of these studies Phinney and Spray (1982) concluded that in maize:

(1) The early 13-hydroxylation pathway is the only path controlling elongation growth. This conclusion arises since only 13-hydroxylated GAs have been identified from maize tassels (Hedden et al., 1982).

(2) GA_1 is the only GA in this pathway that is active per se. This conclusion arises since GA_1 is the only 3β-hydroxylated GA of high biological activity detected in extracts of maize tassels, and since the d_1 mutant, which is blocked for 3β-hydroxylation is a dwarf.

(3) Each of the four mutant genes studied (d_1, d_2, d_3 and d_5) controls a specific and different step in the pathway.

It has also been suggested that GA_1 may be the GA controlling stem elongation in rice (Phinney, 1984, see also Ingram et al., 1984). In rice the d_x gene is responsible for the dwarf phenotype of the cultivar Tan-ginbozu, and the d_y gene for the dwarf phenotype of the cultivars Waito-C and Kotake-Tamanishika. These genes are non-allelic (Murakami, 1972). The dwarf phenotype is expressed from seedling to maturity, when mutants are $\frac{1}{3}$ to $^1/5$ the height of normals (d_x^+ d_y^+) (Phinney, 1984). Both types of mutants will respond to exogenous GAs by normal growth. The structure-activity relationships of exogenous GAs (and GA precursors) suggest that there is an early block in the GA biosynthetic pathway in d_x seedlings, and a block for 3β-hydroxylation in the d_y genotype. Bioassays of extracts failed to detect GAs from d_x tissue, although GA-like substances were detected from d_y and normal type plants (Murakami, 1972).

GA_1 has been detected in extracts of rice stem tissue by GC-SIM (Kuroguchi et al., 1979; Suzuki et al., 1981). The presence of an early 13-hydroxylation pathway is suggested by the GC-MS identification of GA_{19} in shoot extracts, although the tentative identification of GA_4 from seed extracts (Kuroguchi et al., 1979) suggests the additional presence of either an early 3β-hydroxylation pathway or a late-hydroxylation pathway in this tissue. Quantification of GA_{19} and GA_1 in shoots of tall and semi-dwarf cultivars did not provide unequivocal support for the Phinney hypothesis (Suzuki et al., 1981).

In conclusion it seems that GAs are required for normal elongation of shoots in peas, and that only one GA, probably GA_1, is highly active in promoting elongation. Additional support that this hypothesis is tenable comes from work on a variety of other species, in particular maize. However, it is unlikely that GA_1 is universally controlling stem elongation in higher plants since, for example, 13-hydroxylation of GAs significantly reduces their bioactivity in the cucumber hypocotyl bioassay (see Crozier, 1981).

1.5.3. PHOTO-INHIBITION OF STEM ELONGATION

Light causes a characteristic pattern of development in plants termed photomorphogenesis. In the dark a different developmental pattern, termed skotomorphogenesis, is followed (Mohr and Shropshire, 1983). These different developmental strategies are designed to give the maximum chance of survival under different environmental conditions. Plants grown in the

light therefore show a number of structural differences compared to dark grown plants. The most obvious of these are the inhibition of stem elongation and the promotion of leaf and apical hook expansion. Since GAs have been demonstrated to cause stem elongation there has been interest in investigating whether they have a role in mediating the light-induced inhibition of stem growth.

Went (1941) reported that light inhibited stem growth and accelerated leaf growth in peas. Lockhart and Gottschall (1959) reported further investigations into the effect of light on stem elongation. They showed that the stem growth rate declined to zero soon after transfer of dark-grown peas to low or high intensity continuous red light. In the low intensity the growth rate recovered to the rate shown in the dark after 48-72h. There was also some recovery in the high intensity red light, but the new growth rate was less than that shown by dark-grown plants. Lockhart and Gottschall also found that a daily burst of low intensity red light, of up to 100 minutes duration had little effect on stem length. Vince (1967) obtained very similar results to those of Lockhart and Gottschall (1959) using both red and blue lights. However, she found that daily exposure to 15 minutes of blue or red light did cause inhibition on the first day of illumination, but not on subsequent days. Further characteristics of the effect of light are that an 8h photoperiod inhibits stem length of peas to the same degree as longer photoperiods (Lockhart and Gottschall, 1959) and that in some species a period of exposure to far red radiation at the end

of the photoperiod can greatly stimulate stem growth (Downs et al., 1957).

Went (1941) found that when a single burst of light was given the length of some internodes was reduced while others expanded more, which he interpreted as compensatory growth. Thomson and Miller (1961, 1962a, 1962b, 1963) reported a series of investigations into the development of cv. Alaska plants grown in either complete darkness or in daily light periods. They found that light accelerated growth of young internodes, but that internode growth continued longer in dark-grown plants. They postulated that the effect of light was to accelerate the developmental phases. Thus light accelerated growth of the stem apex, all phases in the subsequent development of leaves, and cell differentiation, division and elongation in the internodes. The inhibition of internode growth was visualised as being due to an acceleration of the maturation of the cells, so that the period for internode expansion by cell division and cell elongation was reduced. This concept has been supported by certain workers, but questioned by others (see Gaba and Black, 1983).

Thomson and Miller (1963) found that the internodes of light-grown plants were shorter due to a reduction in both cell length and cell number. This occurred for all stem tissues (pith, cortex, phloem and xylem), but the relative importance of the effect on division or on elongation varied between tissues. GA_3 has been shown to cause similar, but reverse, changes in the internodes of light-grown dwarf peas (Arney and Mancinelli, 1966).

At least three photoreceptors are possibly active in photomorphogenesis; phytochrome, cryptochrome and the photosynthetic pigments (Morgan and Smith, 1981). It is unclear which are involved in the photoinhibition of stem growth in pea seedlings but phytochrome has been implicated (see Galston, 1977). Both phytochrome and cryptochrome have been implicated in the de-etiolation of cucumber seedlings. Cryptochrome has also been implicated in tomato and lettuce (see Gaba and Black, 1983). If different photoreceptors are involved it is possible that they may have different mechanisms of action. For example in cucumber the recovery from cryptochrome-mediated inhibition is much faster than from phytochrome-mediated inhibition (see Gaba and Black, 1983). A number of investigations into the effect of wavelength on the photoinhibition of stem elongation in pea seedlings have been reported (e.g. Parker et al., 1949; Gorter, 1961; Vince, 1967; Elliot, 1979).

Evidence for the possible involvement of GAs in the photoinhibition of stem growth comes from the observation that GAs can stimulate stem growth in light-grown peas. Lockhart (1956) postulated that reduction of stem growth caused by light was due to either i) inhibition of GA biosynthesis, ii) enhanced GA breakdown or iii) a decrease in the sensitivity of the seedlings to GAs. Dark-grown tall peas do respond to GA in the dark (e.g. Reid, 1983; Lockhart, 1956) but the response is less than that of dwarf peas.

There is some confusion in the literature about whether the

effects of the stem dwarfing genes are expressed in the dark as well as in the light. Jones (1973), in a widely cited review, states that tall and dwarf peas grow to a similar height in darkness, citing Lockhart (1956) as evidence of this. However, the only comparison of heights that can be made from Lockhart's paper (figure 1 and figure 3) clearly indicates that the dwarf cultivar, Progress No.9, grows less in the dark than the tall cultivar Alaska. Graebe and Ropers (1978) reach the same conclusions as Jones (1973), citing the results of Sale and Vince (1960) and Gorter (1961) as evidence. Gorter (1961) did find that cv. Alaska and three dwarf cultivars grew to the same height in darkness, but the results of Sale and Vince (1960), Giles and Myers (1966) and Vince (1967) all suggest a difference in height between tall and dwarf cultivars when grown in the dark. Care must be taken, when making comparisons, to exclude light. Butler and Lane (1959) found that development of the first internode of broad bean seedlings in the dark could be affected by exposure to light during seed maturation or during imbibition.

Reid (1983) recently investigated the effect in the dark of individual genes shown to control internode length in light grown peas. He found that all of the three loci examined, Le, La and Na, reduced internode length in the dark as well as in the light. Perhaps interestingly development of leaves and of the apical hook was greatest in nana plants, less in dwarfs and least in the tall and slender plants, when grown in the dark.

Investigations of the levels of GAs in light- and dark-grown plants have yielded conflicting results. Kohler and Lang (1963), Kende and Lang (1964) and Jones and Lang (1968) failed to detect any differences between the GAs extracted from dark- and from light-grown pea plants of both tall and dwarf cultivars. However, Kohler (1965b, 1970) reported that light-grown tall and dwarf cultivars had higher levels of GAs than the corresponding dark-grown plants. The situation is also unclear in other species. Barendse and Lang (1972), using TLC and bioassay, found no difference in the free GA content of light- and dark-grown plants of two cultivars of Pharbitis nil, but levels of bound GAs were apparently higher in light-grown plants. Sponsel (1983) has pointed out that these results are difficult to interpret in terms of the bound GAs known to be endogenous to P. nil. To date probably the most reliable comparison of the GAs from light- and dark-grown plants has been made by Bown et al. (1975). They found that the GA content of light-grown tissue of Phaseolus vulgaris was higher than that of dark-grown tissue.

A different approach to the problem is to study the rate of metabolism of GAs in light- and dark-grown seedlings, but none of the investigations reported has been sufficiently rigorous to allow this comparison to be made (see Bown et al., 1975). Several comparisons of the metabolism of exogenous GAs in light- and dark-grown peas have been made (e.g. Musgrave and Kende, 1970; Kende, 1967; Railton, 1974), but these have not been conclusive. Results obtained with other species have been reviewed by Sponsel (1983).

The only system for which there is good evidence of light having an effect mediated through GA metabolism is the photoperiodic control of bolting in rosette plants. It has been suggested that LD stimulate the conversion of GA_{19} to GA_{20} in shoots of Spinacea oleracea. This hypothesis has been supported by investigations into the endogenous GAs of shoots (Metzger and Zeevaart (1980b) and by studies of the metabolism of $[^2H]GA_{53}$ (Gianfagna et al., 1983). However, in Agrostemma githago the situation was less clear, although there was evidence of changes in GA pool sizes on transfer to LD (Jones and Zeevaart, 1980).

It is unlikely that GAs are the only factors mediating photomorphogenic growth in plants. De Greef and Fredericq (1983) reviewed the role of plant growth substances in photomorphogenesis. There was evidence for the involvement of ethylene, cytokinins, abscisic acid, GAs and auxins, but the mechanisms of action and interactions of these regulators are unknown. It is unclear whether GA application will completely overcome the photoinhibition of stem growth. Vince (1967) found a difference in height between dark- and light-grown peas when both were given saturating doses of GA_3, but this may have been due to inhibition occurring before the GA had an effect. Reid (1983) found that slender plants (which respond only marginally to GA_3, are phenotypically similar to plants treated with saturating doses of GA_3, and are not inhibited by AMO-1618) were significantly shorter in light than in darkness. GA_3 does not prevent the light induced increase in internode number (Vince, 1967).

1.6. SUMMARY AND OBJECTIVES

A number of GAs have been detected in peas, both in seeds and in vegetative tissues (see Table 2). Bioassay evidence has also suggested the presence of a GA of similar polarity to GA_1, but GA_1 has only been identified in peas very recently (Ingram *et al.*, 1983, 1984). This is possibly because dwarf cultivars had been used in previous experiments. The structures of GAs identified as endogenous in peas, and *in vitro* studies, have suggested the presence of two pathways from GA_{12} aldehyde to C_{19}-GAs, an early 13-hydroxylation path and a non-hydroxylated path (see Sponsel, 1983).

Application of GAs to peas can cause dramatic stem elongation (e.g. Brian and Hemming, 1955). Light can cause a marked reduction in the rate of stem elongation (e.g. Went, 1941; Vince, 1967), but most investigations have failed to detect a consistent difference between the GAs extracted from light- and dark-grown tissue or from tall and dwarf cultivars (e.g. Kende and Lang, 1964; Kohler, 1970). This is possibly because of the inadequate nature of the techniques employed (see Crozier, 1981). It is only recently that Reid *et al.* have presented evidence of a difference in GA content between tall and dwarf pea cultivars (Potts *et al.*, 1982a; Potts and Reid, 1983; Ingram *et al.*, 1983, 1984b).

The objectives of the work presented were:

(1) To investigate and identify the endogenous GAs of the tall cultivar Alaska. Previous investigations had only identified the endogenous GAs found in dwarf cultivars. Further, earlier results

had suggested the presence of a biologically active polar compound, similar to GA_1, in peas, but no such compound had been identified until very recently (Ingram et al., 1983, 1984b).

(2) To compare the endogenous GAs of tall and dwarf cultivars, of both light- and dark-grown peas. No consistent differences had been found between the levels of GAs detected in light-grown and in dark-grown tissue (e.g. Kende and Lang, 1964; Kohler, 1970), and only recently have Reid et al. presented evidence of a difference in GAs between tall and dwarf cultivars (Potts et al., 1982a; Ingram et al., 1983, 1984b). Many of the early experiments used crude techniques, which might have been inadequate to detect any differences present. The more sensitive and accurate techniques that have now been developed made a further investigation pertinent.

(3) To study the in vivo pathways of C_{20}-GA metabolism. Until the recent work of Gianfagna et al. (1983) the reports by Durley et al. (1974a,b) of the conversion of $[^3H]GA_{14}$ to $[^3H]GA_1$ represented the only in vivo demonstration of the conversion of a C_{20}-GA to a C_{19}-GA. It was decided to repeat and extend this work, using $[^3H]GA_{12}$ aldehyde as a substrate in addition to $[^3H]GA_{14}$. $[^3H]GA_{12}$ aldehyde has been shown to be synthesised in cell-free systems from peas (Ropers et al., 1978) and is thought to be an intermediate in the synthesis of GAs in peas and in other species (see Hedden, 1983).

(4) To compare the metabolism of $[^3H]GA_9$ and $[^3H]GA_{20}$ in shoots of tall and of dwarf peas. GA_9 and GA_{20} have been identified in peas (see Table 2) and may be the precursors of the other C_{19}-GAs

found in peas. It has been suggested that one cause of dwarfism in peas is the inability to 3β-hydroxylate GAs (see Ingram et al., 1984). A comparison of the in vivo products produced from [^{3}H]GA_{20} and [^{3}H]GA_9, in tall and in dwarf peas, might provide additional information to help assess this hypothesis.

MATERIALS AND METHODS

2.1. PLANT MATERIAL

Seeds of *Pisum sativum* L. cv. Alaska, Meteor and Progress No.9 (Sinclair McGill Ltd., Boston, Lincolnshire) were soaked in running tap water for 6-8h, placed in either light or darkness at 25°C ± 2°C and germinated for 72h between sheets of moist tissue, before being transferred to aerated distilled water culture for further growth.

Dark-grown plants were handled under light from a 30W warm white fluorescent tube, covered in a single layer of a primary green "Cinemoid" filter. Light-grown plants were grown 1m below a bank of 24 "Atlas" warm white (80W) fluorescent lights and twelve 40W incandescent lights giving an intensity of 92Wm^{-2} at plant level. The heights of plants were measured from the cotyledonary node to the terminal visible node.

2.2. APPLICATION OF [^{3}H]GAs, EXTRACTION AND PARTITIONING PROCEDURES

Details of the radioactive [^{3}H]GAs used for feeds are given in table 3. The [^{3}H]GAs were applied to the terminal bud of *Pisum* seedlings in a 5mm^3 droplet of 50% aqueous ethanol. Occasional variations in this procedure are outlined when individual feeds are described.

TABLE 3. Details of [^{3}H]GAs used for feeds.

Gibberellin	Specific Activity	Preparation
[2,3-^{3}H]GA_9	50 CimM^{-1}	Yokota *et al.* (1976)
[17-^{3}H]GA_{12} aldehyde	15 mCimM^{-1}	Cross *et al.* (1968)
[17-^{3}H]GA_{14}	35 mCimM^{-1}	Cross *et al.* (1968)
[2,3-^{3}H]GA_{20}	1.8 CimM^{-1}	Murofushi *et al.* (1977)

Before extraction the area around the point of application of [^{3}H]GAs was usually rinsed with 50% EtOH. The fraction obtained was reduced to dryness *in vacuo* at 40°C and analysed by HPLC. On harvesting dark-grown tissue was frozen in liquid nitrogen in darkness, and taken to the laboratory for immediate extraction.

Plant tissue was extracted by homogenisation with cold MeOH. The homogenate was filtered and the residue repeatedly re-extracted with fresh MeOH until almost homogeneous and colourless. The combined MeOH extracts were reduced to the aqueous phase *in vacuo* at 40°C and an excess of pH8 0.5M phosphate buffer added. The concentration of the tissue equivalent in the buffer was always less than 1g f.wt. cm^{-3}.

In initial experiments the aqueous phase was partitioned against toluene, but it was found that on occasions the toluene removed a variable but significant proportion of the non-polar GAs

from an extract. Petroleum ether (boiling range 40-60°C) was subsequently used. The aqueous phase was partitioned against half volumes of the organic solvent until the bulk of the green colour had been removed.

The aqueous phase was filtered through cellulose powder. Most extracts were then slurried for 30 min with ca. 50mgcm^{-3} insoluble polyvinylpyrrolidone (PVP). The PVP was filtered off and the aqueous phase adjusted to pH2.5 with 50% sulphuric acid, prior to partitioning with 5 x $^{2}/_{5}$ volumes of EtOAc. In some experiments the aqueous phase was subsequently partitioned against 5 x $^{2}/_{5}$ volumes of butan-1-ol.

The EtOAc extracts were combined. Typically the fraction was frozen and the ice removed by filtration. The filtrate was then further dried with anhydrous sodium sulphate, filtered and a small volume of toluene added prior to reducing the EtOAc to dryness in vacuo at 40°C.

Extracts were stored at -20°C. Oxygen-free nitrogen was used to evaporate off small volumes of solvent. All solvents used were either glass distilled or of higher purity.

2.3. DEAE-A25 ANION EXCHANGE CHROMATOGRAPHY

DEAE-A25 Sephadex (Pharmacia Fine Chemicals) was swollen overnight in water and then washed for 1h periods with 0.5M sodium hydroxide, followed by 0.5M hydrochloric acid, before being left overnight in 1M sodium acetate. The gel was subsequently

equilibrated in 0.2M acetic acid:MeOH (1:1 v/v) and poured into a glass column with fritted base. The column size was dependent upon the weight of the extract, ranging from 50 x 10mm for the combined extracts of the [^{3}H]GA_9 feeds (each sample: about 20mg), up to 190 x 40mm for the large scale extraction of the endogenous GAs of cv. Alaska (sample: 7.6g).

Large extracts were dissolved in water and adjusted to pH8 with 0.2M sodium hydroxide. An equal volume of MeOH was then added and the extract applied to the column. Small extracts were dissolved directly in 0.2M acetic acid:MeOH (1:1 v/v) and applied to the column. Neutral and weakly acidic impurities were eluted with four void volumes of 0.2M acetic acid:MeOH (1:1 v/v). The GAs were then eluted with two void volumes of 2M acetic acid: MeOH (1:1 v/v). Fractions were collected and aliquots taken for liquid scintillation counting. The fractions containing the gibberellins were combined and reduced to the aqueous phase *in vacuo* at 40°C. The aqueous phase was adjusted to pH2.5 and partitioned against 5 x $^2/_5$ volumes of EtOAc, which were combined and reduced to dryness.

2.4. GEL PERMEATION CHROMATOGRAPHY

Extracts were dissolved in up to 1.5cm^3 of tetrahydrofuran (THF) and injected onto two 25 x 1000mm columns of Bio-Beads SX-4,

connected in series (see Crozier, 1981). The columns were eluted with THF at a flow rate of 2cm^3 min^{-1}. The THF was freshly distilled after refluxing over copper I chloride for 30 min. Fractions of eluent were collected and assayed for radioactivity. Fractions associated with radioactive maxima were combined and reduced to dryness *in vacuo* at 40°C.

The system has a total volume (V_T) of 630cm^3 and all compounds are eluted before this. [^{3}H]GA_9 and [^{3}H]GA_{43} have retention volumes (V_R) of 550cm^3 and 440cm^3 respectively, with peak widths of *ca.* 40cm^3.

2.5. SEP-PAK C_{18} CARTRIDGES

The Sep-Pak C_{18} cartridge (Waters Associates Inc.) was washed with 2cm^3 MeOH and 5cm^3 water. The extract was dissolved in 5cm^3 of 70% MeOH in pH3.5 ammonium acetate (20mM) and flushed through the cartridge. A further 5cm^3 of solvent was washed through and added to the extract. After removal of the MeOH *in vacuo* at 40°C the sample was frozen and freeze dried. Recoveries of [^{3}H]GA standards were *ca.* 95%.

2.6. PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The sample was dissolved in EtOAc and injected onto the column (either 10 x 450mm Partisil 10µm silica, or 10 x 250mm

Hypersil 5μm silica) via a 330mm^3 sample loop. The silica had a 40% w/v loading of 1M acetic acid, and the column was eluted with varying amounts of EtOAc in hexane at a flow rate of 5cm^3 min^{-1}. The system has been fully described by Reeve and Crozier (1978).

2.7. ANALYTICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Solvents were normally delivered at 1cm^3 min^{-1} by an Altex Model 332 gradient liquid chromatograph. Samples were injected off-column from a 250mm^3 sample loop. For reverse phase HPLC a 250 x 5mm column packed with 5μm ODS-Hypersil was eluted with varying ratios of MeOH in 20mM pH3.5 ammonium acetate. When the absorbance of the column eluent was monitored at 210nm a buffer of 20mM pH3.5 orthophosphoric acid was sometimes substituted for the ammonium acetate buffer. Buffers were purified by passage through a 100 x 10mm column containing 10μm ODS silica gel.

Normal phase analyses were carried out on a 250 x 5mm column containing a 5μm CPS-Hypersil support using a ternary solvent of varying ratios of dichloromethane, hexane and ethanol.

The following detectors were used, either singly or in series:-

a) Adapted ICN Tracerlab Manual Scintillation Spectrometer with a homogeneous 400mm^3 flow cell (See Reeve and Crozier, 1977). A 10s time constant was used. Scintillant was delivered by a Reeve Analytical Ltd. reagent delivery pump. A scintillant composed of 10g 2,5-diphenyloxazole,(PPO), 330cm^3 Triton X-100, 670cm^3 xylene and 150cm^3 MeOH was used for reverse phase analyses. A 3:1 scintillant-eluent ratio was compatible with all mobile phase conditions and gave a counting efficiency of 15-20% for tritium. The mobile phase from normal phase HPLC columns was mixed with a scintillant containing 12g PPO, 150g napthalene, 50cm^3 Triton X-100 and 1.0dm^3 distilled toluene. An efficiency of ca. 25% for tritium was obtained with a 2:1 scintillant-eluent ratio.

b) Perkin-Elmer 650-10S fluorescence spectrophotometer with a 16mm^3 flow cell. Excitation and emission wavelengths were set at 320nm and 400nm respectively for analysis of GA-methoxycoumaryl esters.

c) Pye Unicam PU4020 variable-wavelength single-beam absorbance monitor fitted with a 8mm^3 flow cell, operating at either 210nm or 254nm.

When monitors were used in series peaks were detected by the radioactivity monitor 18s after detection by the spectrophotometers. Retention times given are corrected for this delay.

2.8. BIOASSAYS

a) Barley aleurone bioassay

The method used was similar to that described by Nicholls and Paleg (1963). Seeds of *Hordeum vulgare* cv. Kym were dehusked in 50% sulphuric acid. Four embryo-less half seeds were placed in a $20cm^3$ glass vial and $1cm^3$ of test solution added. GA_3 standards from 4×10^{-3} to $10^0 \mu g cm^{-3}$ were also assayed. All solutions were assayed in duplicate. Reducing sugars were measured after 40h incubation at 25°C, using the Somogyi-Nelson method, as described by Paleg (1960).

b) Lettuce hypocotyl bioassay

The method used was similar to that of Brian *et al.* (1964). Seeds of *Lactuca sativa* cv. Arctic King were germinated on moist filter paper in darkness at 25°C. Test substances were dissolved in $3cm^3$ of distilled water in 4.5cm diameter glass petri dishes containing filter paper. GA_3 standards from 10^{-3} to $10^0 \mu g cm^{-3}$ were assayed. Ten seedlings were placed in each dish. Hypocotyl lengths were measured after 72h incubation in the light at 25°C.

c) Dwarf rice microdrop bioassay

The method used was similar to that of Murakami (1970). Seeds of *Oryza sativa* cv. Tan-ginbozu were soaked for 40h in distilled water then planted on 1% agar in $30cm^3$ Beaton jars.

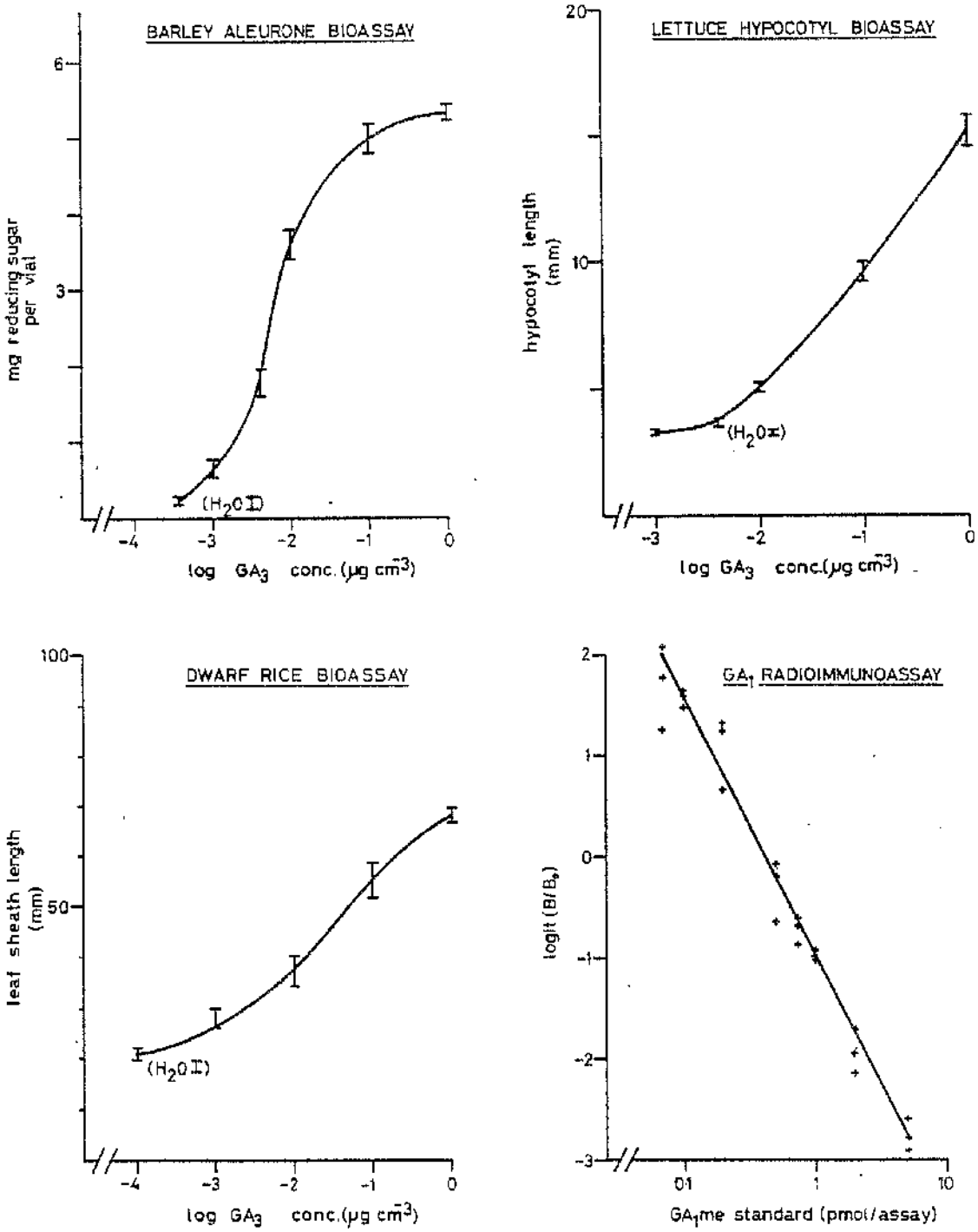


FIGURE 5. Typical bioassay and GA_1 radioimmunoassay standard curves. Vertical bars represent standard errors. B_0 values for the GA_1 RIA were 5448, 5929 and 5322 cpm.

After 48h growth in the light at 32°C 1mm^3 of 50% EtOH containing the test substance was applied to the junction of the blade and sheath of the first leaf. GA_3 standards from 10^{-4} to $10^0 \mu g mm^{-3}$ were also assayed. Five replicate plants were used for each treatment. After a further 72h the length of the first leaf sheath was measured.

Typical standard curves for the bioassays are shown in figure 5.

2.9. GA_1 RADIOIMMUNOASSAY

The radioimmunoassay of Atzorn and Weiler (1983) was used. The incubation mixtures contained 0.1cm^3 methylated sample or standard, 0.5cm^3 buffer (0.01M phosphate, 0.15M NaCl, pH7.4), 0.2cm^3 dilute (diluted 1:7) bovine serum, 0.1cm^3 dilute antiserum and *ca.* 7000cpm [1,2-^{3}H]GA_1 methyl ester (38.2 Cimmol^{-1}). Standards contained from 0.07pmol to 5pmol of methylated GA_1. Samples and standards were assayed in triplicate. After mixing tubes were incubated for 2h at room temperature, and the antibody-antigen complex separated from free antigen by the addition of 1.25cm^3 of 91% saturated ammonium sulphate solution. After 30 min at room temperature the tubes were centrifuged, the supernatants decanted and the pellets resuspended in 1cm^3 of 50% ammonium sulphate. The tubes were re-centrifuged, the supernatant

discarded and the pellet dissolved in 0.25cm^3 distilled water. 1cm^3 scintillant (Koch-Light Ltd. "Minisolve") was added and samples counted for 2 min.

Data was analysed as described by Atzorn and Weiler (1983). Standard curves were plotted as logit relative tracer binding ($^B/_{Bo}$) against the logarithm of the amount of unlabelled methylated GA_1 standard. A typical standard curve is shown in figure 5.

2.10. LIQUID SCINTILLATION COUNTING

Aliquots were counted in 6cm^3 plastic vials containing 5cm^3 of a scintillant composed of 5g 2,5-diphenyloxazole, 0.3g 1,4-Bis-2(4-methyl-5-phenyloxazole) benzene,(dimethyl POPOP), 200cm^3 Triton X-100 and 800cm^3 of distilled toluene. Samples were counted in an LKB 1211 Minibeta liquid scintillation counter.

All counts presented for the [^{3}H]C_{20}-GA feeds are in counts per minute (cpm), with no correction for the counting efficiency. In all the other experiments counting rates were corrected for the counting efficiency and the results are given in disintegrations per minute (dpm). Correction for counting efficiency was either by addition of an internal standard or by the sample channels ratio method. There was very good agreement between the results of these two methods when they were compared.

A standard curve for the sample channels ratio (SCR) method

was obtained using [^{3}H]-hexadecane quenched with varying amounts of chloroform. For the internal standard method 4 aliquots of each sample were counted. Two of these replicates were counted after addition of ca. 30,000 dpm of [^{3}H]GA_9 in 5mm^3 of MeOH. The internal standard method was only used to correct a few of the counts of the [^{3}H]GA_9 feeds.

2.11. PREPARATION OF DERIVATIVES

a) Methoxycoumaryl esters

Methoxycoumaryl esters were prepared as described by Crozier and Durley (1983) and Crozier et al. (1982). Extracts were reacted with 4-bromomethyl-7-methoxycoumarin (BMMC) and 18-Crown-6 in 100mm^3 of dry acetone containing a crystal of potassium carbonate. 1mmol BMMC and 100µmol 18-Crown-6 were used per mg of extract weight. The reaction mixture was incubated for 2h at 60°C, the solvent evaporated off and the residue dissolved in distilled water. GACEs were extracted by partitioning into chloroform.

b) Methyl esters

Compounds were methylated using an etherial solution of diazomethane prepared using a modification of the method of Schlenk and Gellerman (1960). Approximately 0.5g N-methyl-N-nitro-p toluene sulphonamide was placed in a 100cm^3 conical flask with side arm attachment. To this was added 10cm^3 diethyl ether, 1cm^3

methoxyethanol and $3cm^3$ concentrated potassium hydroxide. The flask was stoppered and heated in a hot water bath. The open end of the side arm was placed under ca. $10cm^3$ diethyl ether in a flask cooled in ice. On heating the reaction mixture diazomethane gas and diethyl ether distilled over and dissolved in the cold ether. The solution was used when an intense yellow colour had developed.

Each sample for methylation was dissolved in $100mm^3$ MeOH and $100mm^3$ of diazomethane solution prior to incubation at 0°C for 30 min. The solvent was then evaporated.

2.12. COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY

GC-MS analyses to detect the endogenous GAs of cv. Alaska were carried out by Dr. P. Hedden, East Malling Research Station. Analyses of the fractions from the feed of $[^3H]GA_{14}$ were performed by Dr. L. Rivier, University of Lausanne, Switzerland.

Prior to GC-MS methylated samples were trimethylsilated with N,O-bis (trimethylsilyl)trifluoroacetamide for 30 min at 90°C.

GC-MS analyses to detect endogenous GAs in the cv. Alaska extract (section 3.3.) were carried out using a VG1212 mass spectrometer coupled to a Dani 3800 HR gas chromatograph and a Dani 2015 data system. Samples were injected onto a fused silica column (25m x 0.2mm), coated with a BP-1 stationary phase, connected directly to the ion source. The temperature programme

rose ballistically from 50°C to 240°C, and at 4°C min^{-1} from 240°C to 300°C. The helium inlet pressure was 80KPa. The source ionisation energy, emission current and temperature were respectively 70eV, 100μA and 200°C. There was a 2.2 s scan time. GAs were identified by comparison of the spectra obtained with spectra of authentic GA standards analysed under the same conditions as used for the experimental analyses.

GC-MS analyses of fractions purified from the extract of [^{3}H]GA_{14} fed plants (section 3.5) were carried out using a Hewlett-Packard Model 5985A combined GC-MS. Samples were injected onto a fused silica capillary column (25m x 0.3m), with a stationary phase similar to SE-54 (Hewlett-Packard), connected directly to the ion source. The temperature programme was isothermal at 100°C for 1 min and then 10°C/min to 260°C, with a helium inlet pressure of 50KPa. The ion source ionisation energy was 70eV and the emission current 300μA. The source temperature was 200°C. Mass spectra were collected every 1.2s.

The results from the [^{3}H]GA_{14} analysis were analysed as follows. The mass spectra, of peaks of the total ion current trace that were larger than 0.5% of the base peak size, were compared by the computer with a library containing reference spectra of the MeTMSi derivatives of GA_1 to GA_{62}. These reference spectra were kindly provided by Professor J. MacMillan (University of Bristol). Both forward and reverse library searches were

performed and identifications were based on a similarity index higher than 0.96 (absolute identity had an index of 1) together with direct comparison with authentic standards run under the analytical GC-MS conditions.

RESULTS

3.1. GROWTH OF EXPERIMENTAL PLANTS

Typical growth curves for light and dark-grown peas are shown in figure 6. The growth rates appear to be linear at an early age. It was noticed that light-grown seedlings of the cv. Alaska grown for the earlier experiments were taller than plants of an equivalent age grown for the later experiments. The difference in height was quite large (mean height of 11 early batches, 44 ± 0.9mm; mean height of 8 later batches, 34.1 ± 1.5mm; 40 plants from each batch of ten day old plants measured). No difference in height was apparent between cv. Alaska plants grown in the dark from the earlier and the later batches of seed.

The cause of the discrepancy may be a difference in the seed used, since the cv. Alaska seeds used in the later experiments were of a different batch from the seed suppliers. The first batch of cv. Alaska seeds was used for $[^3H]GA_{14}$ metabolism studies, and also accounted for half the tissue extracted in section 3.3. The later batch of seeds was used for all other experiments. All seedlings of cv. Progress No.9 were grown from a single batch of seeds, as were those of cv. Meteor. No difference in height was found between groups of these seedlings grown at different times.

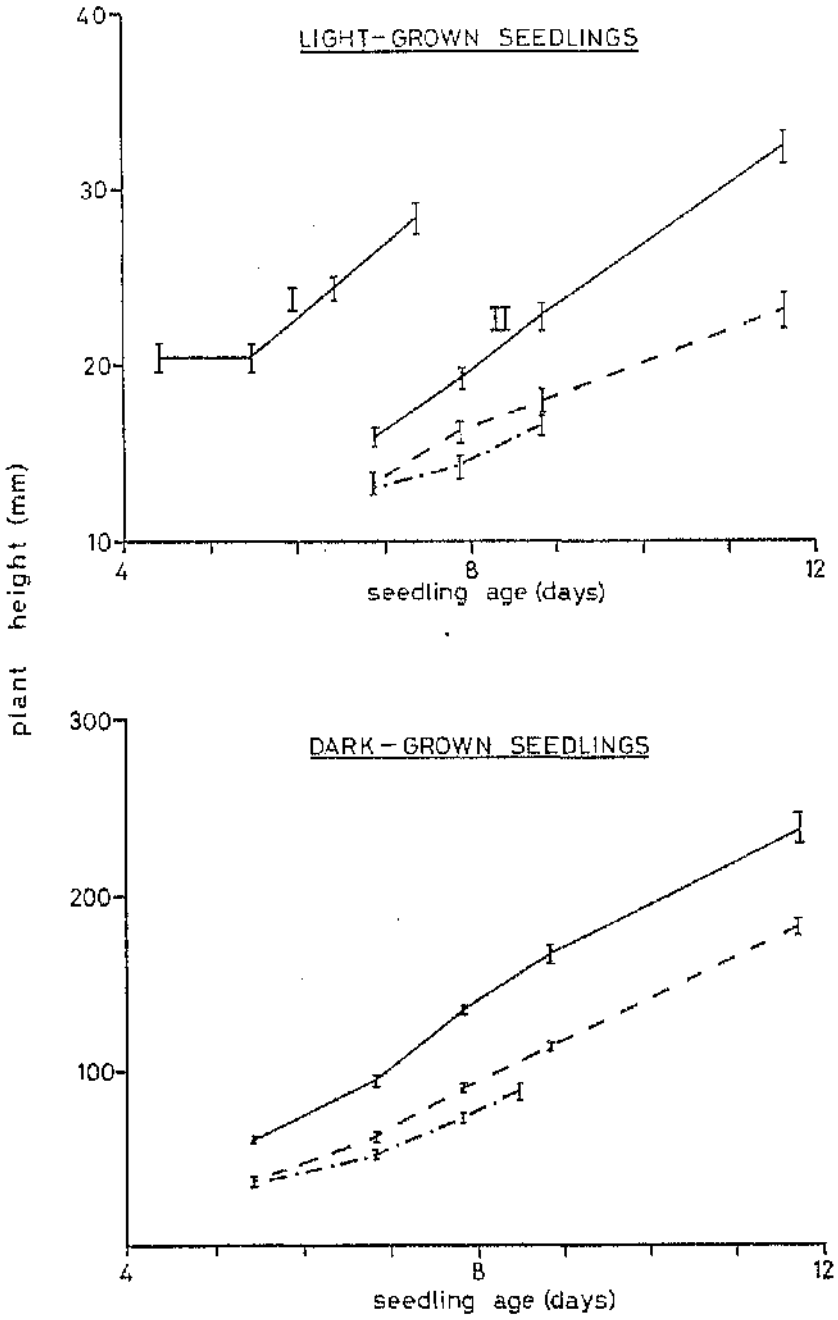


FIGURE 6. Growth of experimental plants. Seedlings were transferred from moist tissue paper to water culture on day 3. The growth curves shown are typical of those measured. Vertical bars show standard errors. 40 plants measured per point.

——— cv. Alaska: I; first batch of seed.
II; second batch of seed.

— — cv. Meteor

—.—. cv. Progress No.9.

3.2. REVERSE PHASE HPLC OF GIBBERELLINS

The availability of a range of GA standards and the ability to accurately determine the retention times of these during reverse phase HPLC made it possible to repeat and extend earlier studies of the reverse phase HPLC elution characteristics of the GAs (e.g. Koshioka et al., 1983). GA standards were detected by on-line monitoring of the radioactivity in, or the absorbance at 210nm of, the column eluent. The detection limit of the latter method was ca. 20ng.

The relative retention characteristics of the GA standards tested are shown in figure 7. The R_{10} value is the percentage of MeOH estimated to give a peak retention time of 10.0 min (see section 3.7.4.). From these results the following conclusions can be drawn (these are similar to those of Koshioka et al., 1983).

For C_{19}-GAs:

(a) A single hydroxyl substitution will reduce the retention time, but the extent of the decrease depends on the substitution position. The effectiveness of the substitution decreases in the order $12\alpha \approx 16\alpha > 13\alpha > 2\beta > 3\beta$. Therefore a C/D ring substitution seems to have a larger effect than A ring substitution.

(b) The effect of further hydroxyl substituents is generally to decrease the retention time further, but this depends on the substitution positions of the hydroxyl groups. Thus if one hydroxyl group is 3β the effectiveness of a second one decreases

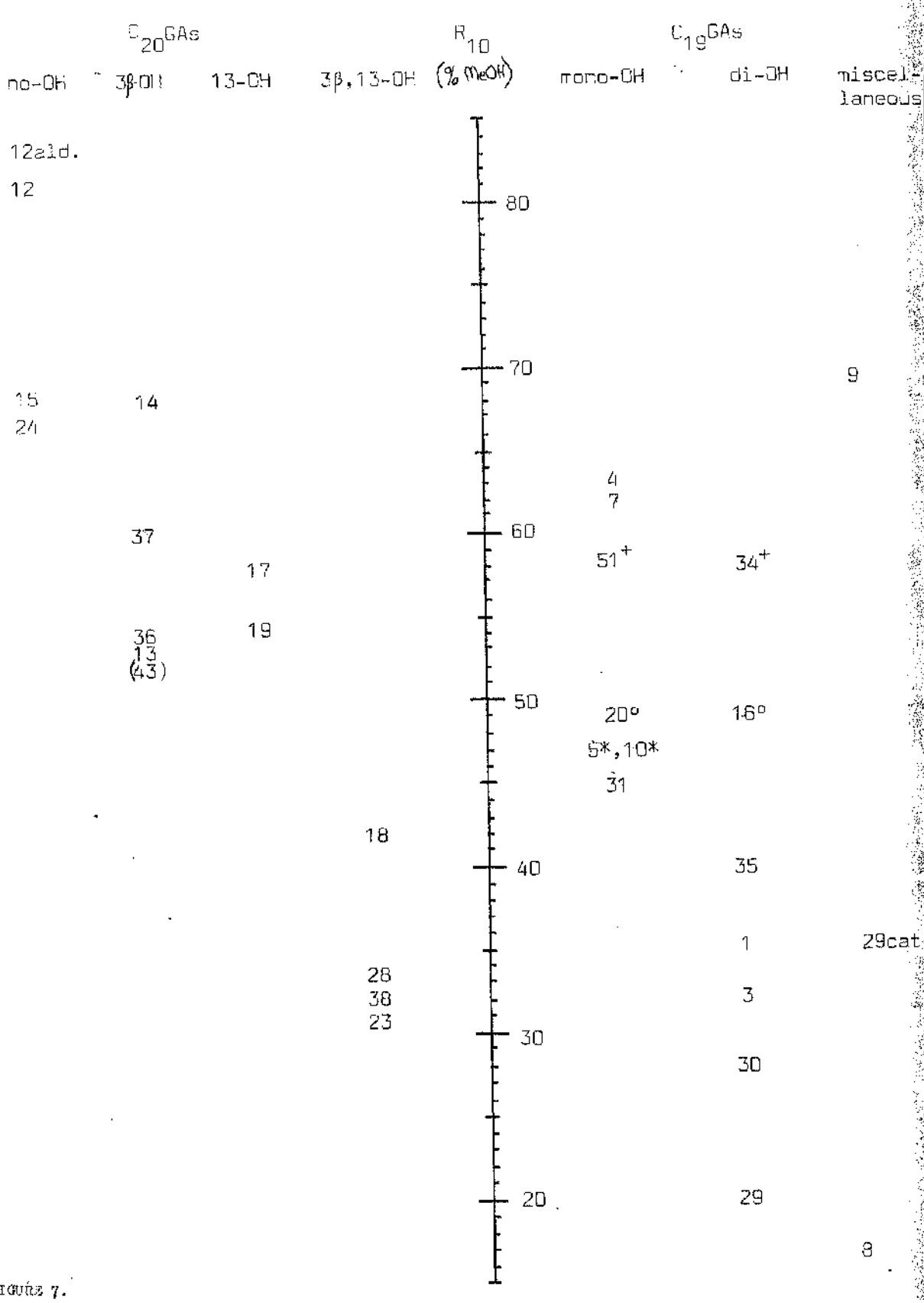


FIGURE 7.

Retention behaviour of GA standards on reverse phase HPLC. C_{20}GAs are shown on the left-hand side, C_{19}GAs on the right-hand side. Each GA is placed adjacent to the central scale at the position corresponding to the percentage of MeOH that would give a R_t of 10min on isocratic reverse phase HPLC analysis (e.g. GA_1, ca. 63%).
+, o, *: these pairs of GAs were shown to be unresolved, even on isocratic analysis.
(GA_{43}): GA_{43} is 2β, 3β-dihydroxy GA_{12}.

in the order 12α > 13α > 11β > 1α > 2β. If the only hydroxyl group is in the A ring then further substitution of the A ring is less effective in reducing the retention time than substitution of the C ring. 2β-hydroxylation causes a larger reduction in retention time than 3β-hydroxylation (e.g. GA_1/GA_{29}, GA_4/GA_{51}). Addition of a 3β-hydroxyl group to a 2β-hydroxylated GA has little effect on the retention time (e.g. GA_{51}/GA_{34}, GA_{29}/GA_8).

(c) The presence of a double bond tends to decrease the retention time (e.g. GA_4/GA_7, GA_5/GA_{20}, GA_1/GA_3).

For C_{20}-GAs:

(a) C-20-methyl GAs elute later than the corresponding C_{19}-δ-lactone GAs (e.g. GA_{12}/GA_9, GA_{14}/GA_4, GA_{18}/GA_1).

(b) Oxidation of the C-20-methyl group to an alcohol can have quite a large effect on retention time, but further oxidation to the aldehyde or acid has less further effect.

(c) The relative effectiveness of different hydroxyl substitutions may be different for C_{20}- and for C_{19}-GAs (e.g. GA_4/GA_{20}, GA_{17}/GA_{13}).

3.3. GC-MS ANALYSIS OF THE ENDOGENOUS GAs OF cv. ALASKA

10.5kg (fresh weight) of ten day old light-grown cv. Alaska seedlings (ca. 11,800 plants) were extracted with the objective of obtaining sufficient quantities of GAs for GC-MS analysis. The plants, grown in nineteen batches, were frozen in liquid nitrogen, freeze-dried and stored at -20°C prior to extraction. Whole seedlings (roots, shoots and cotyledons) were extracted.

The tissue was extracted in five batches, and an internal standard of $[^3H]GA_9$ (total 3.4×10^6 dpm, 10ng) added to two of the batches to quantify losses during work-up. The pH8 aqueous extracts were partitioned against toluene, filtered through cellulose powder, slurried with PVP, adjusted to pH2.5 and partitioned against EtOAc. The EtOAc was dried and evaporated off. The five EtOAc residues were combined, the total weight being 8.0g. There was 60% recovery of the internal standard.

The extract was dissolved in 100cm^3 pH8.0 0.2M ammonium acetate and further partitioned against a half-volume of toluene. ca. 22% of the internal standard partitioned into the toluene, which was discarded. The aqueous phase was adjusted to pH6.5 and applied to a 130 x 40mm column of PVP. The column was eluted with 0.2M pH6.5 ammonium acetate buffer and 10cm^3 fractions were collected. 95% of the recovered $[^3H]GA_9$ eluted in a peak between fractions 15 and 35. Fractions 10-50 were combined and freeze-dried.

The partially purified extract (7.6g) was further purified by DEAE-A25 Sephadex anion exchange chromatography (weight of extract reduced to 350mg, 39% of internal standard recovered), size exclusion chromatography (extract weight reduced to 280mg) and by use of a Sep-Pak C_{18} cartridge. The residual bulk of the extract was composed mainly of a non-volatile component, which was removed by dissolving the extract in $5cm^3$ of pH8 0.5M phosphate buffer and partitioning against 2 x 1 cm^3 toluene, which was discarded. The aqueous phase was then adjusted to pH2.5 and partitioned against EtOAc, which was dried and evaporated off.

The extract (50mg, 27% recovery of internal standard) was dissolved in $270mm^3$ of EtOAc and purified by preparative HPLC using a 450 x 10mm column containing Partisil 10 coated with a 40% w/v 1M acetic acid stationary phase. The column was eluted with a gradient of EtOAc in hexane (figure 8) designed to give maximum separation of GAs.

Seventy successive 2 min fractions were collected, and the solvent evaporated off. Aliquots from each fraction were analysed using three bioassays and the GA_1 radioimmunoassay (GA_1 RIA) of Atzorn and Weiler (1983). The results are shown in figure 9. Subsequently the fractions detailed in Table 4 were analysed by GC-MS.

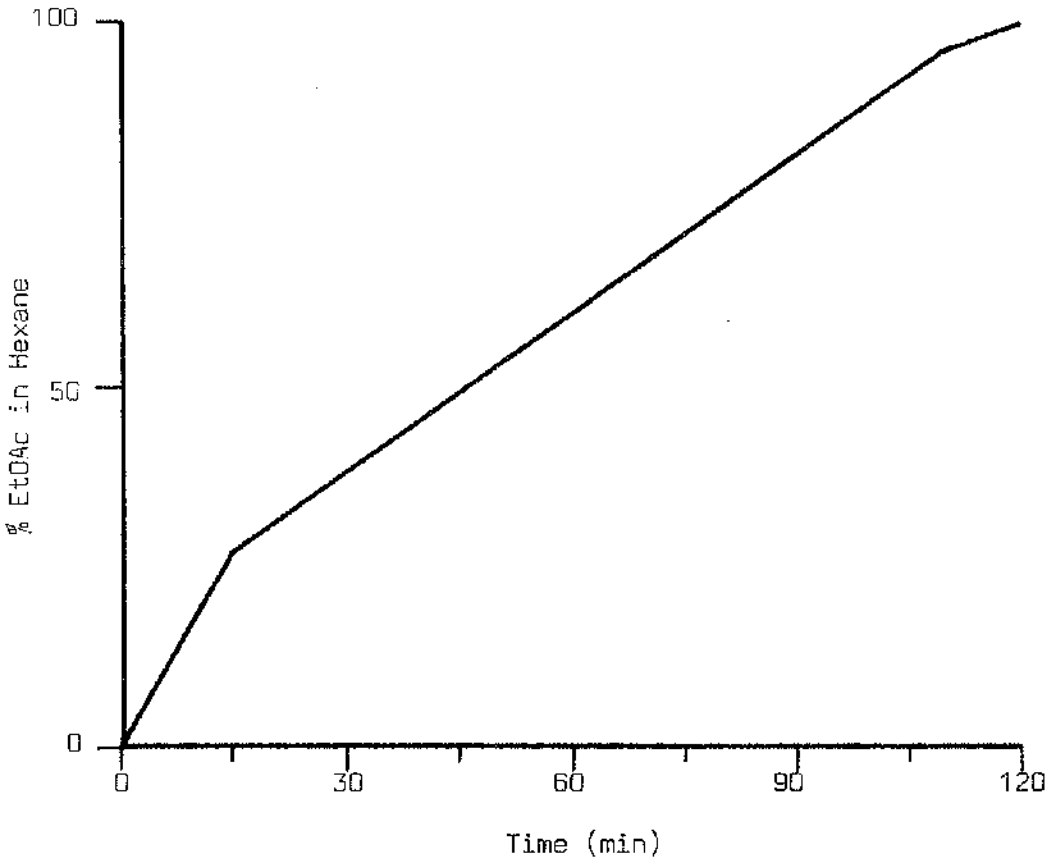


FIGURE 8. Preparative HPLC gradient used to fractionate cv. Alaska extract.

TABLE 4. Details of fractions analysed by GC-MS

Fraction	GA identified
29 and 30 (combined)	GA_{20}
42	?
45 and 46 (combined)	GA_{29}
56	none

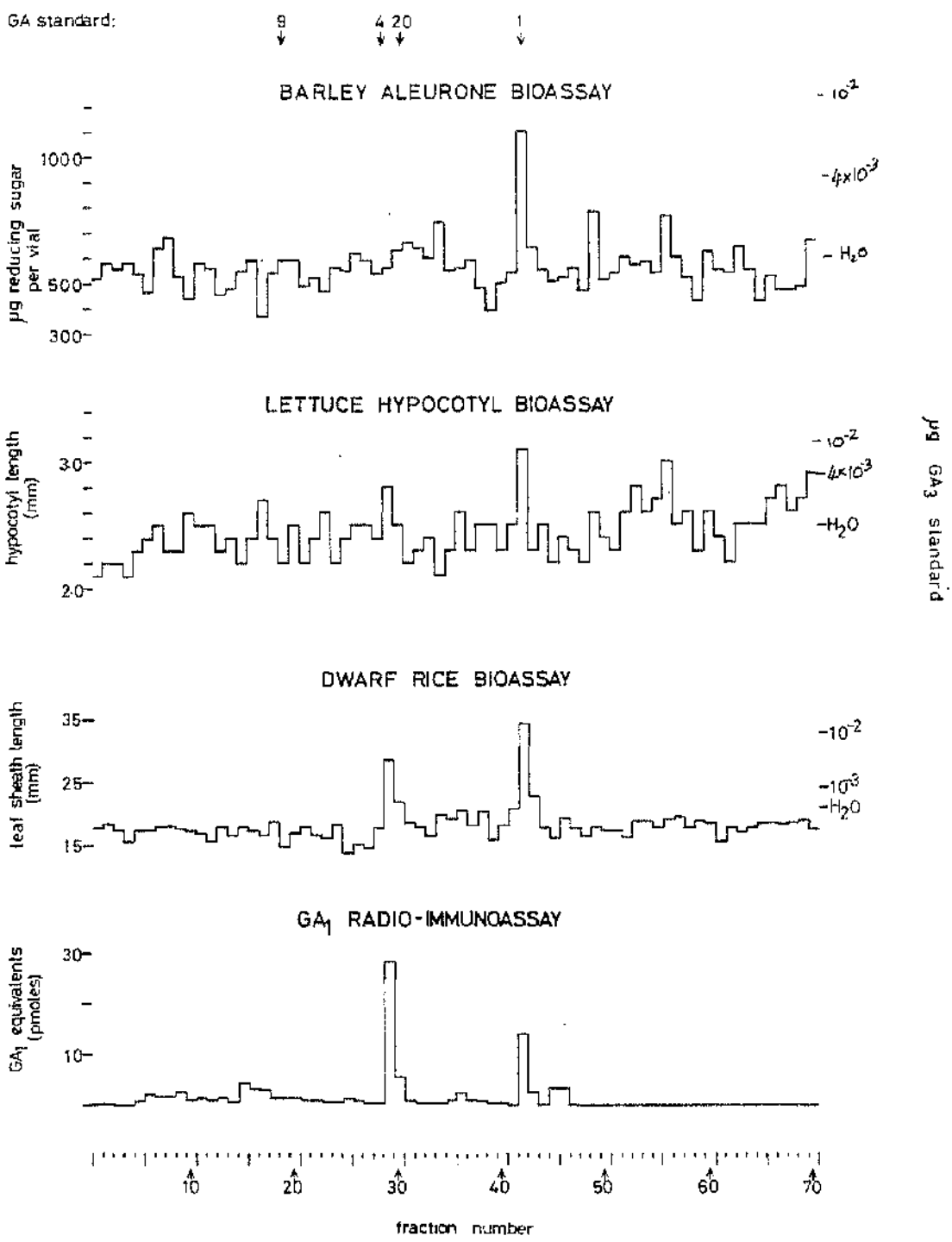


FIGURE 9. GA-like substances extracted from cv. Alaska seedlings. Figure shows bioassay and immunoassay determinations of GA-like substances present in fractions obtained after chromatography of the extract of cv. Alaska on the preparative HPLC system. The amounts of GA_1 and GA_3 standards indicate the GA-like activity in 1% of the total extract. The expected elution positions of GA., GA_4, GA_9 and GA_{20} are shown at the top of the figure.

The [^{3}H]GA_9 internal standard was detected in fractions 17 and 18 with an estimated recovery of ca. 18%. The low recovery is attributed largely to losses into the toluene during partitioning. Since GA_9 is one of the least polar GAs losses of other GAs may not have been so large, and recoveries were possibly greater than 18%.

Fraction 29 showed activity in the lettuce and rice bioassays and in the GA_1 RIA. Fraction 30 also showed activity in the GA_1 RIA. GA_{20} was subsequently identified in these fractions by GC-MS (figure 10). GA_{20} has been previously found in seedlings (see Table 2). If it is assumed that the activity of fractions 29 and 30 in the GA_1 RIA is caused entirely by GA_{20} then, knowing the cross reactivity of GA_{20} in the assay (55%) and the recovery during work up (18%), it is possible to calculate the levels of GA_{20} per seedling as ca. 900pg.

Fractions 45 and 46 were found to contain GA_{29} (figure10). This GA has been previously found in pea seedlings (see Table 2), and exhibits low activity in all four assays used.

Fraction 42 shows GA-like activity in all four assays. GA_1 and other GAs of similar elution characteristics would be expected to occur in this fraction. GA_1 has previously been found in pea seedlings (see Table 2) and is active in all four assays. However, neither GA_1, nor any of the other known GAs was detected by GC-MS analysis of fraction 42. No spectrum contained m/z 506, the molecular ion of GA_1MeTMSi. From the

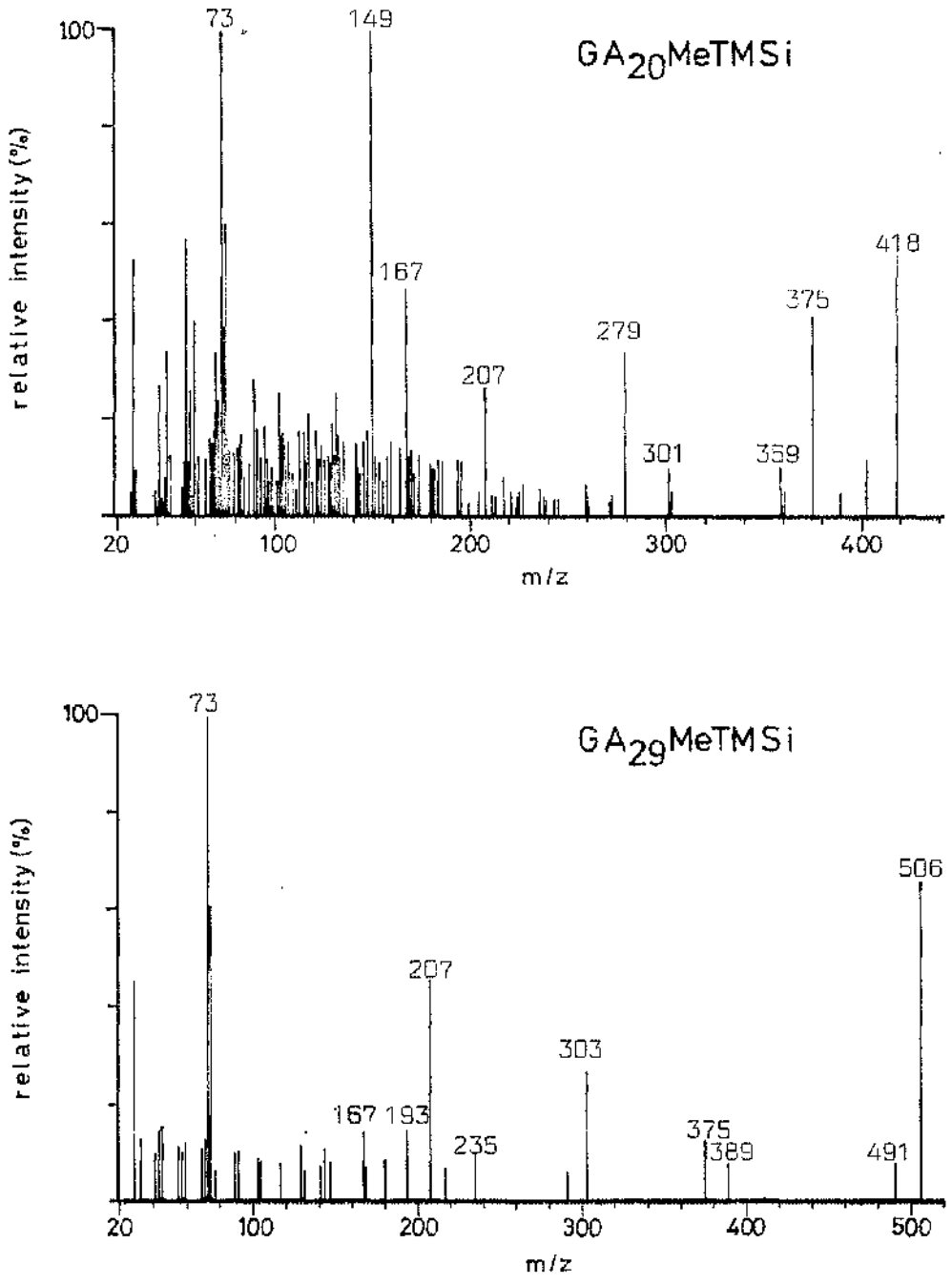


FIGURE 10. Mass spectra of GA_{20} MeTMS and GA_{29} MeTMS, found in ten day old seedlings of cv. Alaska.

bioassay and immunoassay data any known GA, if responsible for the activity seen, should have been present well above the detection limits of the analysis. Therefore it seems unlikely that GA_1, or any other known GA, is responsible for the activity detected by the assays. There is the possibility that the presence of trace quantities of several GAs could account for the activity seen in the assays, but this is unlikely due to the separation of GAs provided by the preparative HPLC.

The only component of fraction 42, that showed a mass spectrum similar to a GA had a GC retention index of 2629, which is lower than that of GA_1 (2693). The mass spectrum obtained from this compound is shown in figure 11. The spectrum is background subtracted, but the peak around scan 190 in the TIC trace was essentially free of contaminants. The presence in the spectrum of a prominent ion at m/z 207 is characteristic of a 13-hydroxylated GA. m/z 462 may represent the molecular ion. If this compound is responsible for the activity seen in the assays then further structural features can be tentatively suggested in view of the known structure-activity relationships of the assays. GAs active in the barley aleurone bioassay typically have a 2-3 double bond or a 3β-hydroxyl group in the A ring. Such a configuration would also be expected to be active in the other assays used. It is possible, however, that a novel and untested configuration could have high activity in the assays and account for the activity seen.

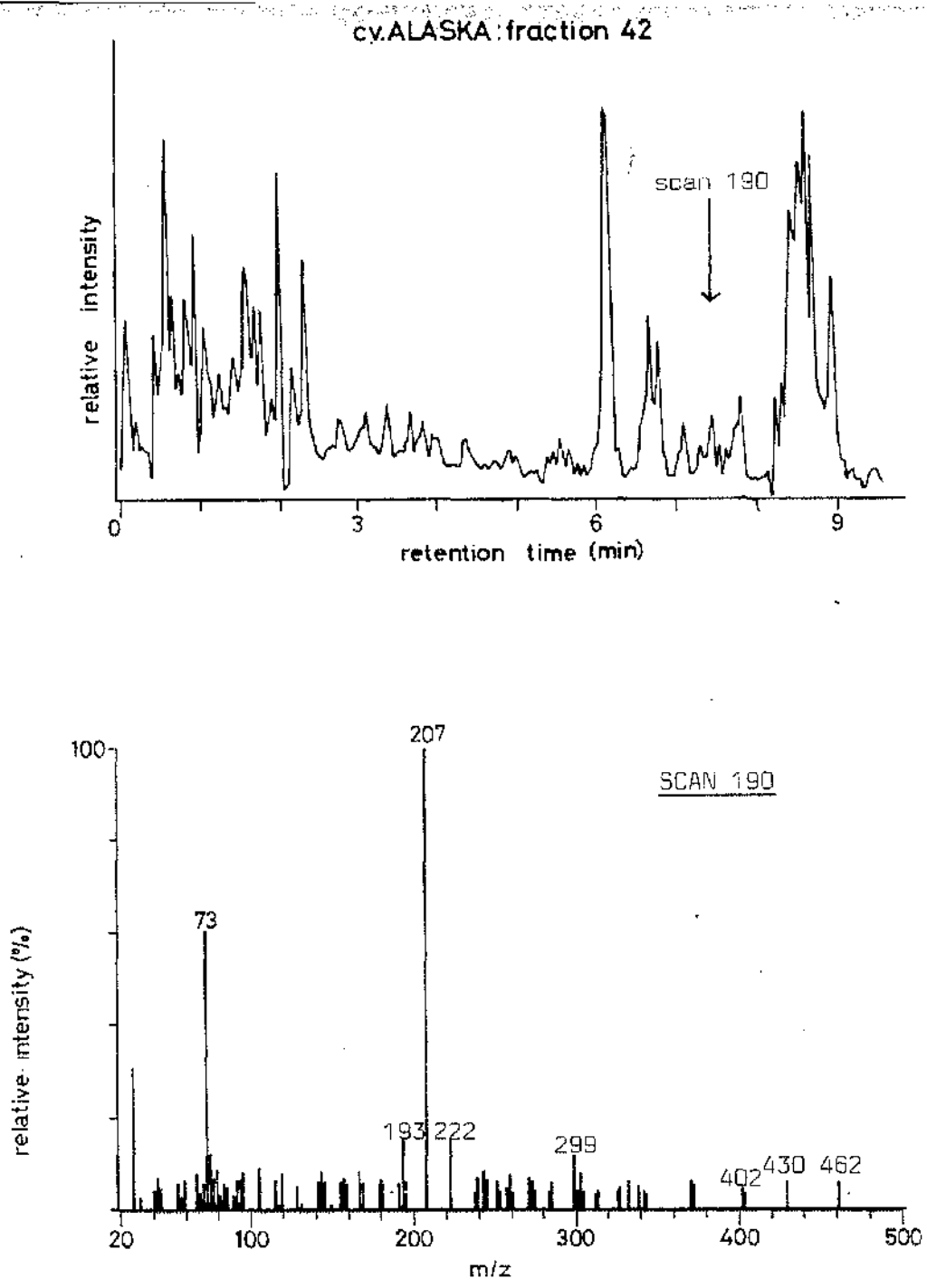


FIGURE 11. GC-MS analysis of fraction 42. This fraction, of an extract of cv. Alaska seedlings, appeared to contain a GA_1-like component, although GC-MS analysis failed to detect GA_1. Upper trace shows the total ion current trace of the fraction. The lower trace shows the mass spectrum of the putative GA-like compound, obtained at the arrowed point of the total ion current trace.

No other fractions showed prominent and consistent activity in the assays. Fraction 56 appeared active in the lettuce and barley bioassays, but GC-MS analysis of this fraction failed to reveal any of the known GAs.

3.4. COMPARISON OF THE ENDOGENOUS GIBBERELLINS OF TALL AND DWARF CULTIVARS BY IMMUNOASSAY.

8 day old shoots of light and dark-grown peas, of cultivars Alaska (tall), Meteor (dwarf) and Progress No.9 (dwarf) were extracted after being excised at the cotyledonary node. The amounts of tissue extracted are detailed in Table 5. [^{3}H]GA_{14} (845,000dpm) was added to each methanolic extract as an internal standard. [^{3}H]GA_{14} was suitable in this regard since it has negligible cross reactivity in the immunoassay employed.

TABLE 5. Details of tissue extracted and the recovery of the internal standard for the GA_1 RIA analysis of endogenous GAs.

	Light-Grown			Dark-Grown		
	Alaska	Meteor	Progress	Alaska	Meteor	Progress
Number of shoots extracted	165	155	204	220	223	222
Weight of tissue extracted (g)	34.0	34.4	37.1	101.8	104.3	94.7
Recovery of internal standard*	22%	37%	21%	35%	50%	54%

* after reverse phase HPLC fractionation

Methanolic extracts were evaporated to ca. 2cm^3, taken up in phosphate buffer, partitioned against petroleum ether, slurried with PVP, adjusted to pH2.5 and partitioned against EtOAc. The crude

EtOAc fractions were purified by DEAE-A25 Sephadex anion exchange chromatography. Potential GA containing fractions were combined and further purified by eluting each through a Sep-Pak C_{18} cartridge.

Each purified EtOAc fraction (ca. 5mg) was fractionated by reverse phase HPLC (see figure 12). Thirty five successive 1 minute fractions were collected for each extract. Aliquots of fractions 23-27 were assayed for radioactivity to determine the recovery of $[^3H]GA_{14}$. The recoveries are detailed in Table 5. The low recovery of both cv. Alaska extracts is due to spillage. The reasons for the low recovery of the extracts of light-grown plants of cultivars Meteor and Progress No.9 are not known.

The HPLC fractions were dried, methylated and 10% aliquots analysed in triplicate using the GA_1 radioimmunoassay (GA_1 RIA) of Atzorn and Weiler (1983). The results, corrected for the recovery of the internal standard and expressed a femtomoles GA_1-like substance per seedling, are shown in figure 12. At least four peaks of GA—like activity are apparent from the extracts of light-grown tissue, around fractions 5, 8, 17 and 21. When considering the possible identities of compounds causing these peaks it is necessary to take into account the elution times of the peaks and the results of cross-reactivity studies that have been made using the GA_1 RIA.

Atzorn and Weiler (1983; personal communication) have

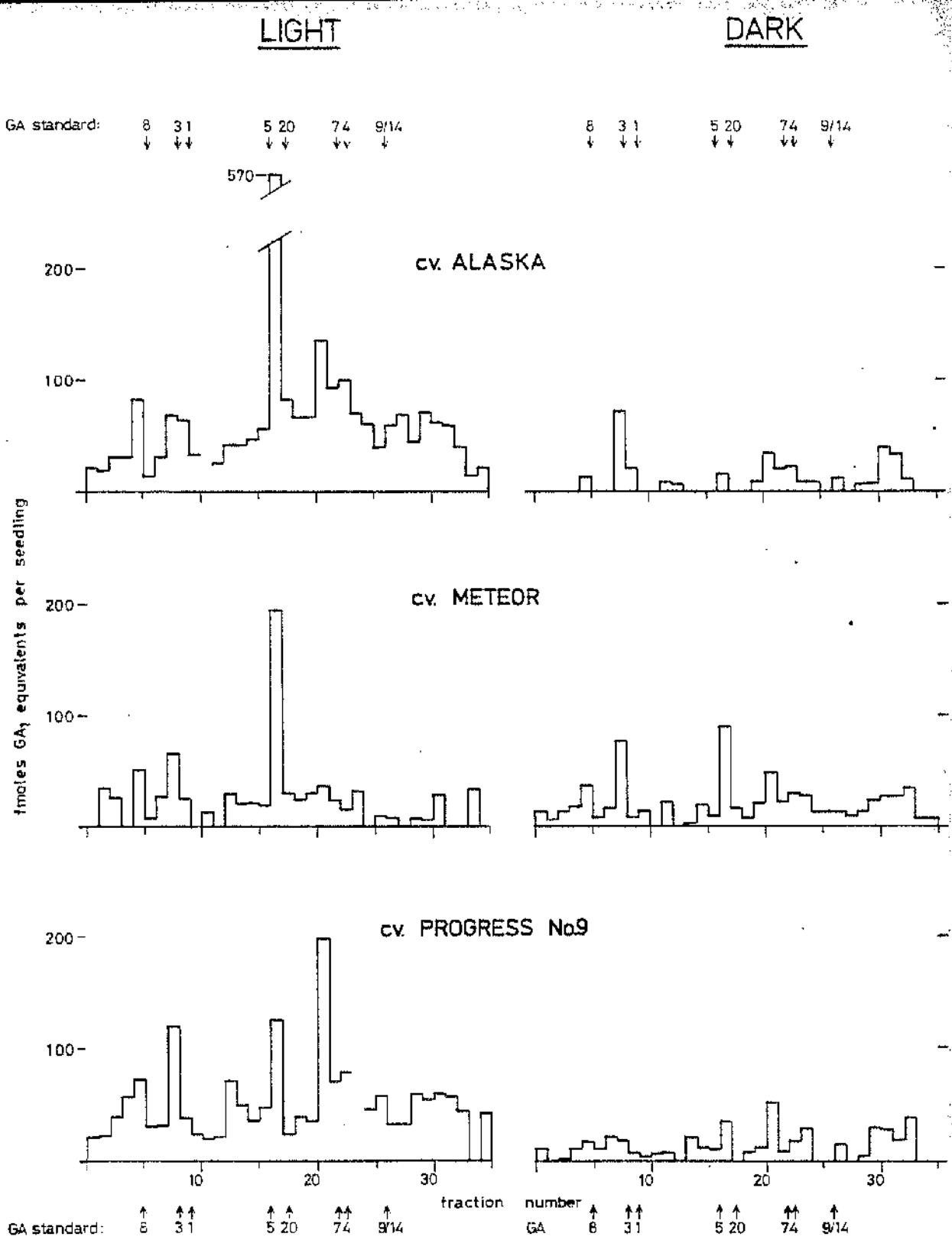


FIGURE 12. GA-like substances in light- and dark-grown Pisum seedlings.

Figure shows the amounts of GA-like substances detected by GA_1RIA in reverse phase HPLC fractions obtained after chromatography of the acidic EtOAc fractions of extracts of light- and dark-grown shoots of the cultivars Alaska, Meteor and Progress No.9. Fraction 11 from light-grown shoots of cv. Alaska, and fraction 24 of cv. Progress No.9, not analysed. The expected elution positions of certain GAs are shown at the top of the figure. HPLC mobile phase: 30-35% MeOH, 0-1 min; 35-50% MeOH, 7-8 min; 50-70%, 14-15 min; 70-100%, 25-26 min; 1cm^3 min^{-1}. 35 x 1 min fractions collected.

studied the cross-reactivities of GAs 1-53 in the GA_1 RIA, with the exceptions of; GA_{11}, GA_{25}, GA_{43}, GA_{45}, GA_{46}, GA_{49}, GA_{50} and GA_{52}. A number of GA conjugates were also tested, some of which were found to be cross-reactive. The results of these cross-reactivity studies are summarised in Table 6 .

TABLE 6. Cross reactivities of GAs and GA conjugates in the GA_1 RIA. Data expressed as % cross reactivities on a molar basis (Courtesy of Dr. Atzorn and Dr. Weiler, Ruhr-Universitat, Bochum).

Free GAs		Conjugated GAs
GA_1	100	GA_1-3-glucoside 6
GA_3	70	GA_1-13-glucoside 10
GA_4	40	GA_1-glucoside ester 5
GA_5	29	GA_3-3-glucoside 2
GA_6	29	GA_3-13-glucoside <1
GA_7	70	GA_3-glucoside ester <1
GA_8	11	GA_4-3-glucoside 2
GA_9	15	GA_4-glucoside ester <1
GA_{10}	6	GA_5-13-glucoside 5
GA_{20}	55	GA_5-glucoside ester 6
GA_{22}	2	GA_7-3-glucoside 3
GA_{31}	20	GA_8-2-glucoside <0.1
GA_{35}	11	GA_{26}-2-glucoside <0.1
Other free GAs tested	1	GA_{29}-2-glucoside <0.1
		GA_{35}-3-glucoside <0.1
		GA_{37}-glucoside ester <0.1
		GA_{38}-glucoside ester <0.1

The cross reactive GAs (except GA_9, GA_{10} and GA_{20}) all have a 3β-hydroxyl group and/or a double bond or an epoxide group (GA_6) in the A ring. 2β-hydroxylation reduces activity. A 13-hydroxyl group usually promotes activity but is not essential. Glucosides have reduced activity, but may still show significant binding. It is likely that the GA-glucoside esters would have been removed from the extracts by the DEAE-A25 Sephadex anion exchange chromatography procedure, although glucoside ethers are still likely to be present (Gräbner et al., 1976).

GA_{29} and GA_8 have both been found in peas and both would be expected to elute around fraction 5. GA_{29} has negligible cross-reactivity so would have to be present in very large amounts to account for the activity seen in figure 12.

The peak in fraction 8 may represent the GA-like compound, of undetermined structure, which was detected in fraction 42 of the cv. Alaska extract previously reported (section 3.3). This compound had a similar retention time to GA_1 during straight phase preparative HPLC, and was reactive in the GA_1 RIA. Alternatively, the peak in fraction 8 may represent GA_1, recently identified as an endogenous GA of peas (Ingram et al., 1983).

Fraction 17, from light-grown tissue of all cultivars, contains a major peak of similar retention time to GA_{20}. In view of the identification of GA_{20} from seedlings of cv. Alaska and cv. Progress No.9, and the high cross-reactivity of GA_{20} in the

GA_1 RIA, it seems likely that this peak is GA_{20}. Fraction 13 of cv. Progress No.9 possibly contains a small peak, but the possible identity of the causative compound is unknown.

The other major peak, in fraction 21, is seen mainly in the extracts of light-grown tissue of cultivars Alaska and Progress No.9. Of the C_{19}-GAs identified from peas only one, GA_{51}, would be expected to chromatograph in this area. However, GA_{51} has a cross-reactivity of under 1% in the GA_1 RIA.

The levels of the compounds detected are summarised in Table 7. These quantitative estimates are very approximate, especially for minor peaks, as no allowance was made for the level of background activity in the assay, or for the possibility that a compound was split between two fractions (with the exception of the peak in fractions 8 and 9 of the light grown cv. Alaska extract). The background activity appears higher in the extracts of light-grown tissue of cultivars Alaska and Progress No.9. This is because the recovery of the $[^3H]GA_{14}$ internal standard in these extracts was low, so that a higher factor was used to correct for losses during extraction.

The amounts of peaks of similar retention times can be compared between extracts, assuming that an identical compound is causing the activity in the different extracts. However, the amounts of different peaks within an extract cannot be compared since they are caused by different compounds which probably have different cross-reactivities.

TABLE 7. Estimates of the amount of GA-like immunoreactive compounds. Figures represent femtomoles per seedling.

Fraction	Tissue	Cultivar Alaska	Meteor	Progress No.9
Fraction 5	Light-grown	80	50	70
	Dark-grown	10	40	20
Fraction 8	Light-grown	130*	60	120
	Dark-grown	70	80	20
Fraction 17	Light-grown	540	190	130
	Dark-grown	-	90	40
Fraction 21	Light-grown	140	40	200
	Dark-grown	30	50	50

* includes fraction 9

If it is assumed that the peak seen in fraction 17 is caused by GA_{20} then it is possible to calculate the absolute amount of GA_{20} present in the tissue (since GA_{20} has a cross-reactivity of 55% in the GA_1 RIA). The results of such a calculation are shown in Table 8. The level of GA_{20} found in light-grown seedlings of cv. Alaska, ca. 300pg per seedling, is lower than the level of ca. 900pg estimated in section 3.3. This difference may arise because whole seedlings were extracted in section 3.3., while only the shoots were extracted in the present experiment. Alternatively the difference may partly reflect the different ages of the tissue when extracted, or may simply arise through experimental error. The levels of GA_{20} are ca. 100 fold less than those estimated by Ingram et al. (1984).

TABLE 8. GA_{20} levels estimated by immunoassay. Data expressed as pg per seedling.

	Alaska	Meteor	Progress No.9
Light-grown plants	330	110	70
Dark-grown plants	-	60	20

The tall cultivar Alaska, and the dwarf cultivars Progress No.9 and Meteor, all contain GA-like substances, and given the errors in the results the differences detected between cultivars may not be significant. It is apparent that the levels estimated in dark-grown tissue appear consistently lower than the levels estimated in light-grown tissue. As this result was seen in extracts of all three cultivars it probably represents a real difference. The difference is much larger when the results are expressed on a unit fresh weight basis.

3.5.1. $[^3H]GA_{14}$ FEEDS: INTRODUCTION

Two major experiments were carried out. In the first $[^3H]GA_{14}$ was applied to plants by two application methods and the products studied after 4 different periods of time, up to 89h after application. Identification of products was not attempted in this time-course experiment. Based on the results of this first experiment a second experiment, with the objective of identifying some of the products produced from $[^3H]GA_{14}$, was conducted. Other investigations confirmed the stability of $[^3H]GA_{14}$ on the plant surface during the feeds, and during the extraction and analytical procedures employed. Prior to all experiments the radiochemical purity of the $[^3H]GA_{14}$ was confirmed by isocratic reverse phase HPLC at 70% MeOH. (Retention time of $[^3H]GA_{14}$: 11.7 min).

3.5.2. $[^3H]GA_{14}$: TIME COURSE FEED

The time course feed compared the metabolism of $[^3H]GA_{14}$ when applied to either the apical bud or the cotyledonary node of the cultivar Alaska. The stability of $[^3H]GA_{14}$ during the analytical procedures was confirmed by adding the label to three extracts during homogenisation. These extracts were partitioned normally to give crude EtOAc fractions, which were then purified by DEAE-A25 Sephadex anion exchange chromatography and by use of Sep-Pak C_{18} cartridges. Analysis of the purified

extracts by isocratic reverse phase HPLC at 70% MeOH showed that [^{3}H]GA_{14} was the only radioactive compound present, confirming that this compound was stable during purification and analysis.

For the time course feed 150,000 cpm [^{3}H]GA_{14} (ca. 2µg), dissolved in 5mm^3 of 50% aqueous ethanol, was applied to each of eighty light-grown 6 day old seedlings. A further 20 plants were treated with 5mm^3 of 50% aqueous ethanol not containing [^{3}H]GA_{14}. The treatments were applied to the apical bud of half of the total number of plants, and injected into the cotyledonary node of the remaining half.

Ten apically treated and ten cotyledonary-node treated plants were extracted after 19, 43, 67 and 89h. Prior to each extraction the area around the point of application of the [^{3}H]GA_{14} was rinsed with ca.10cm^3 MeOH. Reverse phase HPLC analyses, at 70% MeOH, showed that these rinses contained radiochemically pure [^{3}H]GA_{14}, demonstrating that this compound was stable on the plant surface during the feed.

The height of each experimental plant was measured before treatment and 19, 43, 67 and 89h after treatment. From these measurements the growth of the plants after treatment was calculated. The results are shown in figure 13. The mean height of plants before treatment was 22.3 ± 0.4 mm. [^{3}H]GA_{14} application appeared to stimulate stem elongation, but application to the cotyledonary node may reduce growth relative to apical application.

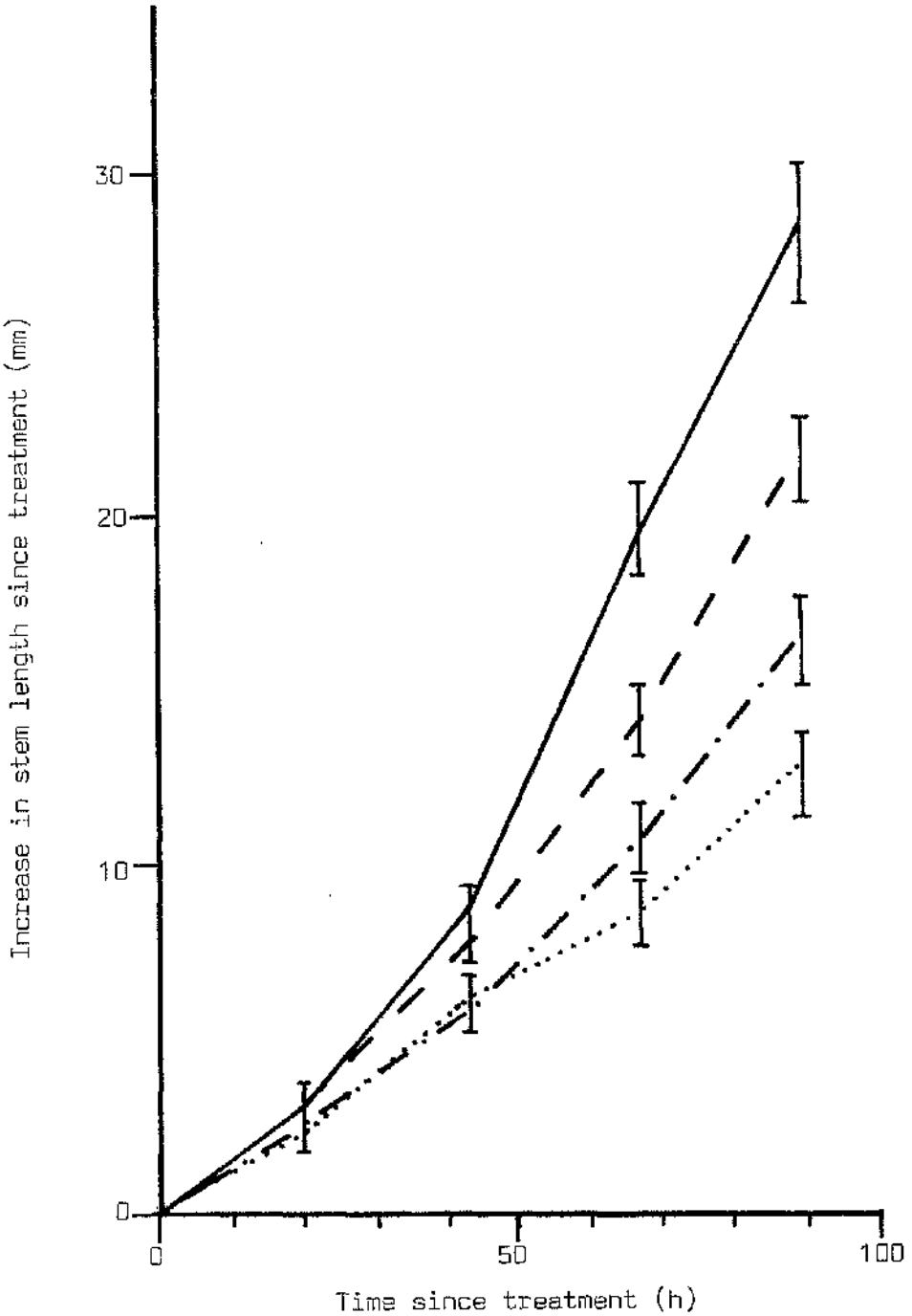


FIGURE 13. Growth of [^{3}H]GA_{14} treated plants.

——— ca. 2µg [^{3}H]GA_{14} applied to apical bud

— — — ca. 2µg [^{3}H]GA_{14} injected into cotyledonary node

—·—·— 50% aqueous EtOH applied to apical bud

····· 50% aqueous EtOH injected into cotyledonary node

(10 plants from each control treatment measured. 40, 40, 30, 20 and 10 plants respectively from each [^{3}H]GA_{14} treatment measured 0, 19, 43, 67 and 89h after treatment).

Whole seedlings were extracted with MeOH which was subsequently evaporated off. An excess of pH8 phosphate buffer was added to the aqueous residue and the extracts purified by partitioning against toluene and by slurrying with PVP. Details of the recoveries of the applied radioactivity are given in Table 9. The errors in the table may be large, since no

TABLE 9. $[^3H]GA_{14}$ Time course: recovery of radioactivity. Data are percentages of the applied radioactivity, calculated from cpm.

Point of application	Bud				Cotyledonary Node			
Duration of feed	19h	43h	67h	89h	19h	43h	67h	89h
Fraction: MeOH rinse	57%	45%	40%	27%	10%	9%	6%	8%
Toluene	-	-	-	8%	-	4%	-	-
Aqueous	3%	4%	2%	3%	4%	3%	6%	6%
EtOAc	11%	19%	19%	20%	25%	19%	27%	21%
Total Recovered	71%	68%	61%	58%	39%	35%	39%	35%

correction was made for quenching which was possibly high. The bulk of the extracted radioactivity was found in the EtOAc fraction and there was no major trend with time towards increasing amounts of radioactivity remaining in the aqueous phase. The toluene phase did not generally contain much radioactivity.

The recovery of radioactivity from both application methods

was low, and it is apparent that the recovery from cotyledonary node treated plants was even lower than that from apically treated plants, largely due to less radioactivity being recovered in the methanolic rinse. The latter is not unexpected since less [^{3}H]GA_{14} would be expected to remain on the plant surface when the label was injected into the cotyledonary node, than when it was applied to the surface of the apical bud. Therefore more radioactivity would have been expected to be extracted from the cotyledonary node treated tissues, although this does not appear to have been so. Therefore the difference in recovery of the applied radioactivity between the two treatments, and the overall low recovery, remain unexplained.

The acid EtOAc fractions were analysed by reverse phase HPLC and the traces are shown in figures 14 and 15. The retention times

TABLE 10. Retention times (min) of [^{3}H]GA standards on reverse phase HPLC. HPLC conditions as figure 14.

Injection number	1	2	3	4
[^{3}H]GA_1	11.1	11.1	10.5	10.9
[^{3}H]GA_4	28.0	-	-	-
[^{3}H]GA_{14}	30.4	30.3	29.9	30.5

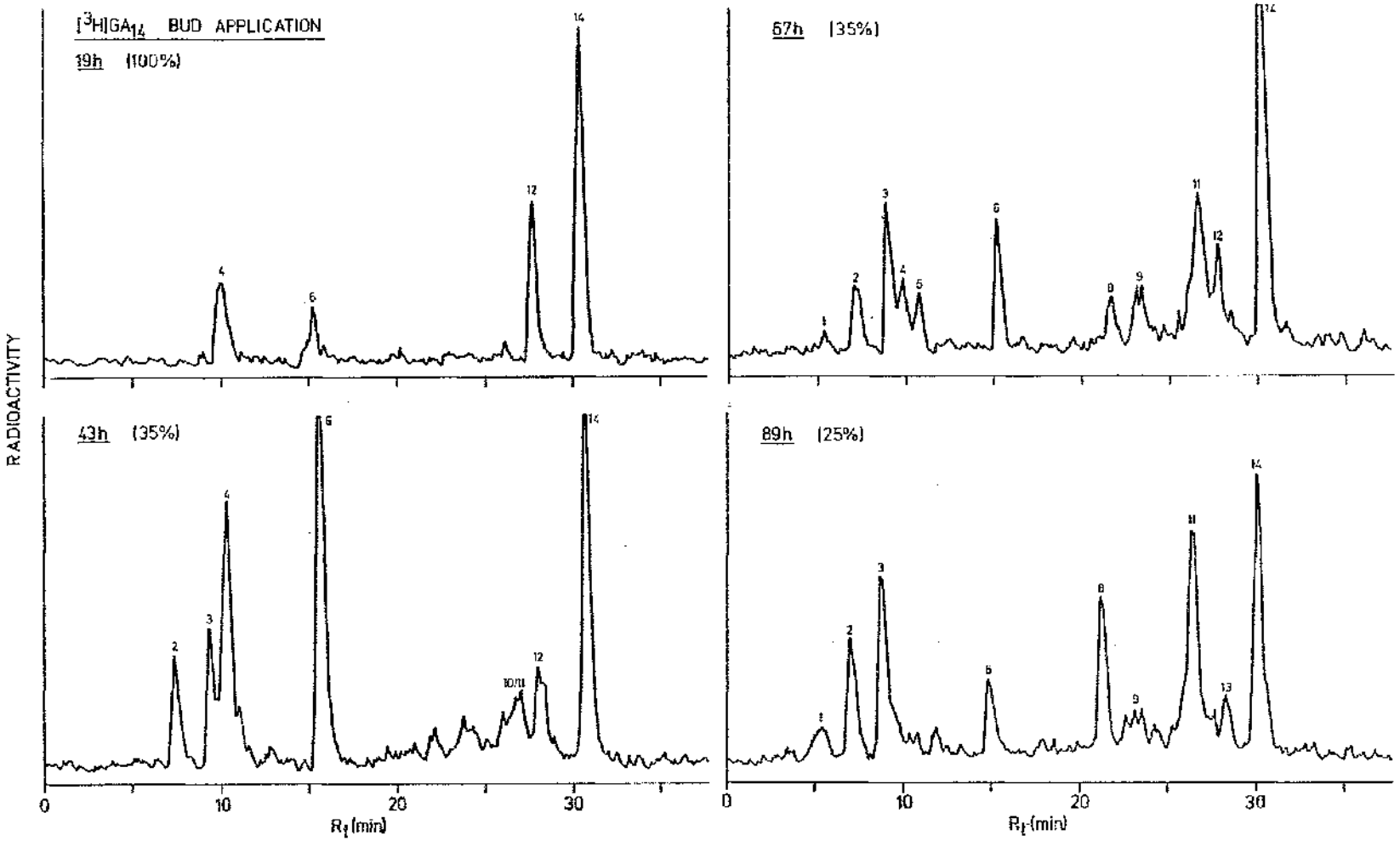


FIGURE 14. Reverse phase HPLC analysis of metabolites from apically applied $[^3H]GA_{14}$. Plants extracted 19, 43, 67 or 89h after treatment. Percentages represent the proportions of the acidic EtOAc fractions injected. Mobile phase: 35-90% MeOH, 0-30 min; 90-100% MeOH, 30-35 min; 0.75cm^3 min^{-1}. Detector: Radioactivity monitor, 10 cps f.s.d., 10s time constant.

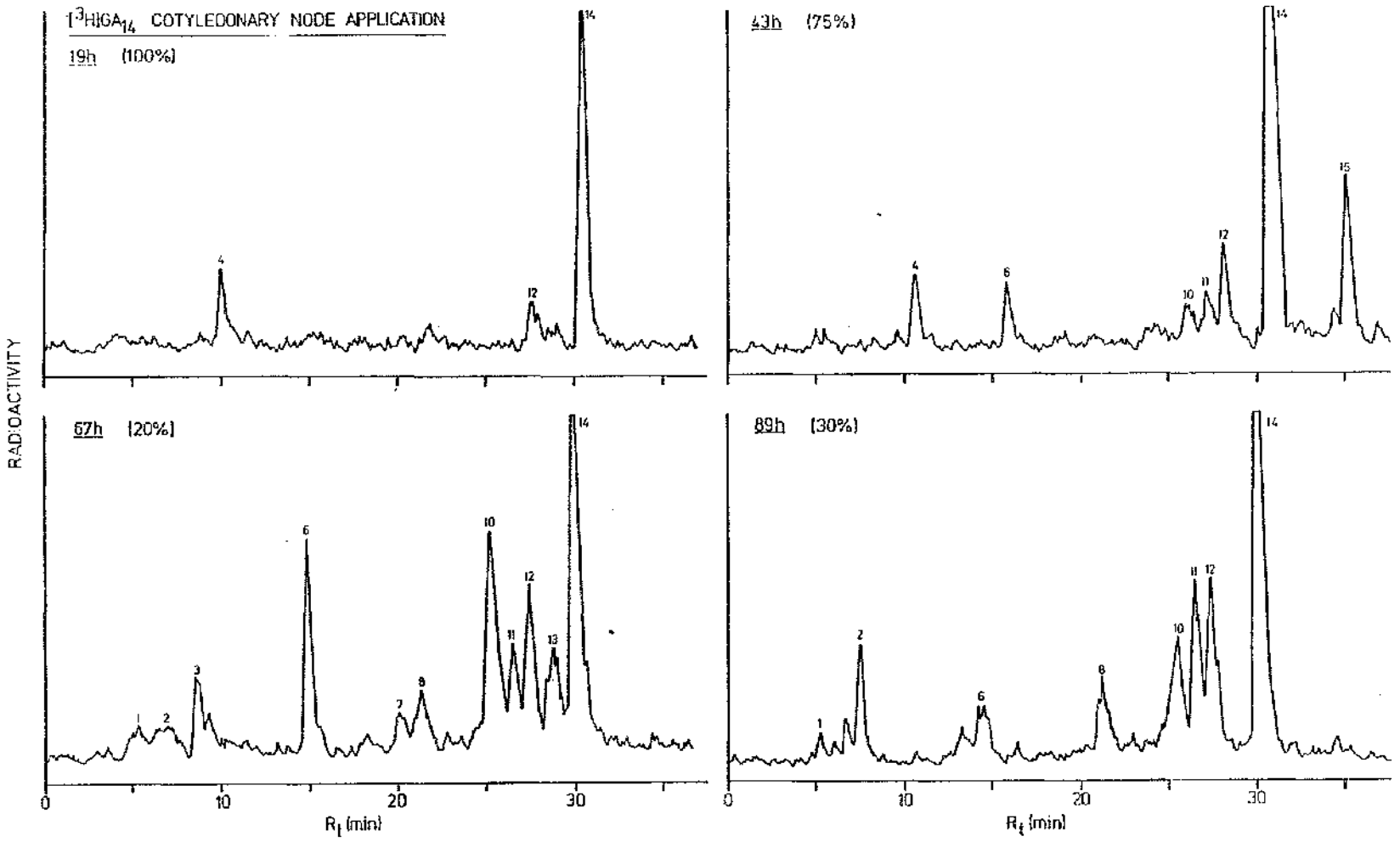


FIGURE 15. Reverse phase HPLC analyses of metabolites from [^{3}H]GA_{14} injected into the cotyledonary node. Plants extracted 19, 43, 67 or 89h after treatment. Percentages represent the proportions of the acidic EtOAc fractions injected. Mobile phase: 35-90% MeOH, 0-30 min; 90-100% MeOH, 30-35 min; 0.75cm^3 min^{-1}. Detector: Radioactivity monitor, 10 cps f.s.d., 10s time constant.

of [^{3}H]GA standards are detailed in Table 10. The peaks observed were numbered in order of decreasing polarity, peaks in different extracts but with similar retention times being allocated the same number. Peak areas were quantified from the chart recorder integrater response. The area of each peak was expressed as a percentage of the total area beneath all the numbered peaks in that extract. This percentage was multiplied by the amount of radioactivity estimated in the crude EtOAc fraction (Table 12) to give the percentage conversion of applied [^{3}H]GA_{14} that each peak represented. These figures, together with the accurate retention times of the peaks, are given in Table 11.

A number of possible sources of error should be borne in mind when considering Table 11. The percentage conversions are dependent on the accuracy of the initial counts of the EtOAc fractions, which were not corrected to dpm. Large losses occurred between the time when these counts were made and the time immediately before HPLC analysis when further counts were made (Table 12). These losses occurred during drying of the EtOAc and during transfer of the extracts between containers, but were much higher than losses observed at a similar stage in subsequent experiments, and may reflect inadequate rinsing of the sodium sulphate used to dry the EtOAc. It is assumed that all components of each extract were lost to a similar degree.

Peak number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Nominal R_t (min)		5.4	7.5	8.8	10.0	10.8	15.5	20.2	21.3	23.5	25.5	26.5	27.3	28.3	30.5	34.3
Bud Application	19h				(10.0) 2%		(15.2) T						(27.7) 2%		(30.3) 7%	
	43h		(7.5) 1%	(9.4) 2%	(10.4) 4%		(15.6) 5%				(25.7-27.5) 2%		(28.2) 2%		(30.7) 5%	
	67h	(5.5) T	(7.3) 1%	(9.0) 1%	(10.0) 1%	(10.8) 1%	(15.2) 1%		(21.7) 1%	(23.3) 1%		(26.7) 3%	(27.8) 3%		(30.2) 7%	
	89h	(5.4) T	(7.0) 2%	(8.8) 3%			(14.8) 1%		(21.2) 2%	(23.0) 1%		(26.4) 5%		(28.2) 1%	(30.1) 4%	
Cotyledonary node Application	19h				(10.1) 3%								(27.6) 2%		(30.2) 21%	
	43h				(10.6) 1%		(15.2) 1%				(26.1) 1%	(27.2) 1%	(28.0) 2%		(30.7) 11%	(35.1) 3%
	67h	(5.2) 1%	(7.5) T	(8.6) 1%			(14.8) 3%	(20.2) 1%	(21.3) 1%		(25.3) 5%	(26.5) 2%	(27.5) 3%	(28.8) 2%	(30.0) 8%	
	89h	(5.3) T	(7.5) 1%				(14.5) 1%		(21.3) 1%		(25.5) 3%	(26.5) 3%	(27.3) 4%		(30.1) 8%	

TABLE 11. Products; $[^3H]GA_{14}$. Bracketed figures represent actual peak retention times (min). Unbracketed figures show the percentage of the applied radioactivity that each peak represents. T represents an estimated percentage conversion of under 0.5%.

TABLE 12. $[^3H]GA_{14}$ Time Course Feed: Counts of radioactivity in the EtOAc fraction (cpm x 10^{-3}). A, counted immediately after partitioning; B, counted after drying and transfer.

Extract	Bud Application				Cotyledonary Node Applic[11]			
	19h	43h	67h	89h	19h	43h	67h	89h
A	170	280	280	300	380	280	410	320
B	20	140	70	150	20	70	170	110

The other main potential sources of error in Table 11 arise from the difficulty of distinguishing poorly resolved peaks, and as a result of the low number of counts present in some peaks. For example, peak 9 in the 89h bud extract possibly represents more than one compound, while the percentage conversion of $[^3H]GA_{14}$ to peak 10 in the 43h extract of cotyledonary node fed plants is estimated from a peak of ca. 30 counts.

Thus $[^3H]GA_{14}$ was metabolised to a range of more polar products, the pattern of which appeared broadly similar from both bud and cotyledonary node fed plants. Peaks 4 and 12 appeared to be major early products and peak 4 was further converted. The pattern of occurrence of peak 12 was less clear. In both time courses peak 6 was a major product, chromatographically well

separated from other products, and was further metabolised. Peaks 10 and 11 were late products, sometimes poorly resolved. Peak 10 only appears as a major product from the cotyledonary node feeds. Other peaks generally appear to be late products, with peak 3 appearing much more prominent from bud fed plants. No peak of similar retention time to GA_1 was observed, with the possible exception of peak 5. This was a minor peak observed only in the 67h bud extract, although possibly present but incompletely resolved in the 43h bud extract.

From this experiment it was concluded that $[^3H]GA_{14}$ was metabolised by cv. Alaska to more polar compounds, more numerous than those reported by Durley *et al.* (1974a,b) from cv. Meteor. However, the results do need to be interpreted with some caution due to the unexplained low and variable recovery of applied label after extraction and during work-up. Based on the result of this experiment a large-scale experiment, with the objective of conclusively identifying some of the products observed, was conducted. The $[^3H]GA_{14}$ was applied to the apex of the plants, since this was easier than cotyledonary node injection and had given higher recoveries of the applied radioactivity. It was decided to harvest the plants after *ca.* 43h since peak 6 was a major product at this time. Peak 6 was potentially a good peak to attempt to identify since it was chromatographically well separated from other products and since it appeared to be further converted, and thus it might provide a clue to the identity of further products.

3.5.3. GC-MS ANALYSIS OF $[^3H]GA_{14}$ METABOLITES

120,000cpm (ca. 1.6µg) of $[^3H]GA_{14}$ was applied to the apex of each of 255 six day old light-grown pea plants, cv. Alaska. The plants were extracted 43h after feeding. Prior to extraction the area around the point of application of ten plants was rinsed with 100% MeOH which was subsequently analysed by reverse phase HPLC at 70% MeOH (R_t $[^3H]GA_{14}$: 11.7 min). $[^3H]GA_{14}$ was the only radiolabelled compound present.

The plants were extracted with MeOH, which was evaporated off to leave an aqueous residue. After addition of excess phosphate buffer this was partitioned against toluene and slurried with PVP. The distribution of recovered radioactivity after partitioning was: toluene phase, 1%; residual aqueous phase, 6%; acidic EtOAc phase, 90% (percentages calculated from cpm and expressed as a percentage of applied radioactivity). The recovery of applied label was high and better than from the extracts of the time course feeds. The much higher recovery of radioactivity in the EtOAc phase was probably because only ten of the plants had their apical regions rinsed with MeOH prior to extraction. This MeOH rinse removed 43% of the radioactivity that had been applied to these plants, a similar proportion to that removed during the time course feeds. A high proportion of the radioactivity in the EtOAc fraction was therefore expected to be unmetabolised $[^3H]GA_{14}$.

Isocratic reverse phase HPLC analysis of the crude acidic EtOAc fraction (250mg) suggested that approximately 15% of the radioactivity in this fraction represented products more polar than [^{3}H]GA_{14}, while the remainder was [^{3}H]GA_{14}. The crude acidic EtOAc fraction was purified by DEAE-A25 Sephadex anion exchange chromatography. A major peak (16.7×10^6 cpm, 60mg) and a later eluting minor peak (6×10^5 cpm) were detected. The reverse phase HPLC profile of the minor peak differed to that of the crude EtOAc fraction. The minor peak contained two polar compounds, eluting after 9.3 min and 10.5 min when analysed on a gradient of 25-90% MeOH over 30 min at a flow rate of 0.75cm^3 min^{-1}. These peaks possibly represented products 3 and 4 observed in the original time course.

The major peak, which had a reverse phase HPLC profile similar to that of the crude EtOAc fraction, was purified by elution through a Sep-Pak C_{18} cartridge. To separate the metabolites from the unmetabolised [^{3}H]GA_{14} the whole fraction (29mg) was purified by isocratic reverse phase HPLC at 65% MeOH. An initial fraction (5.8×10^6 cpm) containing products more polar than [^{3}H]GA_{14} was collected, together with a series of later fractions (10.9×10^6 cpm), during which a rapid gradient to 100% MeOH was run. Isocratic reverse phase HPLC analysis of the later fractions showed no trace of any radioactive component apart from [^{3}H]GA_{14}. The initial fraction (containing the metabolites) was further analysed.

Aliquots of the metabolite fraction were analysed immediately and after the fraction had been dried and transferred to a small vial. Both analyses are shown in figure 16. It is apparent that the peaks eluting prior to 29 min were similar in both analyses, but that the less polar peaks were not present in the second analysis. Subsequently the whole fraction was injected and chromatographed using the same gradient as in figure 16. Nineteen 2 min fractions and then a single 9 min fraction were collected. The distribution of radioactivity was as expected from figure 16B. Fractions were combined as detailed in Table 13.

TABLE 13. Fractions combined after analysis of the metabolite fraction.

Fractions	Weight (mg)	Radioactivity (cpm)
3- 5	15	89,000
7- 9	8	277,000
10-12	3	185,000
13-15	3	162,000

The peaks observed in figure 16 with retention times 7.8, 10.2, 15.8 and 28.5 min may respectively represent peaks 2, 4, 6, and 12 detected in the original time course experiment. The percentage conversions of applied [^{3}H]GA_{14} to these peaks were respectively 0.2%, 0.06% 0.7% and 0.4%. These values are considerably

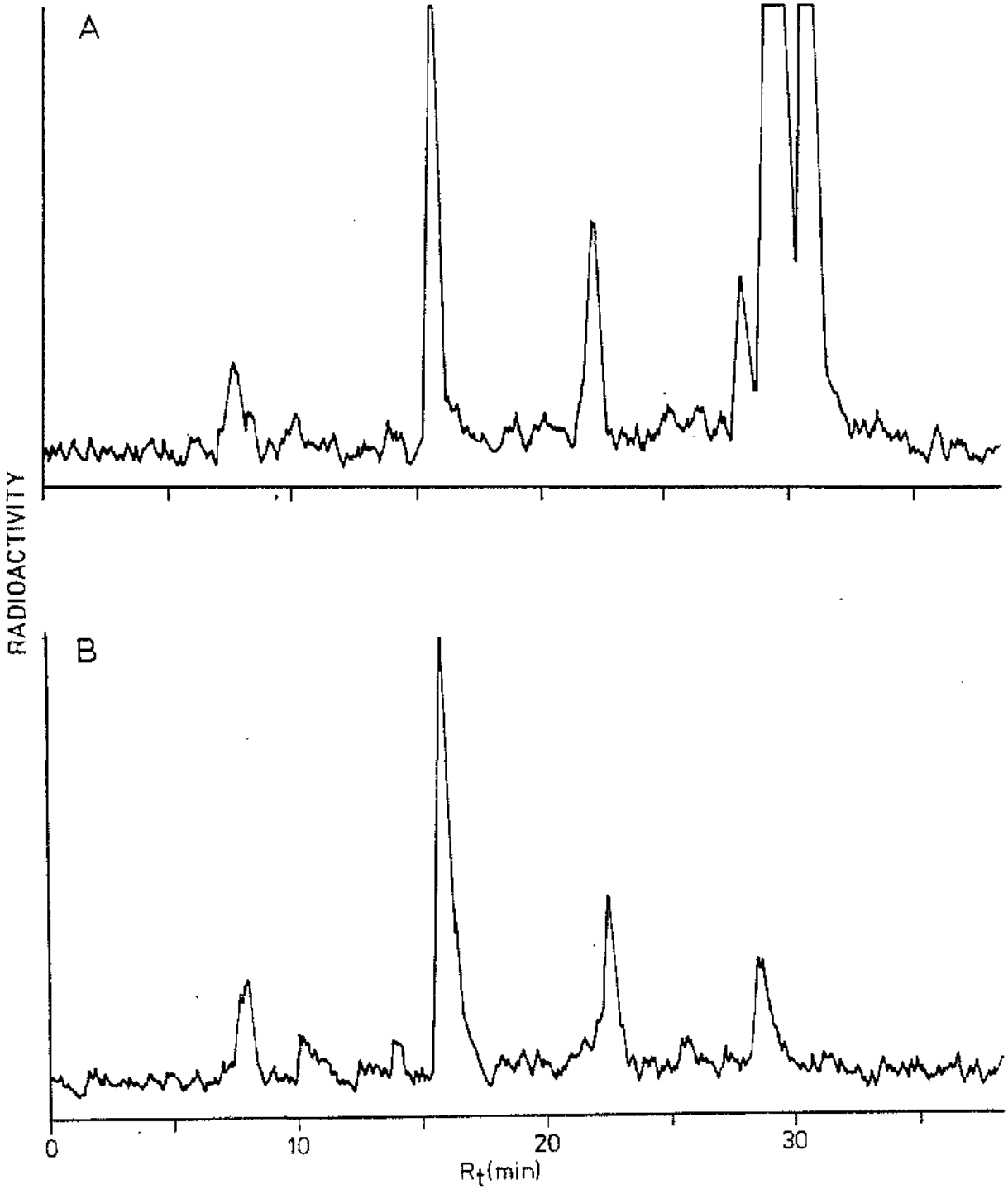


FIGURE 16. Reverse phase HPLC analyses of [^{3}H]GA_{14} metabolites. Gradient analysis of the [^{3}H]GA_{14} metabolite fraction (see pages 108 and 109 for details of this fraction). A: 1.5% of fraction before drying and transfer. B: 1% of fraction after drying and transfer. Mobile phase: 35-90% MeOH, 0-30 min; 90-100% MeOH, 30-35 min; 0.75cm^3 min^{-1}. Detector: Radioactivity monitor, 100 cps f.s.d., 10s time constant.

less than the conversions estimated during the time course analysis, but this is probably because the latter values were estimated as a proportion of the activity of the crude EtOAc fractions, while in this experiment the amounts were quantified directly. The relationship of the peak with a retention time of 22.5 min (0.5% of applied radioactivity) to those detected in the time course is not known. No peak of similar retention time was detected in the time course 43h bud extract, but peaks 8 and 9 had the retention times closest to 22.5 min.

It is apparent from Table 13 that the recovery of radioactivity (750,000 cpm) was considerably lower than the 5.8×10^6 cpm originally estimated to be present in the metabolite fraction. This discrepancy is difficult to explain. It is possibly due to the loss of the less polar peaks seen in figure 16A during drying and during transfer between containers. However, such complete selective loss of the less polar peaks is considered unlikely. Alternatively a mistake may have been made when originally estimating the radioactivity as 5.8×10^6 cpm. The presence of the less polar peaks in figure 16A may be an artifact caused by carry-over, on the injector and column, of $[^3H]GA_{14}$ from the preparative run of the whole extract. Such carry-over is possible since the analysis in figure 16A followed immediately after the injection of the whole of the major peak collected from the Sephadex column.

Each of the combined fractions detailed in Table 13 was

analysed in a similar manner. Initially aliquots were examined by isocratic reverse phase HPLC and co-injected with standards representing possible products. After such preliminary analyses the whole of each fraction was injected. Fractions were collected and those corresponding to radioactive peaks were kept, while the remainder were discarded. Radioactive peaks were methylated and aliquots re-analysed by isocratic reverse phase HPLC prior to injection and purification of the whole methylated sample. The purified methylated peaks were derivatised to their corresponding TMSi ethers and analysed by GC-MS. Details of the analysis of each of the combined fractions are given below. When percentages are given in this section referring to preparative runs of fractions, the figures represent percentages of the total amount of radioactivity recovered from the column during the preparative run.

Fraction 3-5

When analysed isocratically at 25% MeOH two radioactive peaks were detected (figure 17). The major peak probably corresponds to the peak eluting after 7.8 min in figure 16, and eluted between GA_8 and GA_{23}. The minor peak, eluting between GA_{23} and GA_{38}, probably represents the peak eluting after 10.2 min in figure 16.

The whole sample was injected at 25% MeOH. The peaks were collected (82%) and methylated, while other recovered radioactivity

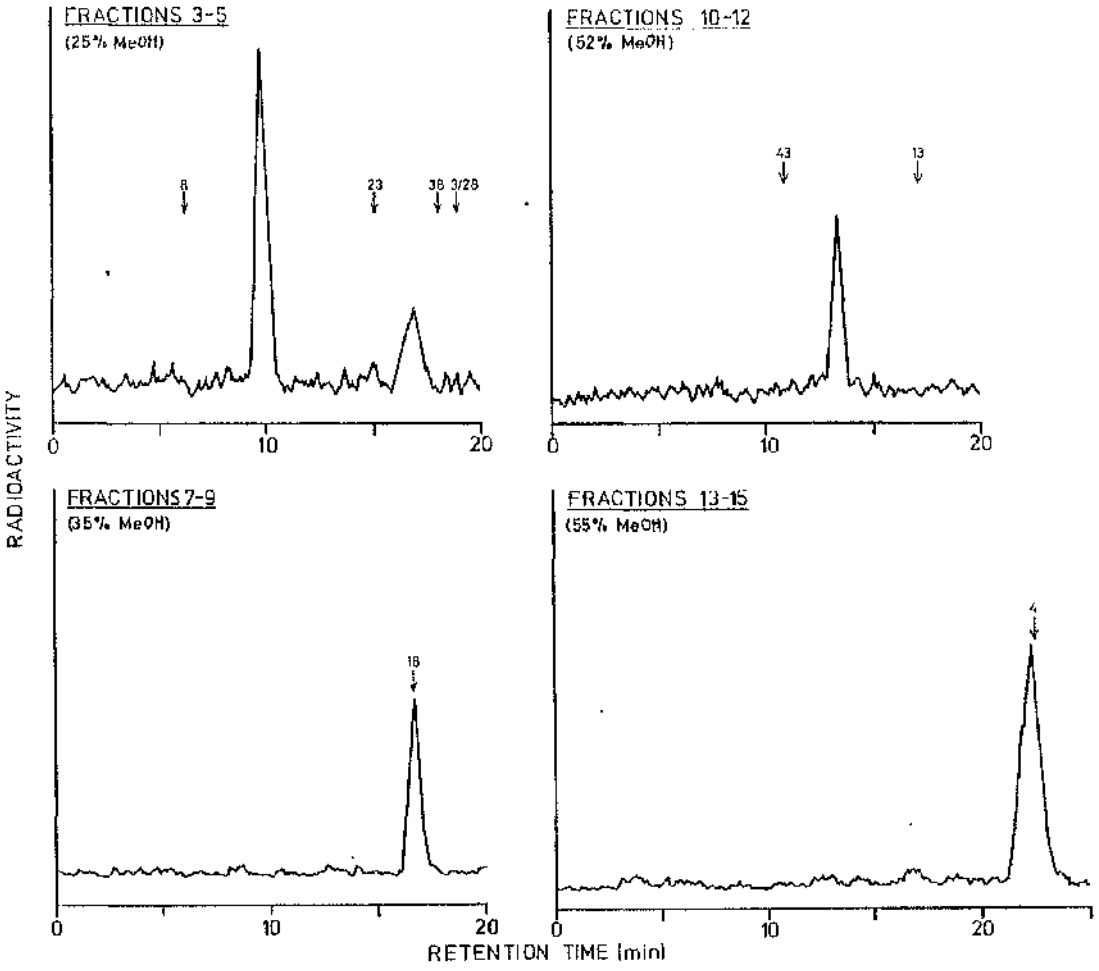


FIGURE 17. Isocratic reverse phase HPLC analyses of [^{3}H]GA_{14} metabolites. See text for details of fractions. Arrows indicate the elution positions of authentic GAs when co-injected with metabolites. Mobile phase: isocratic, % MeOH as indicated in figure; 1cm^3 min^{-1}. Detector: Radioactivity monitor, 10s time constant, f.s.d. settings detailed below:

Fractions 3- 5: 10% aliquot, 10 cps f.s.d.
Fractions 7- 9: 4% aliquot, 30 cps f.s.d.
Fractions 10-12: 3% aliquot, 10 cps f.s.d.
Fractions 13-15: 12% aliquot, and ca. 4 Kcpm [^{3}H]GA_4, 30 cps f.s.d.

(18%) was discarded. The major peak (60%) was re-injected at 40% MeOH. Successive one minute fractions were collected but no distinct peak was detected. The minor peak (22%) was not further analysed.

Fraction 7-9

This fraction contained a single radioactive peak, probably the one with a retention time of 15.8 min in figure 16. The peak co-chromatographed with GA_{18} (figure 17). The whole fraction was run isocratically at 40% MeOH and the peak was collected (81%) and methylated. The derivatised peak was re-run at 55% MeOH. A single peak, retention time 9.2 min, was detected and collected (78%). GC-MS analysis of a proportion of this peak, estimated to contain ca. 800ng of radioactive metabolite, confirmed the presence of GA_{18} (figure 18). No other known GAs were identified.

Fraction 10-12

This fraction contained a single peak, probably the one with a retention time of 22.5 min in figure 16. The peak eluted between GA_{13} and GA_{43} (figure 17). The whole fraction was injected at 48% MeOH, the peak collected (81%) and methylated. Other recovered radioactivity (19%) was discarded. Isocratic analysis of the methylated peak at 70% MeOH showed a peak with a retention time of 7.1 min. It is possible that other earlier eluting radioactive compounds were present, but the small amount of sample available precluded

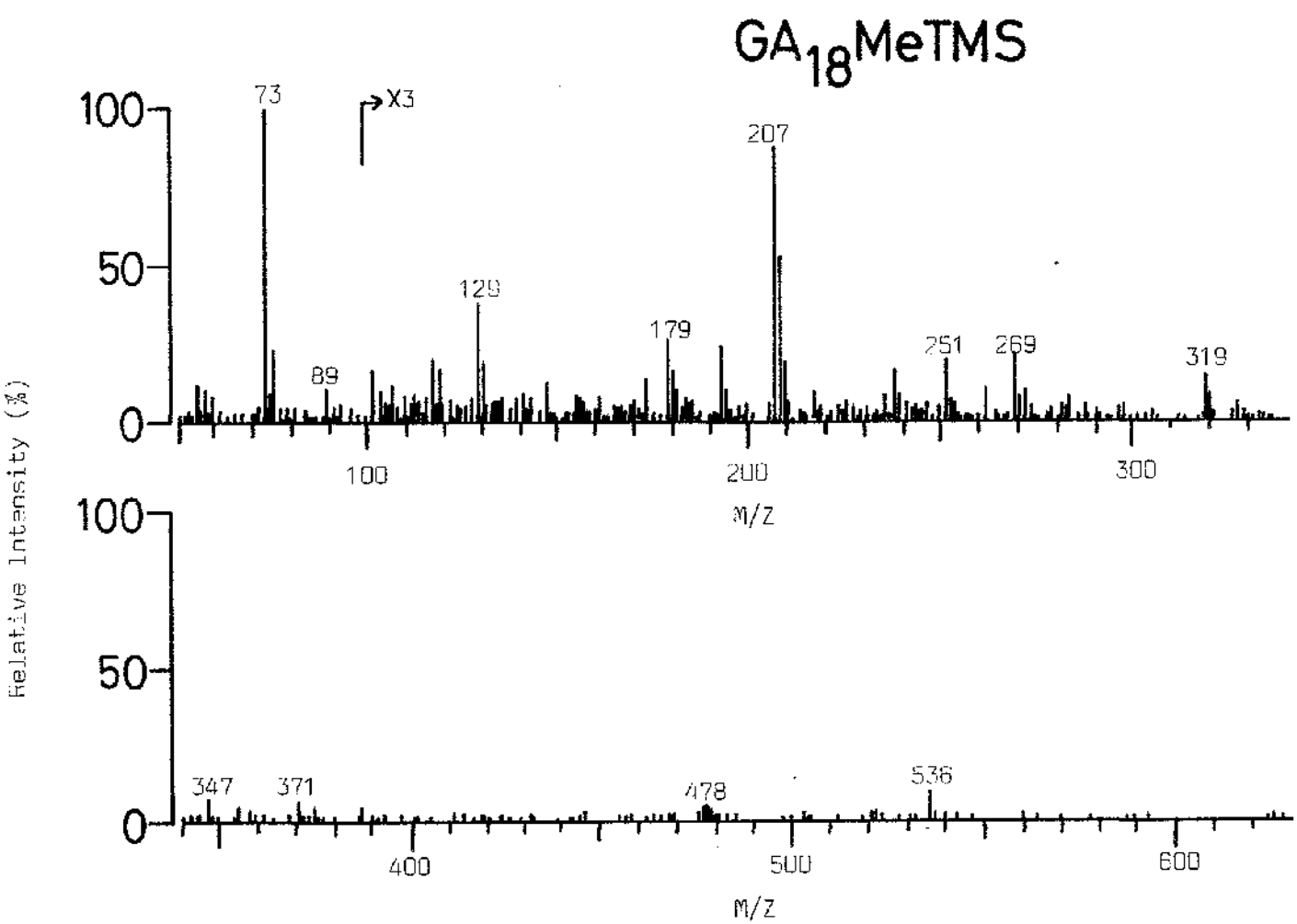


FIGURE 18. Mass spectrum of GA_{18} MeTMS: obtained from an extract of 255 cv. Alaska seedlings which had been fed ca. 408µg [^{3}H]GA_{14}.

further investigations. The whole fraction was injected and the peak collected (66%) while otherradioactivity (34%) was discarded. GC-MS analysis of the peak (ca. 450ng of radioactive metabolite) failed to detect any of the known GAs.

Fractions 13-15

Reverse phase HPLC analysis showed a single peak which eluted close to GA_4, but after GA_7. The peak is probably the one with a retention time of 28.5 min in figure 16.

The unknown peak co-chromatographed with [^{3}H]GA_4 (figure 17), but the resultant peak was broader than expected. This suggests that the major component of the unknown peak was not [^{3}H]GA_4, but does not exclude the possibility that some [^{3}H]GA_4 was present.

The whole fraction was injected and the peak collected (70%) while other collected radioactivity (30%) was discarded. The peak was methylated and re-run at 80% MeOH. The major peak had a retention time of 10.6 min. As in fractions 10-12 it is possible that other earlier eluting radioactive compounds were present, but again the small amount of sample available precluded further injections. The whole methylated sample was injected at 80% MeOH and the major peak (55%) collected, while other collected radioactivity (45%) was discarded. GC-MS analysis of the peak, estimated to contain ca.100ng of radioactive metabolite failed to detect any of the known GAs.

Therefore GA_{18} was the only one of the known GAs found in the fractions. The GA_{18} found by GC-MS could be a metabolite of $[^3H]GA_{14}$ or alternatively may represent an endogenous GA. The former is considered more likely since strong chromatographic evidence for the presence of $[^3H]GA_{18}$ in sufficient amounts to give a mass spectrum was obtained. Further, 3β, 13-hydroxylated C_{20}-GAs have not been found in peas, although such compounds could represent a possible biosynthetic path to GA_1, recently reported in peas by Ingram et al. (1983). Even if GA_{18} is an endogenous GA in peas the small amount of tissue extracted would probably exclude it as the source of the GA_{18} seen.

Four other peaks were observed in figure 16. It is possible that the peaks with retention times 7.8, 22.5 and 28.5 min separated into a number of components after methylation, but this is not certain since the low levels of radioactivity prevented definitive chromatographic analysis. The identity of all these products remains unknown. A large number of GAs are excluded on the basis of their HPLC retention times (see section 3.2), including those identified by Durley et al. (1974a,b). No GAs, apart from GA_{18} were identified by GC-MS, although this may have been due to the small masses of radioactive metabolites present. The peaks with retention times of 7.8 and 10.2 min in figure 16 may correspond with the components that were present in the minor (later eluting) peak from the Sephadex column. If this is so, these compounds may not be free GAs. It is possible, for example, that they could

represent acidic conjugates.

3.5.4. $[^3H]GA_{14}$ FEEDS: CONCLUSION

$[^3H]GA_{14}$ was metabolised to a range of more polar products by cv. Alaska seedlings. Only one of these was identified, as GA_{18}. The identity of the other products is unknown, but some of them were shown to be dissimilar to the products found from $[^3H]GA_{14}$ by Durley *et al.* (1974a,b). GA_{18} appears to be further metabolised. The results confirm the *in vivo* capacity of cv. Alaska seedlings to 13-hydroxylate a C-20-methyl GA, although the substrate used has not been found as an endogenous constituent in peas.

3.6.1. $[^3H]GA_{12}$ ALDEHYDE EXPERIMENTS: INTRODUCTION

Two time course studies of $[^3H]GA_{12}$ aldehyde/metabolism, after application to the apices of pea plants, are reported. However, the interpretation of the results of these studies is difficult since only low levels of poorly-resolved products were detected and as $[^3H]GA_{12}$ aldehyde was found to be unstable during extraction.

3.6.2. STABILITY OF $[^3H]GA_{12}$ ALDEHYDE

The $[^3H]GA_{12}$ aldehyde had been stored crystallised at -20°C for 6 years prior to use and approximately 60% had broken down. Most of the breakdown products were more polar than $[^3H]GA_{12}$ aldehyde when analysed by either reverse phase HPLC or by the straight phase preparative HPLC system. $[^3H]GA_{12}$ accounted for between 2% and 5% of the total radioactivity present. Chromatographically pure $[^3H]GA_{12}$ aldehyde and $[^3H]GA_{12}$ were obtained by successive purifications, initially by preparative straight phase HPLC and then by reverse phase HPLC. The identity of each of these compounds was confirmed by co-chromatography with unlabelled standards.

The purified $[^3H]GA_{12}$ aldehyde was stored crystallised at -20°C. Breakdown continued to occur and re-purification was carried out when necessary. Prior to each experiment the radiochemical purity of the $[^3H]GA_{12}$ aldehyde to be used was confirmed by reverse phase HPLC at 80% MeOH (retention time $[^3H]GA_{12}$ aldehyde: 12.7 min). The purified $[^3H]GA_{12}$ was stored in MeOH at -20°C and appeared stable.

To check the stability of $[^3H]GA_{12}$ aldehyde during the purification and analysis of extracts 3.8×10^5cpm of $[^3H]GA_{12}$ aldehyde was added to each of two extracts during homogenisation. After removal of the MeOH *in vacuo* at 40°C the extracts were dissolved in pH8 0.5M phosphate buffer and were partitioned twice against $^3/_5$ volumes of petroleum ether. The aqueous phase was then acidified and partitioned five times against $^2/_5$ volumes of EtOAc. Analysis of the EtOAc fractions by reverse phase HPLC at 80% MeOH showed that extensive breakdown of the $[^3H]GA_{12}$ aldehyde had occurred during extraction (figure 19).

It was found that most of the $[^3H]GA_{12}$ aldehyde is removed from a pH8 aqueous extract by partitioning against petroleum ether. Thus in the above extraction the petroleum ether phase contained 2.5×10^5cpm, while the EtOAc phase contained only 7×10^4cpm. Toluene was also found to remove most of the $[^3H]GA_{12}$ aldehyde from a pH8 aqueous extract, and a K_d of 0.35 was found between pure pH8 buffer and toluene (K_d = concentration in aqueous phase/concentration in organic phase). Much less $[^3H]GA_{12}$ partitioned into the organic solvent under similar circumstances. A K_d of 10.9 was found between pure pH8 buffer and toluene for $[^3H]GA_{12}$.

$[^3H]GA_{12}$ aldehyde did not appear to breakdown on the plant surface during the feeds. Immediately prior to extraction the point of application of $[^3H]GA_{12}$ aldehyde was washed with 50% aqueous MeOH. These rinses were analysed by isocratic reverse phase HPLC and found to contain only $[^3H]GA_{12}$ aldehyde.

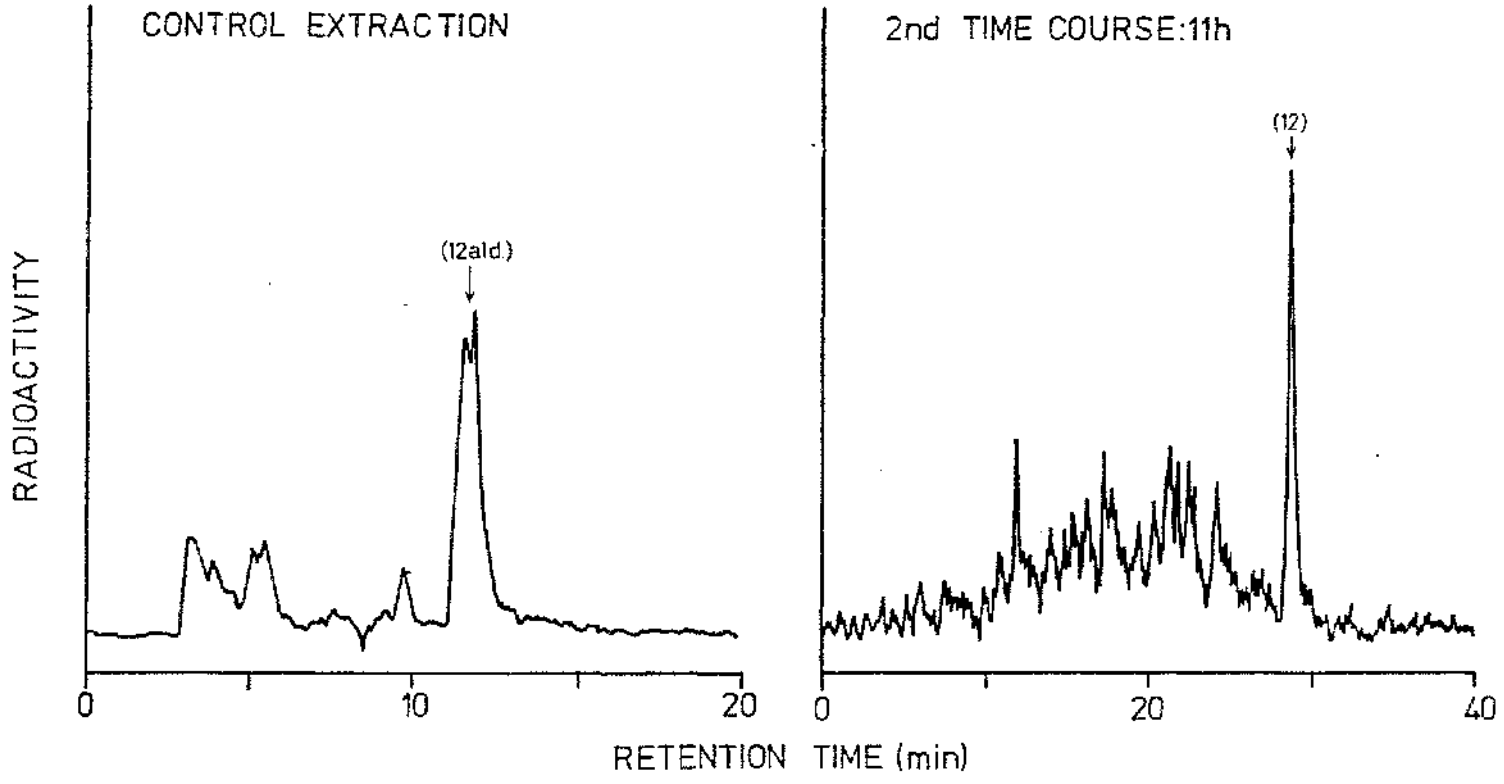


FIGURE 19. $[^3H]GA_{12}$ aldehyde feed: reverse phase HPLC analyses of acidic EtOAc fractions. Control extraction: Analysis of an aliquot of the acidic EtOAc fraction obtained after $[^3H]GA_{12}$ aldehyde had been added to a crude methanolic tissue homogenate. Mobile phase: 80% MeOH, isocratic; $1cm^3\ min^{-1}$. Detector: Radioactivity monitor, 30 cps f.s.d., 10s time constant. 2nd time course, 11h: 100% of acidic EtOAc fraction. Mobile phase: 40-100% MeOH, 0-30 mins; $1cm^3\ min^{-1}$. Detector: Radioactivity monitor, 10 cps f.s.d., 10s time constant.

3.6.3. [^{3}H]GA_{12} ALDEHYDE TIME COURSE FEEDS

Two time course feeds were carried out using seven day old plants of the cultivar Alaska. Ten plants were used per treatment and each plant received *ca.* 1.5 x 10^5cpm [^{3}H]GA_{12} aldehyde (*ca.* 3.5μg). In the second experiment some plants also received 9.5μg GA_3. This was applied in an attempt to prevent the possible rapid further metabolism of products from [^{3}H]GA_{12} aldehyde, by saturating the enzyme systems with GA_3. The amounts of [^{3}H]GA_{12} aldehyde applied in the experiments are detailed in Table 14.

Whole plants were extracted after rinsing the apices with 50% aqueous MeOH. The extracts were purified by partitioning the pH8 aqueous phase against toluene and by slurrying with PVP. The crude acidic EtOAc fractions were analysed by reverse phase HPLC. Details of the recovery and distribution of radioactivity after partitioning are given in Table 14.

It is apparent from Table 14 that the recovery of applied label was high in both experiments. A large proportion of the recovered radioactivity was in the toluene phase, possibly indicating that a large proportion of the [^{3}H]GA_{12} aldehyde was unmetabolised. As in the [^{3}H]GA_{14} experiments the figures must be treated with some caution since no correction was made for the counting efficiency before calculating the percentages presented in Table 14.

Only a small amount of radioactivity was found in the EtOAc fractions, and this was not increased by use of a GA_3

Amount of $[^3H]GA_{12}$ applied (cpm x 10^{-5})	First time course 1.5	1.5	1.5	1.5	Second time course 1.1	1.3	1.1	1.3
Amount of GA_3 applied (µg)	-	-	-	-	-	9.5	-	9.5
Duration of feed (h)	17	45	67	87	11	11	23	23
Fraction: 50% MeOH rinse	42%	34%	21%	19%	60%	38%	44%	57%
Toluene	27%	39%	47%	40%	35%	31%	32%	36%
Aqueous (pre BuOH partition)	14%	7%	10%	7%	-	-	-	-
Aqueous (post BuOH partition)	-	2%	3%	2%	2%	2%	3%	1%
BuOH	-	10%	-	5%	-	4%	4%	3%
EtOAc	5%	5%	5%	5%	5%	4%	4%	4%
Total Recovered	88%	85%	83%	71%	102%	79%	87%	101%

TABLE 14. $[^3H]GA_{12}$ aldehyde time course feeds. Recovery of applied label. Data represent percentages of applied radioactivity, calculated from cpm.

"cold trap". The crude EtOAc fractions had similar profiles when analysed by reverse phase HPLC, although the very low levels of radioactivity present made comparisons difficult. Typically the major peak had a R_t similar to that of [^{3}H]GA_{12}. Little or no [^{3}H]GA_{12} aldehyde was present. Each EtOAc fraction also contained low levels of a range of incompletely resolved products, all more polar than [^{3}H]GA_{12}. Apart from the presence of the [^{3}H]GA_{12}-like component no other peak was consistently detected in the profiles of the crude EtOAc fractions. The profile of the EtOAc fraction of the 11h extract from the second feed is shown in figure 19. The trace illustrates the large number of poorly resolved compounds, more polar than [^{3}H]GA_{12} aldehyde, which were detected in the EtOAc fractions. The products present in the extract shown are more easily distinguished from each other than in most of the analyses of the EtOAc fractions.

It appears that the bulk of the applied [^{3}H]$GA_{12}^{\text{aldehyde}}$/possibly remained unmetabolised, despite ca. 50% not being removed by the MeOH rinses of the plant surface (i.e. apparently ca. 50% was absorbed). The products observed in the EtOAc fraction were present in very low amounts and only a single peak, with a retention time similar to [^{3}H]GA_{12} aldehyde, was consistently found. In view of the previous results showing that [^{3}H]GA_{12} aldehyde broke down significantly during extraction it is considered quite likely that some of the products observed were the result of non-metabolic conversions.

3.7.1. $[^3H]GA_9$ FEEDS: INTRODUCTION

Time course feeds were made to investigate whether the metabolism of $[^3H]GA_9$ differed between light-grown plants of the cultivars Alaska, Meteor and Progress No.9; and between light- and dark-grown plants of cv. Alaska. Preliminary experiments investigated the stability of $[^3H]GA_9$ during analysis, the translocation of the applied label and the effect of dose on the pattern of metabolites. Subsequently the results of the time course feeds are reported. After analysis of the individual extracts selected extracts were bulked and further analysed in an attempt to identify the radioactive compounds present. Analysis of the bulked extracts is also reported.

3.7.2. $[^3H]GA_9$ FEEDS: PRELIMINARY EXPERIMENTS

The radiochemical purity of the $[^3H]GA_9$ was confirmed by isocratic reverse phase HPLC at 65% MeOH (R_t $[^3H]GA_9$ *ca.* 9.5 min) prior to all experiments.

The stability of $[^3H]GA_9$ during extraction was investigated. $[^3H]GA_9$ was added to duplicate extracts in phosphate buffer and the EtOAc fraction, obtained after partitioning, was analysed by reverse phase HPLC at 65% MeOH. $[^3H]GA_9$ was the only radioactive compound present. Possible breakdown was also investigated under more severe conditions by adding $[^3H]GA_9$ to a crude methanolic tissue extract, which was evaporated to dryness *in vacuo* and then

left on the rotary film evaporator for a further 25 min. The residue was taken up in 25cm^3 of phosphate buffer, partitioned against petroleum ether and filtered through cellulose powder. The aqueous phase was reduced to ca. 1cm^3 in vacuo at 40°C, ultrasonicated, diluted, re-partitioned against petroleum ether, adjusted to pH2.5 and partitioned against EtOAc. Isocratic reverse phase HPLC of the crude EtOAc fraction showed ca. 25% breakdown to more polar products. A reverse phase HPLC gradient analysis of the extract is shown in figure 20, the retention times of the peaks were: [^{3}H]GA_9, 34.7 min; breakdown products, 24.5, 27.6, 28.0 and 29.4 min.

The translocation of applied label was investigated by feeding [^{3}H]GA_9 to the apices of eleven day old light-grown seedlings, cv. Alaska. Since after 3 days very little of the label was translocated out of the shoots (Table 15), only the shoots, excised at the cotyledonary node, were extracted in further experiments. Recovery of the applied label was high.

The effect of dose was investigated by treating light-grown 7 day old seedlings, cv. Alaska, with 1·2 x 10^{6}dpm [^{3}H]GA_9, 3.4ng, diluted with either 0, 28, 280 or 2800ng of unlabelled GA_9. After 23h the shoots of ten plants receiving each dose were excised at the cotyledonary node, extracted and the crude EtOAc fractions analysed by reverse phase HPLC. The distribution of radioactivity is shown in Table 16. Reverse phase HPLC traces

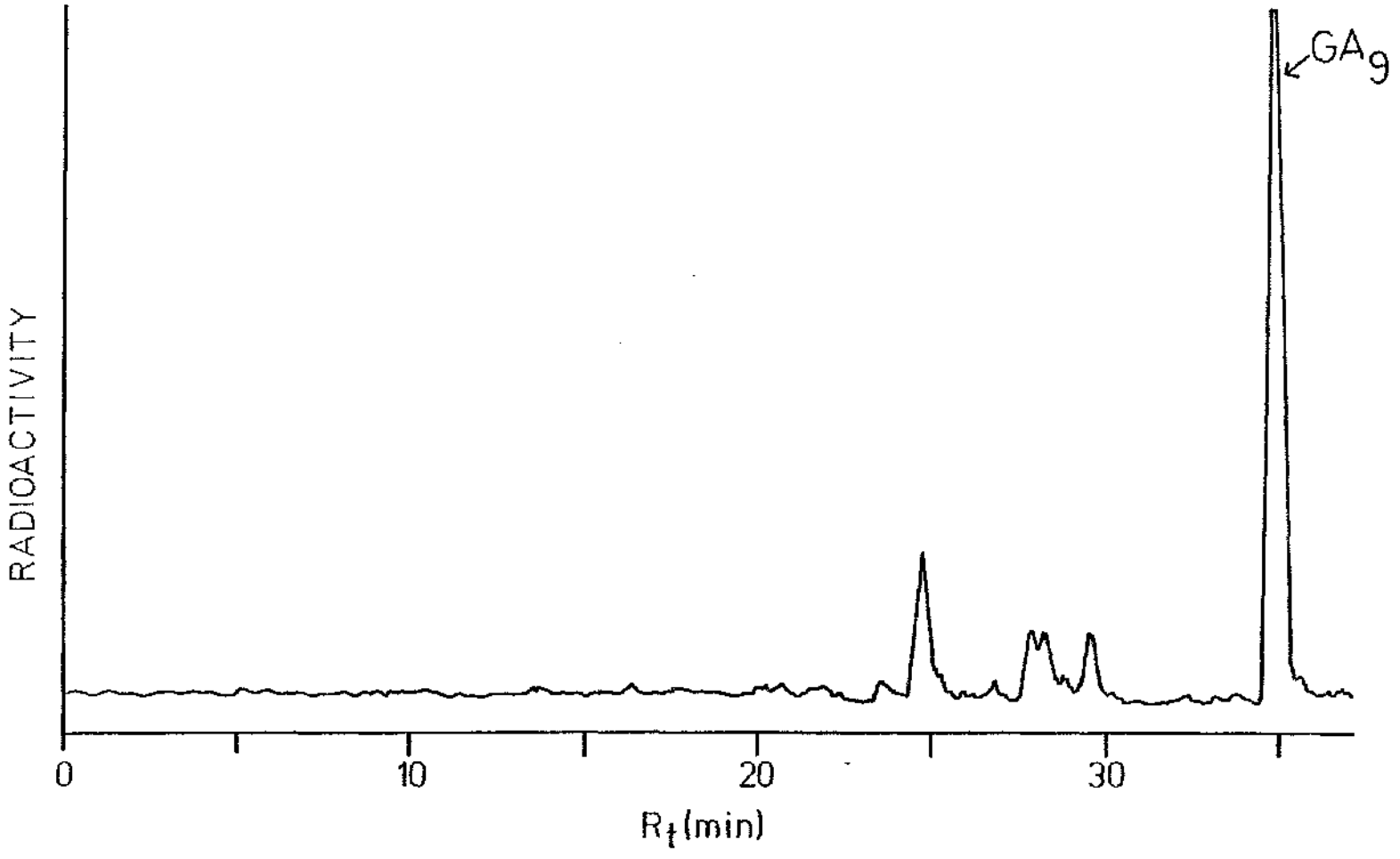


FIGURE 20. Reverse phase HPLC analysis of breakdown products of [^{3}H]GA_9. [^{3}H]GA_9 was added to a crude methanolic tissue homogenate (see pages 125 and 126). The trace shows the radioactive compounds subsequently detected in the acidic EtOAc fraction. Mobile phase: 25-70% MeOH, 0-30 min; 70-100% MeOH, 30-33 min; 1cm^3 min^{-1}. Detector: Radioactivity monitor, 30 cps f.s.d., 10s time constant.

TABLE 15. [^{3}H]GA_9: Distribution of label 3 days after application to the apex.

Plant Part Extracted	% Of Applied Activity
50% MeOH wash of apical region	42.7%
Terminal expanded leaf and apical bed	36.2%
Remainder of shoot	3.9%
Cotyledons	0.2%
Roots	0.3%
Total recovery	83.3%

are shown in figure 21 and summarised in Table 18. The methods used to number the peaks and to estimate the percentage conversions of applied radioactivity are discussed when the time course [^{3}H]GA_9 feeds are considered (section 3.7.3.). Prior to extraction the apices of the plants receiving each dose were rinsed with 50% aqueous MeOH, which was subsequently analysed by isocratic reverse phase HPLC at 65% MeOH. [^{3}H]GA_9 was the only radioactive compound detected, indicating that it was stable on the plant surface during the feeds.

The distribution of radioactivity on partitioning, and the reverse phase HPLC profiles of the extracts appear similar at all doses, suggesting that the amount of GA_9 applied, from 3.4ng to 2800ng per plant, did not significantly affect the pattern of metabolism.

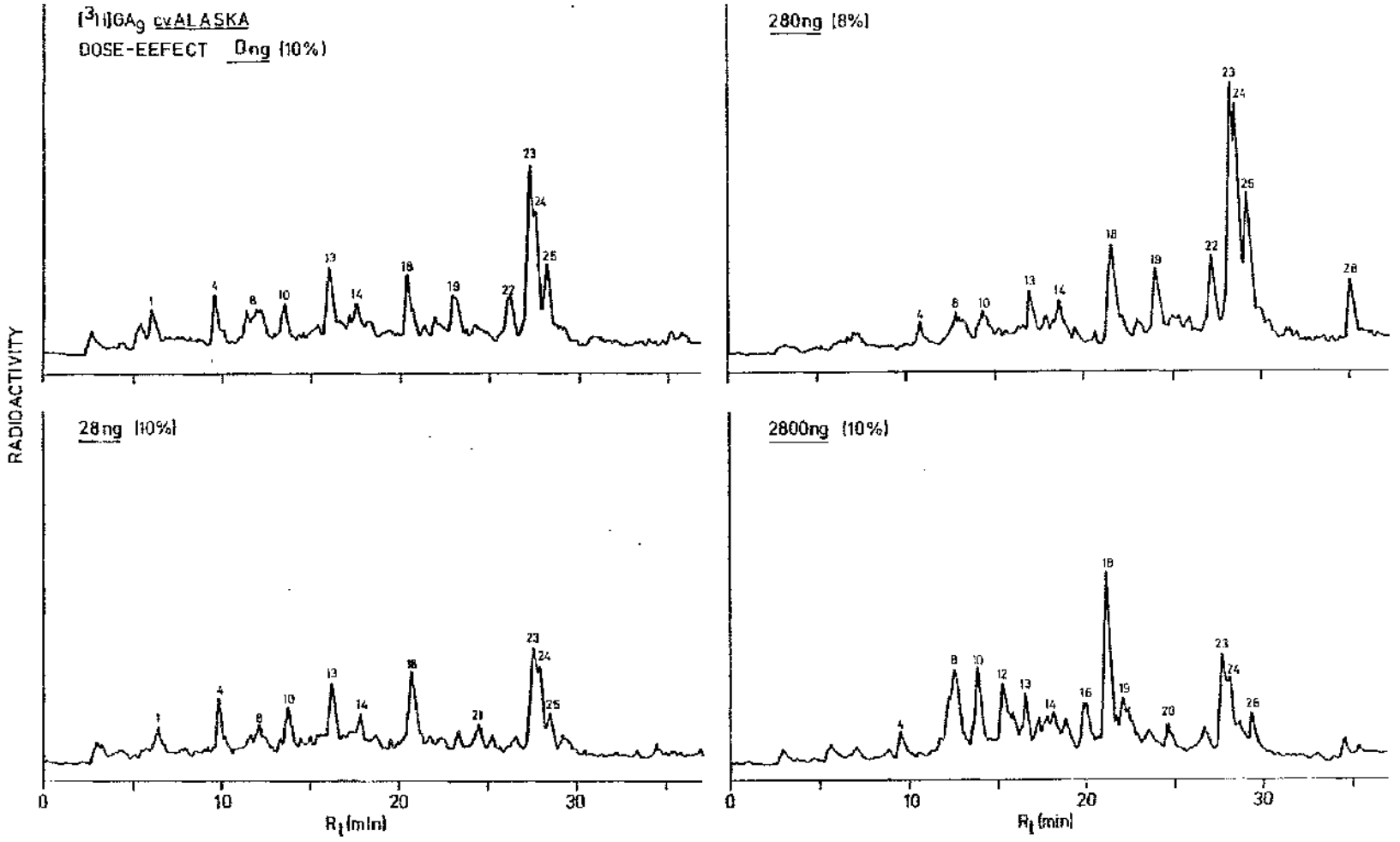


FIGURE 21. Reverse phase HPLC analyses of metabolites from [^{3}H]GA_9 dose-effect experiment. Percentages represent the proportions of the acidic EtOAc fractions injected. Each plant received ca. 3.4ng [^{3}H]GA_9 diluted with an amount of unlabelled GA_9, as indicated. Mobile phase: 25-70% MeOH, 0-30 min; 70-100% MeOH, 30-33 min; 1cm^3 min^{-1}. Detector: Radioactivity monitor, 30 cps f.s.d., 10s time constant.

TABLE 16. [^{3}H]GA_9: Dose effect experiment, distribution of radioactivity after partitioning. All figures are percentages of applied radioactivity calculated after correction for quenching.

Additional GA_9 received (ng)	Fraction				Total
	50% MeOH	Toluene	Aqueous	EtOAc	
0	11%	0%	21%	30%	62%
28	11%	1%	21%	26%	59%
200	-	1%	23%	45%	69%
2800	32%	0%	12%	28%	72%

By mistake a 50% aqueous MeOH rinse was not made of the plants treated with 28Cng. However, from the partitioning data it is apparent that the radioactivity not washed off appeared in the EtOAc fraction, as would be expected. The peaks detected by reverse phase HPLC show some variation in size between extracts, but allowing for the errors involved in estimating the amounts of the products these variations are not considered significant. Consequently it was conluded that the dose of [^{3}H]GA_9 applied, from 3.4ng to 2800ng did not markedly affect the pattern of metabolism observed.

3.7.3. $[^3H]GA_9$ TIME COURSE FEEDS: INITIAL ANALYSES OF INDIVIDUAL EtOAc FRACTIONS

Two time course feeds were conducted in which $[^3H]GA_9$ was applied to the apices of young peas. In each experiment plants were harvested after approximately 1, 8, 24 and 48h. Only the shoots, excised at the cotyledonary node, were extracted.

In the first experiment (feed I) each eight day old seedling of the cultivar Alaska, grown in either the dark or in the light, received 10×10^6dpm (2.9ng) of $[^3H]GA_9$. Ten light-grown and ten dark-grown plants were extracted 1, 6.5, 27 and 53h after treatment. In the second experiment (feed II) each seven day old light-grown seedling of the cultivars Alaska, and Meteor/Progress No.9 received 9.6×10^5dpm (2.7ng) of $[^3H]GA_9$. Fifteen plants of each cultivar were extracted after 1, 8.5, 25 and 48.5h. During the purification of extracts of feed I the pH8 aqueous phases were partitioned against toluene. Petroleum ether was used instead of toluene for feed II. Subsequently the aqueous phases in both experiments were acidified and partitioned against EtOAc.

The distribution of recovered radioactivity on partitioning is shown in Table 17 for both feeds. It is apparent that the recovery of radioactivity was high in all extracts, with the exception of the 1h dark extract of cv. Alaska, ca. 50% of which was spilt. Recoveries in excess of 100% reflect errors in the

Experiment	[^{3}H]GA$_9$ Feed I								[^{3}H]GA$_9$ Feed II (light)											
	cv. Alaska (dark)				cv. Alaska (light)				cv. Alaska				cv. Meteor				cv. Progress No.9			
Extract / Fraction	1h	6.5h	27h	53h	1h	6.5h	27h	53h	1h	8.5h	25h	48.5h	1h	8.5h	25h	48.5	1h	8.5h	25h	48.5h
Toluene/ Petroleum ether	5	4	1	2	9	5	3	2	5	2	2	1	4	2	2	3	3	2	1	1
Aqueous	1	7	28	30	1	10	33	35	1	18	42	52	1	17	37	41	1	16	36	45
EtOAc	45	87	72	44	110	91	78	54	89	72	43	30	97	76	43	36	91	75	44	33
Total Recovered	51	98	101	76	120	106	114	91	95	92	87	83	102	95	82	80	95	93	81	79

TABLE 17. [^{3}H]GA$_9$: Feed I and Feed II: Distribution of radioactivity after partitioning. All figures are percentages of applied radioactivity, calculated after correction for quenching.

counting of the radioactivity. The partitioning data suggested that the [^{3}H]GA_9 was extensively metabolised to more polar compounds during the feeds. Thus, as the duration of the feeds became longer, the radioactivity recovered in the organic phases decreased, while the radioactivity recovered in the aqueous phase increased. No significant differences were apparent between cultivars or between light- and dark-grown seedlings of cv. Alaska. The recovery of the applied label decreased as the duration of the feeds increased, perhaps reflecting conversion to volatile compounds (e.g. water).

Aliquots of the crude EtOAc fractions of all the extracts were analysed by reverse phase HPLC. Representative traces for each time course are shown in figures 22 to 26. The radioactive peaks detected were numbered after comparing the traces from all the [^{3}H]GA_9 time course feeds with each other, and with those from the [^{3}H]GA_9 dose-effect experiment. Peaks detected in different analyses, but which had similar retention times were allocated the same number, in order of increasing retention time. Two peaks allocated the same number had similar retention times but may not have necessarily represented the same compound. The comparison of traces was complex due to the large number of products detected and due to small variations in the retention times of peaks caused by the use of different columns and, to a lesser extent, by inter-run differences on a single column. Some correction was made for these variations by analysing certain extracts on two columns, and by comparing the elution positions of peaks after aligning prominent constituents, usually peaks 23, 24 and 28. Each of the latter peaks was assumed to be representing the same compound in the different extracts. The numbering of peaks

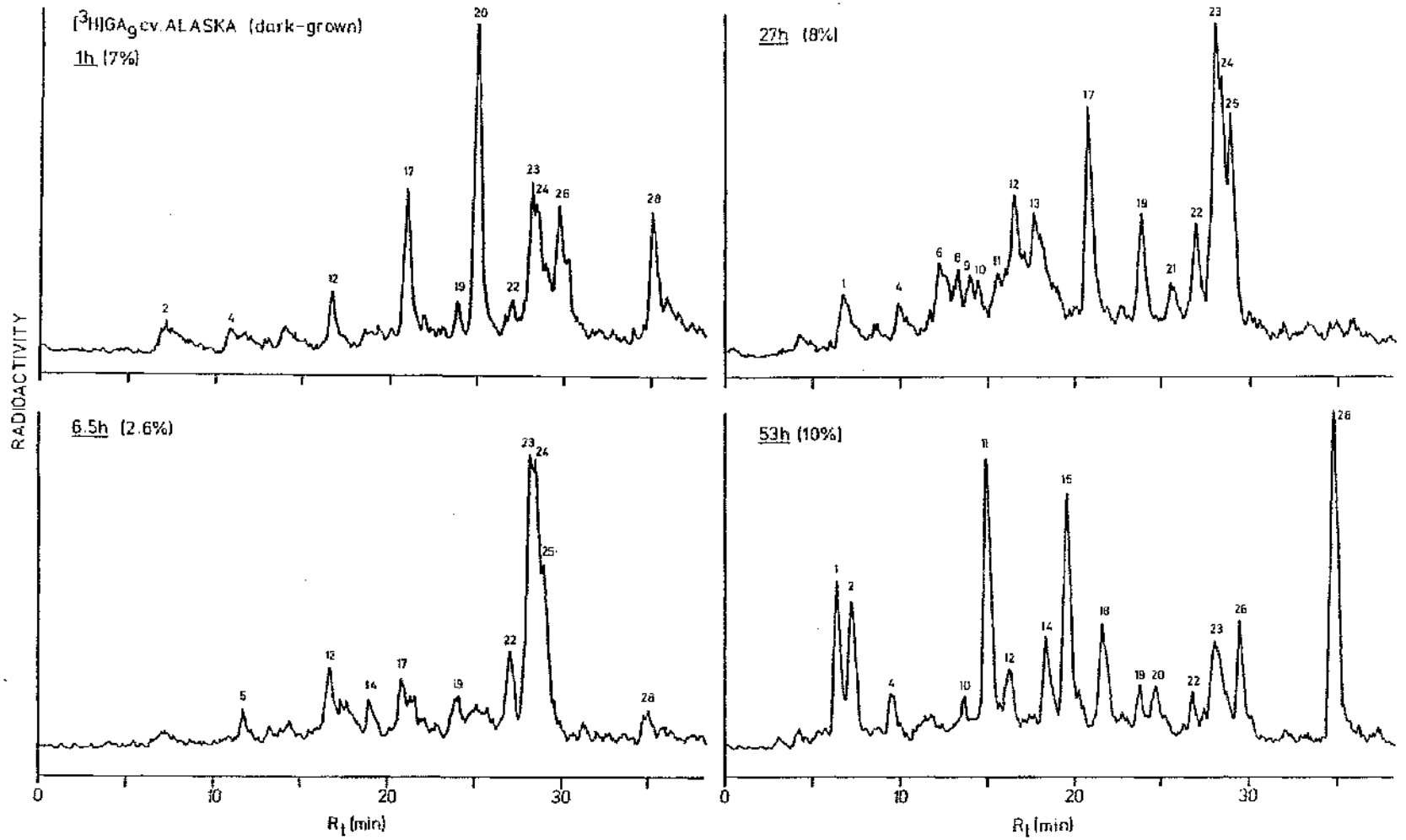


FIGURE 22. Reverse phase HPLC analyses of metabolites from [^{3}H]GA_9 fed to dark grown peas, cv. Alaska (feed 1). Plants were extracted 1, 6.5, 27 or 53h after treatment. Percentages represent the proportions of the acidic EtOAc fractions injected. Mobile phase: 25-70% MeOH, 0-30 min; 70-100% MeOH, 30-35 min; 1cm^3 min^{-1}. Detector: Radioactivity monitor, 30 cps f.s.d., 10s time constant.

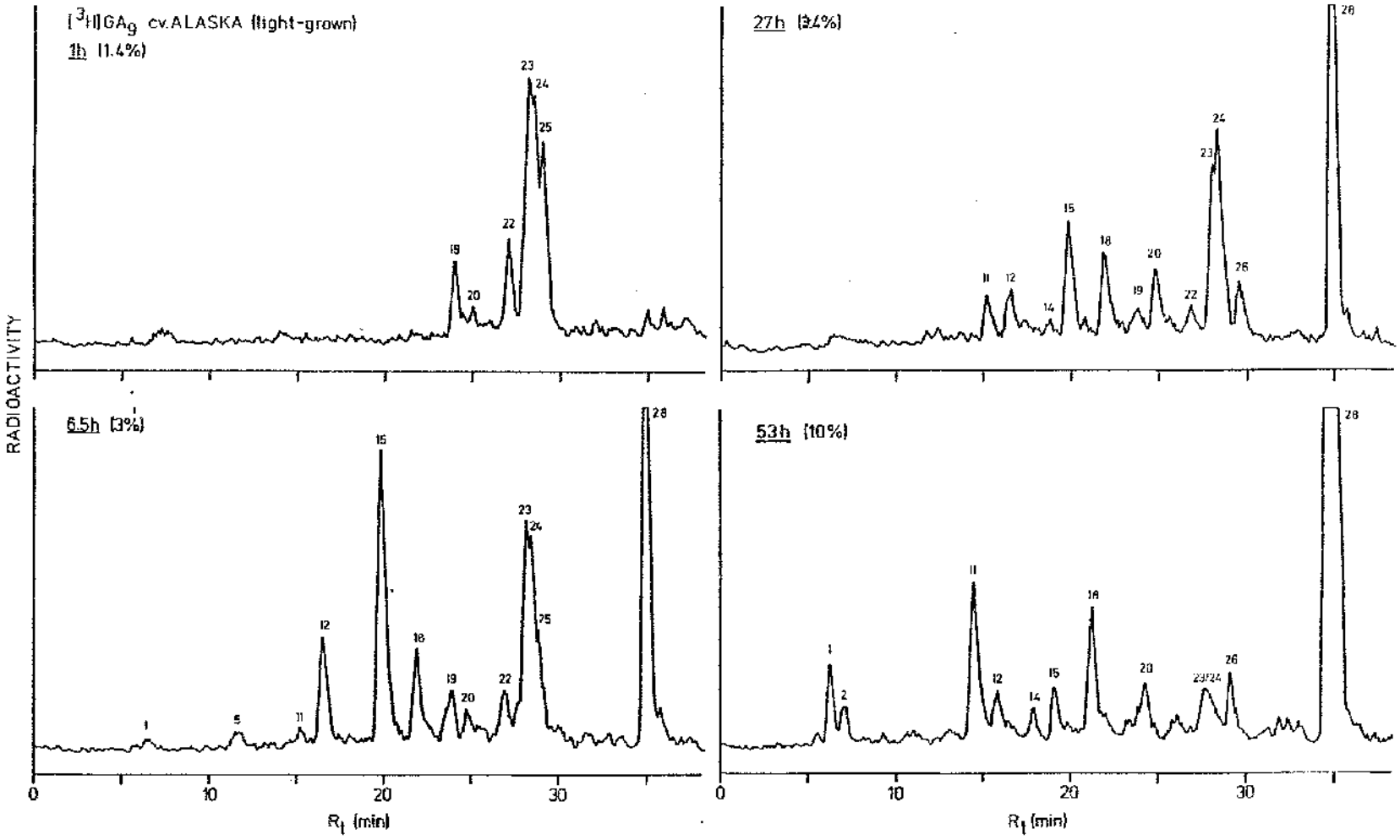


FIGURE 23. Reverse phase HPLC analyses of metabolites from [^{3}H]GA_9 fed to light-grown peas, cv. Alaska (feed 1). Plants extracted 1, 6.5, 27 or 53h after treatment. Percentages represent the proportions of the acidic EtOAc fractions injected. Analytical conditions - as figure 22.

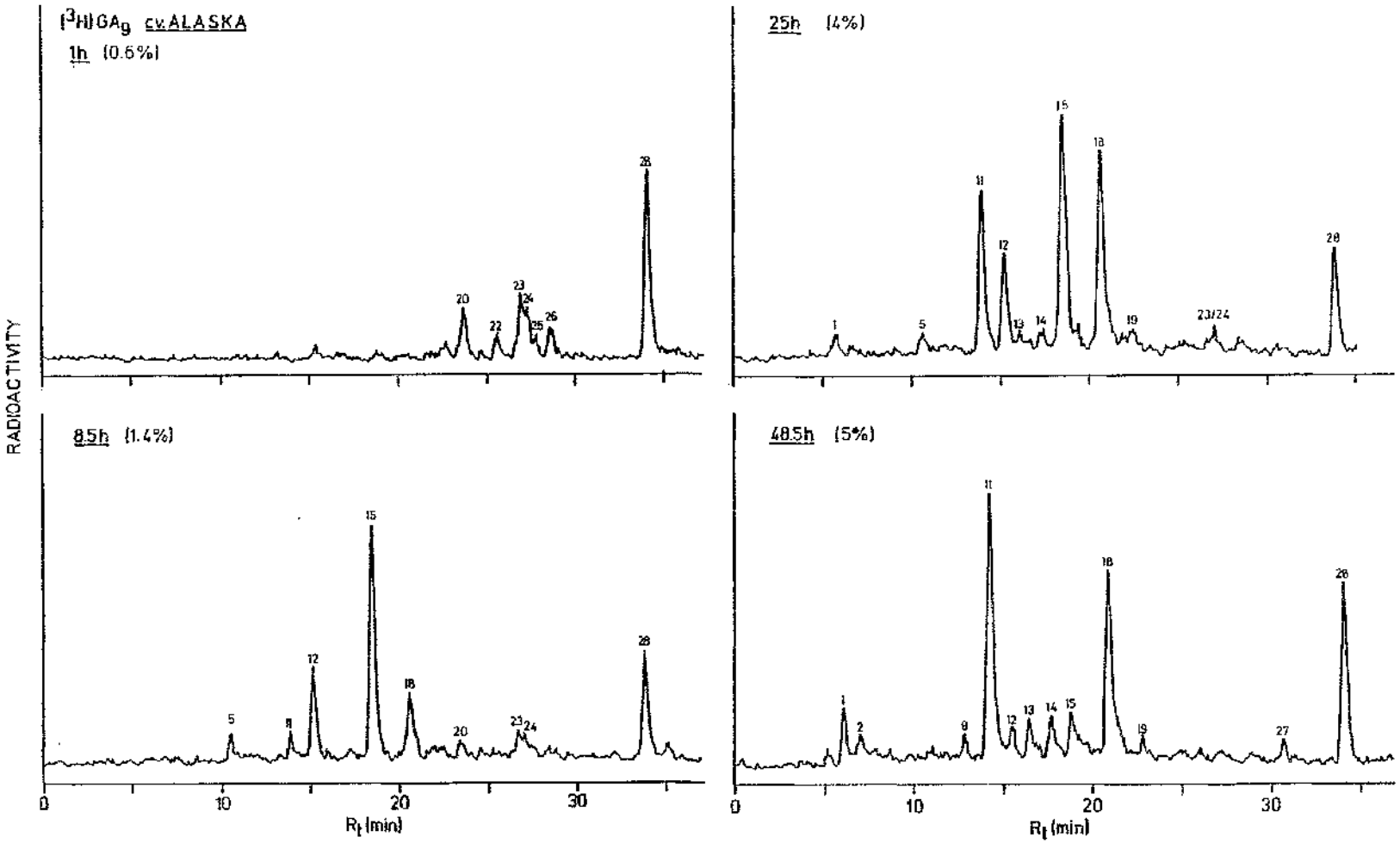


FIGURE 24. Reverse phase HPLC analyses of metabolites from [^{3}H]GA_9 fed to light grown peas, cv. Alaska (feed II). Plants extracted 1, 8.5, 25 and 48.5h after treatment. Percentages represent the proportions of the acidic EtOAc fractions injected. Analytical conditions - as figure 22.

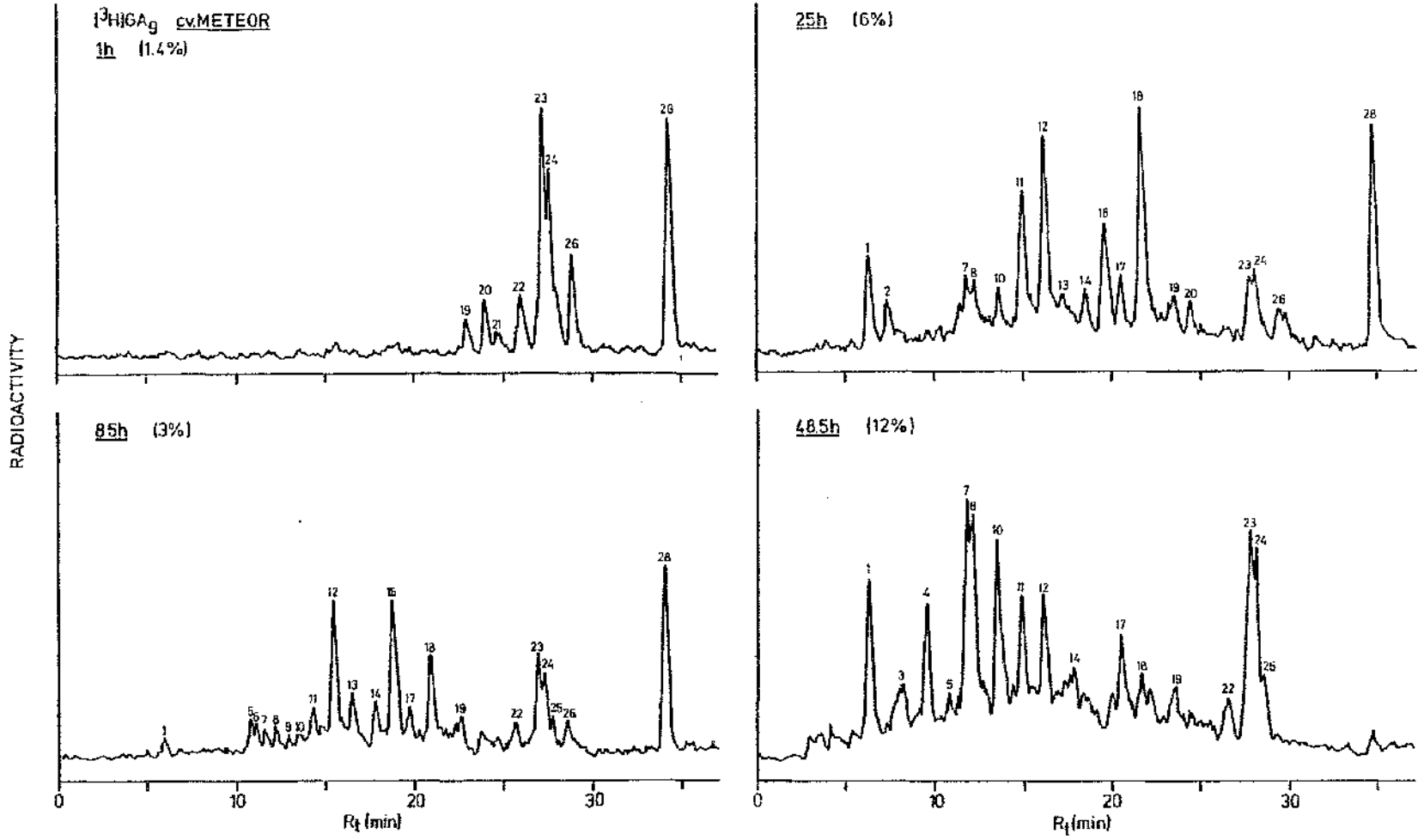


FIGURE 25. Reverse phase HPLC analyses of metabolites from [^{3}H]GA_9 fed to light grown peas, cv. Meteor (feed II). Plants extracted 1, 8.5, 25 and 48.5h after treatment. Percentages represent the proportions of the acidic EtOAc fractions injected. Analytical conditions - as figure 22.

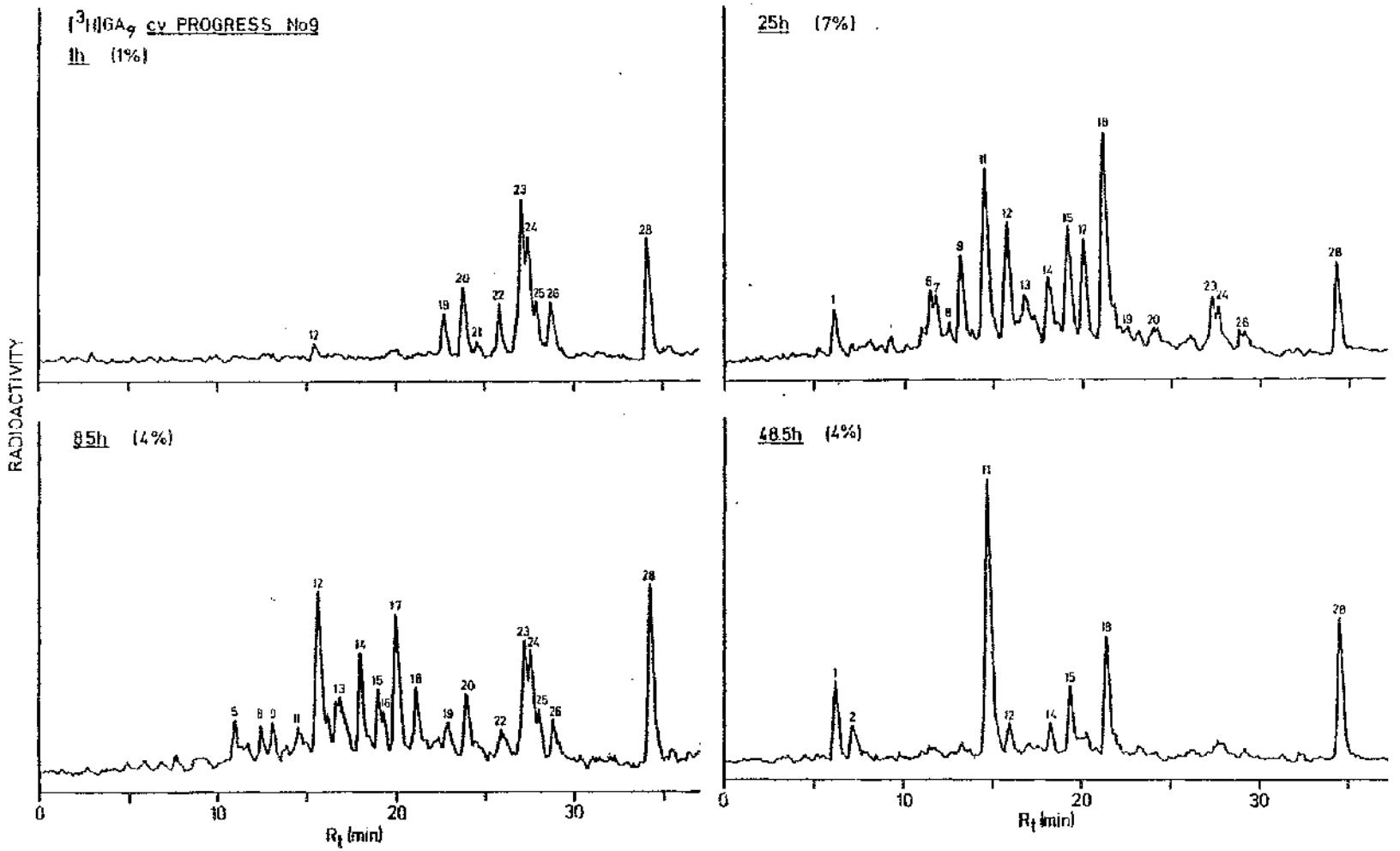


FIGURE 26. Reverse phase HPLC analyses of metabolites from [^{3}H]GA_9 fed to light grown peas, cv. Progress No.9 (feed II). Plants extracted 1, 8.5, 25 and 48.5h after treatment. Percentages represent the proportions of the acidic EtOAc fractions injected. Analytical conditions - as figure 22.

prior to number 11 was almost completely arbitrary and implies little relationship. In this part of the chromatograms a large number of small peaks were detected. These were often poorly resolved and it is possible that more or less than ten products were present. The arbitrary numbering of these peaks from 1 to 10 is intended simply as a reminder that a range of polar compounds were present.

The amounts of the various products detected were estimated as follows. The total of all the peak heights in a trace was found and this was divided into the total radioactivity detected in the EtOAc phase to give the radioactivity that each mm of peak height represented. Each peak height was then multiplied by this figure (radioactivity per mm of peak height) to give an estimate of the amount of product that each peak represented. This is expressed in the results tables as a percentage of the radioactivity applied. There are a number of possible sources of error inherent in each calculation:-

a) The calculation assumed that peak height is proportional to peak size. This is true for completely resolved peaks retained on a linear gradient. However, the size of incompletely resolved peaks (e.g. peaks 23 and 24) will tend to have been overestimated as no allowance was made for incomplete resolution.

b) The choice of a background level of radioactivity, from which peak heights were measured, was sometimes difficult.

c) A full scale deflection at the sensitivity used (30 cps f.s.d.) corresponded to ca. 900 counts. For smaller deflections the number of counts is reduced proportionately. The normal statistical errors associated with counting radioactivity apply.

d) Small peaks were not measured, so consequently the sizes of larger peaks will have been overestimated as a fraction of the whole.

It is apparent from Table 18 and from the traces in figures 22 to 26 that the [^{3}H]GA_9 was metabolised to increasing amounts of more polar (earlier eluting) compounds as the duration of the feeds increased. This observation is consistent with the trend suggested by the partitioning data. The amount of unmetabolised [^{3}H]GA_9 (peak 28) recovered from light-grown plants generally decreased with time in feed II, but remained constant in feed I. The absence of any [^{3}H]GA_9 in the 1h extract light-grown peas of feed I is surprising and appeared inconsistent with the results from the rest of the extracts in that time course. There was generally less [^{3}H]GA_9 remaining unmetabolised in extracts of dark-grown tissue than in the extracts of light-grown tissue. This does not necessarily indicate that there is faster endogenous metabolism in the dark, since a number of other factors such as penetration and transport could affect the metabolism of the applied label. The percentage of the applied [^{3}H]GA_9 that was metabolised was always high.

Peak Number		1	2	3	4	5	6	7	8	9	10	11	12	13
R_t Column 1		6.6	7.5			11.6						15.2	16.5	17.3
R_t Column 2		5.8	6.8	8.2	9.6	10.8	11.1	11.6	12.2	12.9	13.5	14.0	15.3	16.4
ALASKA I (Light)	1h													
	6.5h	(6.5) 1				(11.6) 1						(15.3) 1	(16.5) 5	
	27h											(15.3) 3	(16.6) 3	
	53h	(6.3) 2	(7.1) 1									(14.5) 5	(15.9) 1	
ALASKA I (Dark)	1h		(7.1) 1		(10.9) 1								(16.6) 2	
	6.5h					(11.8) 2							(16.7) 6	
	27h	(6.7) 1			(9.9) 1		(12.3) 3		(13.4) 3	(14.0) 3	(14.5) 3	(15.6) 2	(16.5) 5	(17.7) 5
	53h	(6.3) 3	(7.2) 3		(9.6) 1						(13.7) 1	(14.9) 6	(16.3) 1	
ALASKA Dose-Response	3.4ng	(6.0) 1			(9.6) 2				(12.0) 1		(13.5) 1			(16.0) 3
	28ng	(6.4) 1			(9.9) 2				(12.1) 1		(13.8) 2			(16.2) 3
	280ng				(10.8) 1				(12.8) 1		(14.4) 1			(17.0) 2
	2800ng				(9.5) 1				(12.6) 3		(13.9) 3		(15.3) 2	(16.5) 1

Peak Number		1	2	3	4	5	6	7	8	9	10	11	12	13
R_t Column 1		6.8	7.5			11.6						15.2	16.5	17.3
R_t Column 2		5.8	6.8	8.2	9.6	10.8	11.1	11.6	12.2	12.9	13.5	14.0	15.3	16.4
ALASKA II (Light)	1h													
	8.5h					(10.5) 3						(13.9) 3	(15.2) 10	
	25h	(5.7) 1				(10.6) 1						(13.9) 7	(15.2) 4	(16.1) 1
	48.5h	(5.9) 1	(6.8) 1							(12.7) 1		(14.2) 8	(15.4) 1	(16.4) 1
METEOR II (Light)	1h													
	8.5h	(5.9) 1				(10.8) 2	(11.1) 1	(11.6) 1	(12.3) 1	(12.9) 1	(13.5) 1	(14.3) 2	(15.5) 6	(16.7) 3
	25h	(6.2) 2	(7.2) 1					(11.7) 2	(12.3) 1		(13.5) 1	(14.9) 4	(16.1) 5	(17.2) 1
	48.5h	(6.2) 2		(8.2) 1	(9.6) 2	(10.8) 1		(11.8) 4	(12.1) 3		(13.5) 3	(14.9) 2	(16.2) 2	
PROGRESS II (Light)	1h												(15.5) 1	
	8.5h					(11.0) 2			(12.5) 2	(13.1) 2		(14.5) 1	(15.7) 7	(16.8) 3
	25h	(6.1) 1					(11.5) 2	(11.8) 2	(12.6) 1	(13.2) 3		(14.5) 5	(15.7) 4	(16.8) 2
	48.5h	(6.2) 3	(7.1) 1									(14.7) 11	(16.0) 2	

TABLE 18. (continued overleaf)

Peak Number		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
R_t Column 1		18.7	19.8		20.6	21.8	23.6	24.8	25.6	26.9	28.0	28.3	28.9	29.6		34.8
R_t Column 2		17.1	18.5		19.6	20.7	22.3	23.5		25.6	26.7	27.1	27.8	28.6	30.0	33.9
ALASKA I (Light)	1h						(23.9) 9	(25.1) 4		(27.1) 12	(28.2) 32	(28.5) 30	(28.9) 24			
	6.5h		(19.8) 15			(21.9) 5	(23.9) 3	(24.8) 2		(26.9) 3	(28.1) 12	(28.4) 11	(28.8) 6			(35.0) 27
	27h	(18.8) 1	(19.9) 7			(22.0) 4	(24.0) 2	(24.9) 4		(26.9) 2	(28.1) 9	(28.4) 11		(29.6) 3		(35.0) 30
	53h	(18.0) 1	(19.1) 1			(21.3) 4		(24.3) 2				(27.7) 1		(29.1) 2		(34.8) 28
ALASKA I (Dark)	1h				(20.8) 6		(23.8) 2	(24.7) 12		(27.0) 2	(28.0) 6	(28.3) 5		(29.6) 5		(35.0) 5
	6.5h	(19.0) 3			(20.8) 5		(24.0) 3			(27.0) 7	(28.1) 22	(28.4) 22	(28.9) 14			(35.0) 2
	27h				(20.8) 8		(23.8) 5		(25.6) 2	(27.0) 4	(28.2) 11	(28.5) 10	(29.0) 8			
	53h	(18.5) 2	(19.5) 5			(21.5) 3	(23.7) 1	(24.7) 1		(26.8) 1	(28.1) 2			(29.4) 2		(34.8) 7
ALASKA Dose-Response	3.4ng	(17.5) 1				(20.5) 3	(23.0) 2			(26.1) 2	(27.2) 7	(27.5) 5	(28.3) 3			
	28ng	(17.8) 2				(20.7) 4			(24.5) 1		(27.5) 5	(27.9) 4	(28.5) 2			
	280ng	(18.7) 2				(21.5) 4	(24.0) 3			(27.2) 3	(28.2) 10	(28.5) 10	(29.2) 6			(35.0) 3
	2800ng			(20.0) 2		(21.1) 6	(22.0) 2	(24.5) 1			(27.7) 3	(28.1) 2		(29.3) 1		

Peak Number		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
R_t Column 1		18.7	19.8		20.6	21.8	23.6	24.8	25.6	26.9	28.0	28.3	28.9	29.6		34.8
R_t Column 2		17.1	18.5		19.6	20.7	22.3	23.5		25.6	26.7	27.1	27.8	28.6	30.0	33.9
ALASKA II (Light)	1h							(23.7) 9		(25.6) 4	(26.8) 13	(27.2) 10	(27.8) 4	(28.6) 6		(33.9) 37
	8.5h		(18.4) 26			(20.6) 7		(23.5) 2			(26.7) 3	(27.0) 3				(33.8) 12
	25h	(17.4) 1	(18.5) 10			(20.6) 8	(23.3) 1				(27.0) 1					(33.8) 4
	48.5h	(17.1) 1	(18.8) 1			(20.9) 6	(22.8) 1								(30.0) 1	(34.0) 5
METEOR II (Light)	1h						(22.9) 3	(24.0) 5	(24.7) 2	(26.0) 6	(27.3) 24	(27.6) 18		(28.7) 10		(34.3) 23
	8.5h	(17.8) 3	(18.8) 8		(19.7) 2	(20.9) 5	(22.6) 2			(25.7) 2	(27.0) 5	(27.3) 4	(27.7) 2	(28.6) 2		(34.0) 10
	25h	(18.5) 1	(19.4) 3		(20.4) 2	(21.5) 5	(23.5) 2	(24.4) 1			(27.7) 2	(28.0) 2		(29.5) 1		(34.6) 5
	48.5h	(17.8) 1			(20.4) 2	(21.6) 1	(23.5) 1			(26.3) 1	(27.6) 3	(28.0) 3	(28.6) 1			
PROGRESS II (Light)	1h						(22.7) 5	(23.8) 8	(24.5) 2	(25.8) 6	(27.0) 19	(27.4) 14	(27.9) 7	(28.7) 6		(34.1) 14
	8.5h	(18.0) 5	(19.0) 3	(19.3) 2	(20.0) 6	(21.0) 3	(23.0) 2	(23.9) 3		(26.0) 2	(27.2) 5	(27.5) 5	(28.0) 2	(28.8) 2		(34.2) 8
	25h	(18.1) 2	(19.2) 3		(20.1) 3	(21.2) 6	(22.5) 1	(24.0) 1			(27.3) 2	(27.7) 1		(29.0) 1		(34.3) 3
	48.5h	(18.2) 1	(19.3) 3			(21.2) 5										(34.4) 6

TABLE 18. (continued) [^{3}H]GA_9 Time course and dose-effect feeds. Peak sizes and retention times. The bracketed figures are the retention times (min) of peaks detected on gradient analysis as shown in figure 21-26. The unbracketed (lower) figures represent the percentage conversions of applied radioactivity into each peak, calculated as described in the text. Typical retention times of peaks on two of the columns used during the analyses are also shown.

The peaks numbered 23, 24 and 25 appeared to be early products, which were further metabolised, in all the time courses. This suggests that these peaks represented identical compounds in all the extracts. Peak 25 appeared absent from the 11h dark extract of cv. Alaska, but this was probably due to incomplete resolution, during HPLC analysis, from peak 24. All three peaks were incompletely resolved and so their sizes will have tended to have been slightly overestimated by the method of calculation used.

Peaks 20, 23, 24 and 26 had similar retention times to three of the peaks observed in the acidic EtOAc fraction of the control extract shown in figure 20. A possible interpretation of the reduced amounts of these peaks present in the later extracts of the time course feeds is, that they represented breakdown products of $[^3H]GA_9$ and that the amounts present in extracts decreased as the amount of unmetabolised $[^3H]GA_9$ decreased. This is generally consistent with the data in Table 18, although the 53h light extract of cv. Alaska in feed I contained a large amount of unmetabolised $[^3H]GA_9$ but only low amounts of peaks 23, 24 and 26. However, it is considered unlikely that breakdown made a significant contribution to the amounts of peaks 20, 23, 24 and 26 observed in the time course extracts. This is because the control extract was treated much more severely than the time course extracts but contained less of these products.

The pattern of metabolism appeared very similar in the two feeds to light-grown plants of cv. Alaska, both in terms of the metabolites detected, and the distribution of these products in the extracts made at the different times. Thus in both feeds peaks 12 and 15 appeared to be intermediates which were further converted during the feeds. The amount of peak 18 detected was roughly constant after 6h. This may indicate that further metabolism did not occur or that the rates of synthesis and metabolism of this product were similar. Peak 18 had a similar retention time to [^{3}H]GA_{20} (see Table 19), and is thought to be the peak later identified as [^{3}H]GA_{20} from the cv. Metcor and cv. Progress No.9 extracts of feed II. Peak 11 seemed to be present in increasing amounts as the duration of the feeds increased. It is possible that the levels of the more polar compounds found in the EtOAc phase of the extracts may be considerably less than the amount of the product actually produced, since a significant proportion may have remained in the aqueous phase on partitioning.

TABLE 19. Retention Times (min) of [^{3}H]GA Standards: Analysed by reverse phase HPLC using the gradient: 25-70% MeOH, 0-30 min: 70-100% MeOH, 30-33 min.

GA_8	GA_1	GA_5	GA_{20}	GA_4	GA_9
5.4	11.4	19.7	21.0	30.6	34.0
5.6	11.6	20.0	21.2	30.6	33.9
5.5	11.5	19.8	21.2	30.6	34.0

Comparison of the pattern of metabolism of $[^3H]GA_9$ by light- and dark-grown plants, cv. Alaska, reveals that it was generally similar, although the amounts of unmetabolised $[^3H]GA_9$ that remained do seem to have differed. A larger number of more polar compounds were noted in the 27h dark extract than in most of the other extracts of cv. Alaska. Larger numbers of more polar compounds were also detected in many of the other extracts (especially of cv. Meteor and cv. Progress No.9) that were not dominated by one or two large peaks. The lack of dominating metabolites meant that a larger proportion of the extract could be injected (before full-scale-deflection was obtained), so that there was more likelihood of minor constituents being detected. Therefore the differences in the peaks detected may not always indicate differences in the pattern of metabolism.

The extracts of dark-grown tissue of cv. Alaska contained a compound similar to peak 12, detected in the extracts of light-grown tissue, but peak 15 appeared to be absent. It is not clear whether peak 17 was caused by a different compound to that causing peak 18. In the case of the extracts of light- and dark-grown tissue, cv. Alaska, it is considered unlikely, since only one peak was detected when the extracts were combined (Table 21), and as each extract contained one or the other, but never both compounds. (For the opposite reasons it appears that peak 17 is distinct from peak 18 in the extracts of cultivars Meteor and Progress No.9). It is not clear from the results whether there was any difference

in the rate or the pattern of metabolism of [^{3}H]GA_9 applied to plants grown in either light or darkness.

Comparison of the metabolites from [^{3}H]GA_9 fed to light-grown cv. Alaska seedlings (feeds I and II) with those of the dose-effect experiment shows that the products seem to have been generally similar. It was found difficult to number some of the peaks in the dose-effect experiment and it is possible, for example, that some of the peaks numbered 13 were actually peak 12. The failure to match the peaks observed with those present in other extracts probably results from chromatographic differences. The important conclusion of the dose effect experiment is that the dose of GA_9 applied did not appear to markedly affect the pattern of metabolism of the [^{3}H]GA_9.

Comparison of the metabolites in the extracts from the three cultivars used in feed II suggests that they were similar. Peaks 22, 23, 24 and 25 appear as major early products from all cultivars. Peak 18 seemed to be a major product from all three cultivars, and is probably the one that corresponded to the [^{3}H]GA_{20}, which was subsequently shown to be present in the cv. Meteor and cv. Progress No.9 extracts. Peak 12 was detected in quite large amounts in the 8.5h extracts of all three cultivars. Lower amounts were detected in later extracts, indicating that it was further metabolised. Peaks 11 and 15 were also important products in all cultivars.

More metabolites were generally detected in extracts of cv. Meteor and cv. Progress No.9 than in the extracts of cv. Alaska. This may reflect a difference in the pattern of metabolism, or simply fortuitous choices of sampling times. It is therefore unclear from the results obtained whether the metabolism of [^{3}H]GA_9 differed in the three cultivars used. However, further analyses of the extracts from cv. Meteor and cv. Progress No.9 suggested that the products formed by these two cultivars had very similar retention times and were present in similar amounts, suggesting that the pattern of [^{3}H]GA_9 metabolism was the same. It is also evident that the pattern of [^{3}H]GA_9 metabolism in seedlings is more complex than previous reports in the literature had suggested.

3.7.4. [^{3}H]GA_9 TIME COURSE FEEDS: ANALYSES OF COMBINED EtOAc EXTRACTS

After analysis of the individual extracts had been completed the following groups of extracts were bulked to give four combined extracts: all extracts, feed I; all cv. Alaska extracts, feed II; all cv. Meteor extracts, feed II; all cv. Progress No.9 extracts, feed II (figure 27). The combined extracts were purified by DEAE A-25 anion exchange chromatography. Radioactive fractions eluting from the Sephadex columns were combined, and the MeOH evaporated off *in vacuo* at 40°C, prior

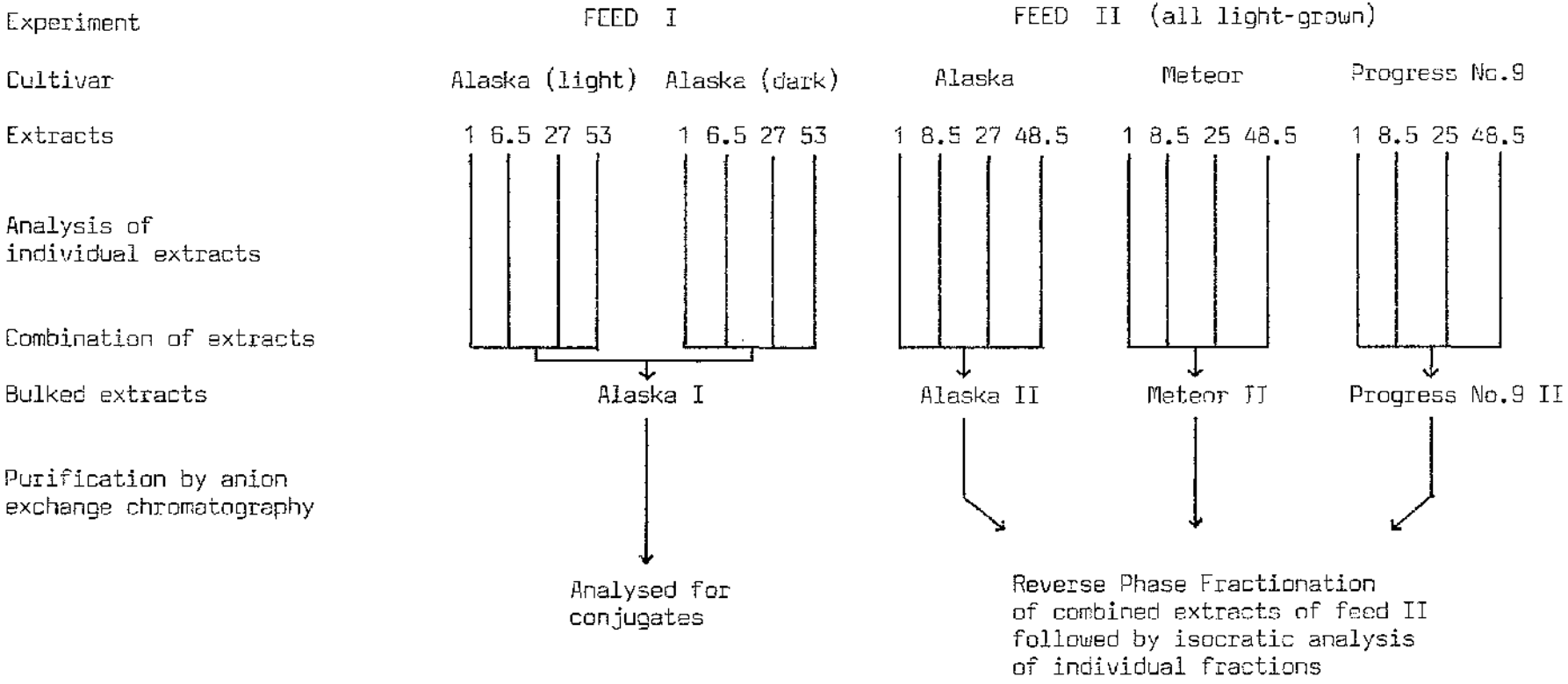


FIGURE 27. Summary of the method used to analyse the extracts from $[^3H]GA_9$ feeds.

to partitioning against EtOAc. The combined extract, Alaska II, was left on the evaporator for longer than normal, the volume being reduced from ca. 25cm^3 of 2M acetic acid:MeOH (1:1 v/v) to ca. 2cm^3. Details of the radioactivity in the extracts before and after anion exchange chromatography are given in Table 20.

TABLE 20. [^{3}H]GA_9: The amount of radioactivity in the combined extracts before and after Sephadex anion exchange chromatography (dpm)

Combined Extract	Pre-Sephadex	Post-Sephadex	
		Discarded	Retained
Alaska I	5.0×10^7	8×10^5	2.5×10^7
Alaska II	3.0×10^7	2.0×10^6	1.8×10^7
Meteor II	2.6×10^7	2.0×10^6	1.7×10^7
Progress No.9 II	2.9×10^7	2.0×10^6	1.6×10^7

It appears from Table 20 that ca. 40-50% of the radioactivity present in the extracts was lost during anion exchange chromatography. However, the actual losses at this stage were not as great as appears. This is because the estimate of the pre-Sephadex radioactivity is based on counts of the original EtOAc phases. Although corrected for the aliquots taken for analysis of the individual extracts, losses also occurred during drying and transfers between containers. Losses might also be

expected on recovery from the Sephadex column, as discussed below.

After purification by anion exchange chromatography the combined extracts were analysed by reverse phase HPLC, and the traces are shown in figure 28. The expected elution profiles were calculated from the distribution of radioactivity in the individual extracts prior to bulking (Table 18), (e.g. for Alaska II, the 1, 8.5, 25 and 48.5h extracts). Correction was made for the proportion of individual extracts used prior to bulking. The expected profiles and the profiles obtained are detailed in Table 21.

With the exception of Alaska II there is broad agreement between the expected and the actual distributions of radioactivity. The Alaska II extract shows a shift to much more polar radioactivity, and a large unexpected peak 17. It is likely that this extract broke down during the excessive period that it was left on the rotary film evaporator after recovery from the anion exchange column. Smaller discrepancies between the expected and actual distributions of radioactivity do exist in the remainder of the extracts. These are attributed to i) errors in the calculation of the expected distribution of radioactivity, ii) polar compounds remaining in the aqueous when partitioning against EtOAc after ion exchange chromatography, and/or iii) the possible separation of small amounts of radioactivity from the main peak of radioactivity eluting from the ion exchange column.

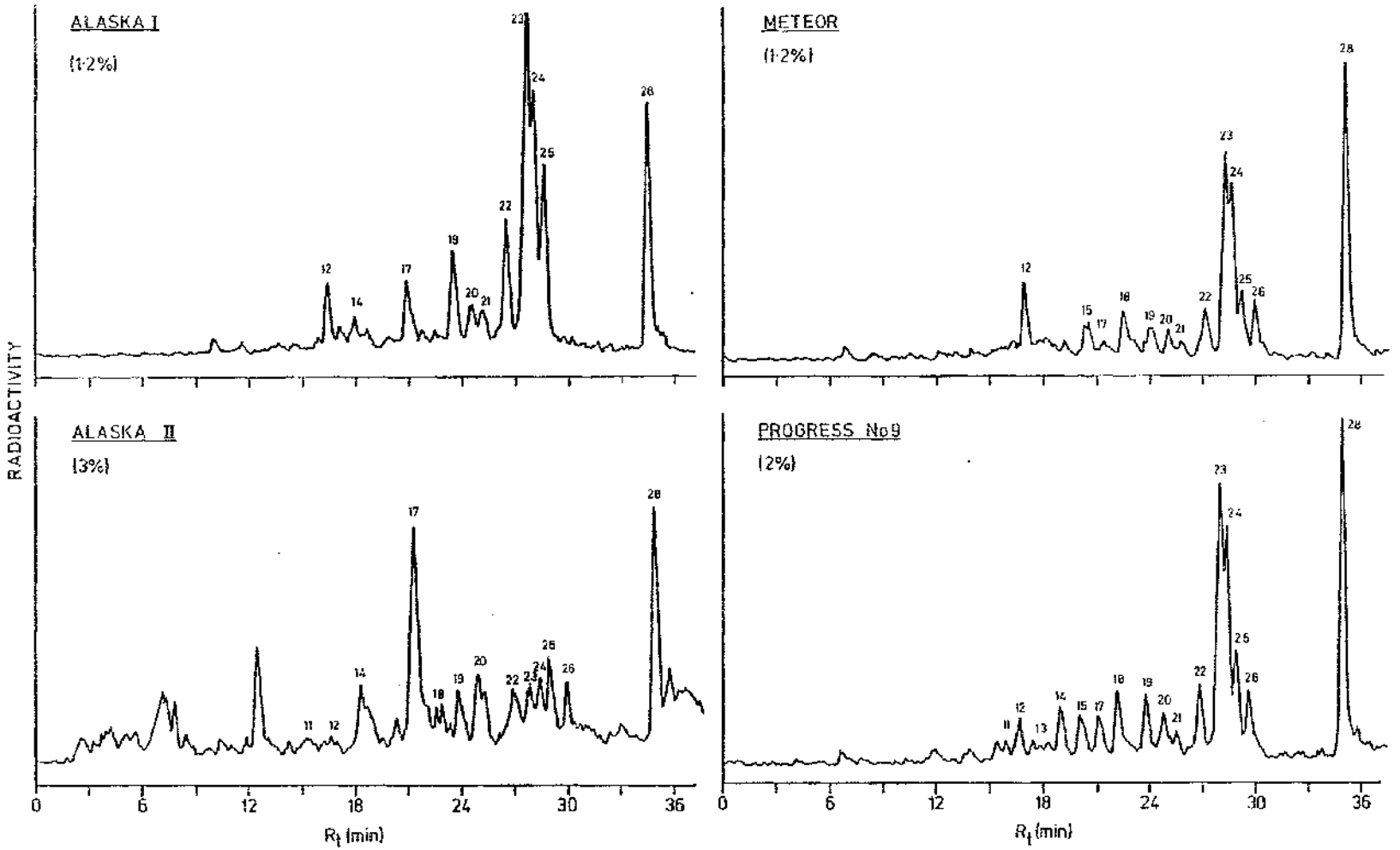


FIGURE 28. Reverse phase HPLC analyses of metabolites in the combined [^{3}H]GA_9 fractions. See text for details of fractions. Percentages represent the proportions of the fractions injected. Mobile phase: 25-70% MeOH, 0-30 min; 70-100% MeOH, 30-35 min; 1cm^3 min^{-1}. Detector: Radioactivity monitor, 30 cps f.s.d., 10s time constant.

	Peak Number	1-10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Approximate Expected R_t (min)		6.7-14.1	15.5	16.8	17.8	19.2	20.1		21.1	22.2	23.8	24.8	25.8	26.9	28.0	28.4	28.9	29.8		34.9
ALASKA (light and dark) FEED I	Expected	5%	2%	5%		1%	5%		3%	3%	5%	2%		5%	17%	16%	9%	2%		17%
	Actual	2%		(16.4) 4%		(18.5) 4%			(20.9) 4%		(23.4) 6%	(24.5) 2%	(25.3) 2%	(26.5) 8%	(27.5) 22%	(27.8) 17%	(28.5) 12%			(34.4) 16%
ALASKA FEED II	Expected	1%	8%	7%	1%		16%			9%	1%	5%		2%	8%	6%	2%	3%		30%
	Actual	23%	(15.7) 1%	(16.7) 1%		(18.8) 5%			(21.5) 15%	(22.7) 3%	(23.7) 4%	(25.0) 5%		(27.0) 5%	(27.8) 4%	(28.4) 5%	(28.8) 6%	(29.1) 4%		(34.9) 17%
METEOR FEED II	Expected	10%	3%	6%	2%	2%	5%		2%	5%	3%	3%	1%	4%	16%	12%	1%	6%		19%
	Actual	1%		(16.7) 6%			(20.1) 3%		(21.3) 2%	(22.3) 5%	(23.8) 3%	(24.8) 2%	25.6) 2%	(26.9) 4%	(28.0) 19%	(28.3) 16%	(29.0) 6%	(29.7) 5%		(34.8) 27%
PROGRESS No.9 FEED II	Expected	8%	7%	6%	2%	4%	4%	1%	4%	6%	3%	6%	1%	4%	12%	10%	4%	4%		14%
	Actual	4%	(15.7) 2%	(16.1) 3%	(17.4-18.3) 2%	(19.0) 3%	(20.0) 3%		(21.1) 3%	(22.1) 4%	(23.8) 4%	(24.8) 3%	25.6) 2%	(26.8) 5%	(27.9) 17%	(28.2) 15%	(28.9) 7%	(29.6) 4%		(34.9) 21%

TABLE 21. Comparison of expected and actual distributions of radioactivity in combined [^{3}H]GA_9 extracts when analysed by reverse phase HPLC. The bracketed figures represent the actual peak retention times (min). The percentage figures are the percentage that each peak represents of the total radioactivity detected by reverse phase HPLC. The reverse phase HPLC gradient was the same as in figure 28.

The combined extract, Alaska I was examined to determine whether there were any conjugated products present amongst the radioactive metabolites. An aliquot was analysed by gel permeation chromatography on the 2 x 1M Bio-Beads SX-4 column. 93% of the recovered radioactivity eluted between [^{3}H]GA_1 and [^{3}H]GA_9 standards. This suggests that conjugated metabolites were only minor components, if present at all, in the extract. However, this does not completely exclude the possibility that conjugated products were produced from [^{3}H]GA_9, as certain conjugates (e.g. GA glucoside esters) could have been removed from the extract by the purification procedures prior to analysis.

The whole of each of the combined extracts from feed II was purified by reverse phase HPLC using a gradient of: 25-70% MeOH, 0-30 min; 70-100%MeOH, 30-33 min. Fourteen 3 minute fractions were collected, and a larger fifteenth fraction as the column was run at 100% MeOH. The distribution of radioactivity in the collected fractions is shown in Table 22, and is very similar to that predicted from figure 28. The difference between the amount of radioactivity recovered from the Sephadex column and the amount recovered after reverse-phase fractionation is partly accounted for by the aliquots analysed by reverse-phase HPLC (e.g. figure 28).

Most of the fractions detailed in Table 22 were subsequently analysed by isocratic reverse phase HPLC. Radioactive peaks detected were co-chromatographed with a range of appropriate GA standards. Fractions containing radioactive peaks with retention times closely matching those of GA standards were derivatised to form methoxycoumaryl esters. The derivatised fractions were

Fraction Number / Extract	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Residue	Total
Alaska	0.20	0.15	0.49	0.19	0.60	0.40	1.13	1.13	1.08	1.31	0.60	1.40	1.36	0.53	0.37	0.23	11.17
Meteor	-	0.03	0.10	0.12	0.15	0.83	0.81	1.01	0.91	3.82	0.17	2.11	0.28	0.06	0.04	0.11	10.55
Progress No.9	0.01	0.02	0.13	0.08	0.18	0.65	0.94	1.14	0.99	3.89	0.29	1.58	0.31	0.07	0.03	1.37	11.68

TABLE 22. Radioactivity collected after reverse phase HPLC fraction of the combined extracts of feed II.

All figures are d.p.m. x 10^{-6}.

subsequently co-chromatographed with GACE standards, initially using reverse phase HPLC and subsequently, if necessary, using normal phaseHPLC.

The results of the analysis of each fraction from cv. Meteor and cv. Progress No.9 will be discussed in detail. The results of the analyses of the cv. Alaska fractions are not further considered as a result of the likelihood that extensive breakdown had occurred in this extract. None of the peaks detected from the cv. Alaska extract co-chromatographed with any of the GA standards tested.

To estimate the amount of radioactivity that each peak detected by isocratic analysis represented, the heights of all the radioactive peaks detected during the analysis of each fraction were totalled. The height of each peak was then expressed as a proportion of the total, and this proportion was multiplied by the total amount of radioactivity in the fraction. Sources of error in such estimations are similar to those involved when estimating peak size from gradient analyses (see section 3.7.3.). However, there is an additional factor because as the retention time on isocractic analysis increases the peak shape changes, becoming broader and shorter. Although this will tend to lead to an under-estimation of the size of later eluting peaks this effect is probably not important in the analyses reported, as the peaks within a fraction all have similar retention characteristics.

The retention time of a peak is dependant on the amount of MeOH in the mobile phase. When comparing the elution characteristics of peaks detected it is useful if one of these variables is standardised. For this purpose the percentage of MeOH that would give a retention time of 10.0 min was found for each peak. This value was called the R_{10}. It was estimated from a calibration curve, prepared from standard GAs, of retention time against percentage of MeOH. In practice, estimates of R_{10} values were consistent to within 1-1.5% MeOH, provided that the retention time from which they were estimated was not too short (less than about 6.5 min) or too long (over about 14 min).

Except for early eluting peaks the R_{10} is directly related to the retention time of a peak when analysed on a linear gradient. Therefore, using the estimates of R_{10} values and of peak sizes made during the isocratic analyses it was possible to construct models of the anticipated gradient elution profiles of the combined extracts of cv. Meteor and cv. Progress No.9, prior to reverse phase HPLC fractionation. These are shown in figure 29. It is useful to refer to this figure when considering the analyses of the individual fractions, in order to see the relative sizes of the peaks discussed. The analysis of the individual fractions will now be presented in detail.

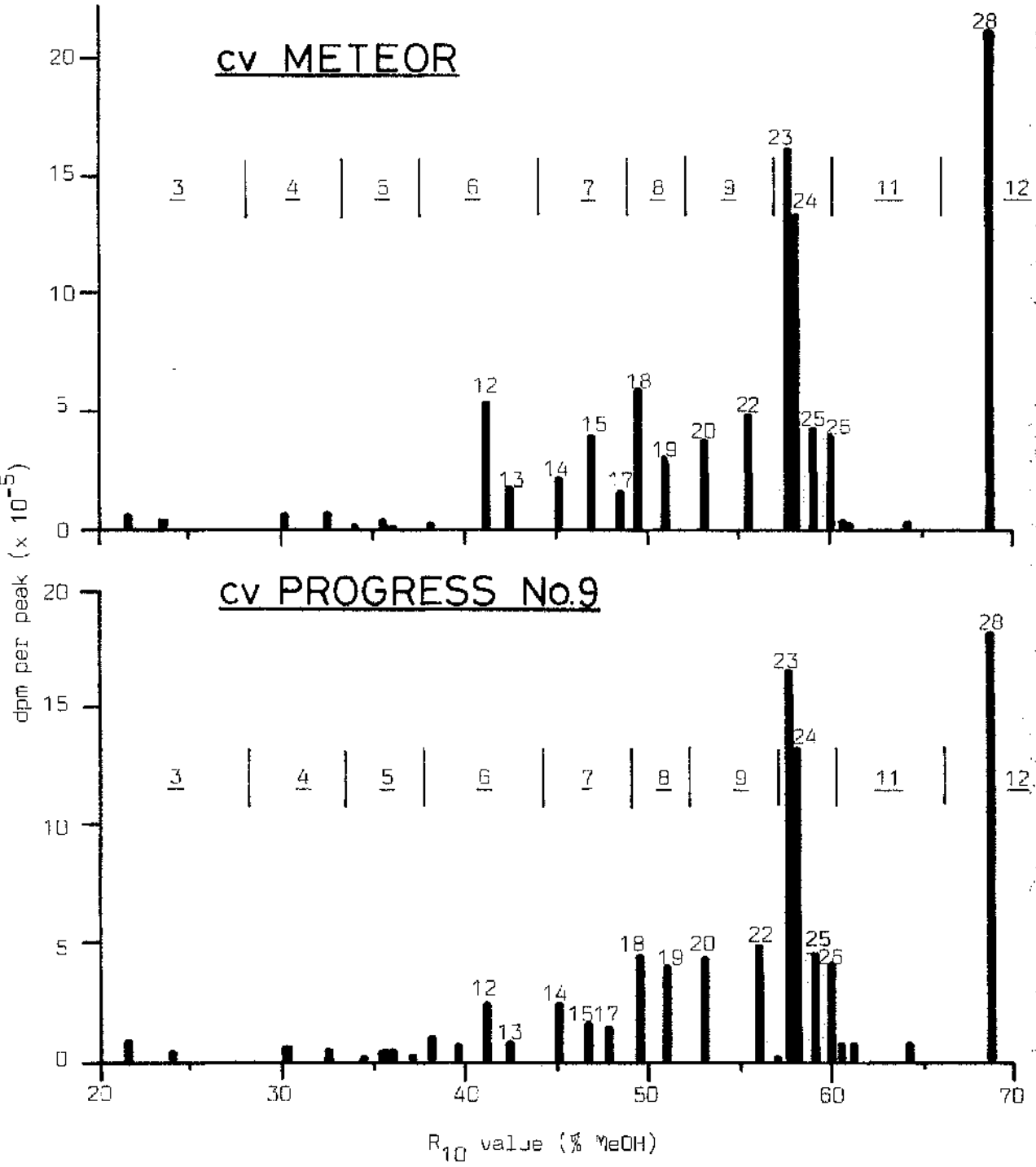


FIGURE 29. R_{10} values and sizes of [^{3}H]GA_9 metabolites detected from cv. Meteor and cv. Progress No.9 on isocratic analyses of fractions 3 to 12.

Fraction 1

Not analysed

Fraction 2

Not analysed

Fraction 3

This fraction was similar from both cv. Meteor and cv. Progress No.9, both fractions containing two radioactive peaks. The early eluting peak from cv. Progress No.9 co-chromatographed with GA_{29} (Table 23), but derivatisation was not possible due to the low levels of radioactivity present. Therefore [^{3}H]GA_{29} was possibly a minor product. GA_8 elutes before GA_{29} so is unlikely to have been present in the extracts in detectable quantities.

TABLE 23. Fraction 3 Retention times (min) of unknown products and standards when co-chromatographed.

	% MeOH	GA_{29}	Unknown
cv. Meteor	25%	-	8.0, 9.7
cv. Progress No.9	25%	-	8.0, 9.7
cv. Progress No.9	20%	11.1	11.1, 13.9

Fraction 4

This fraction was similar from both cultivars. Two peaks were detected which did not co-chromatograph with GA_{30}, GA_3 or GA_{29} catabolite (Table 24).

TABLE 24. Fraction 4 Retention times (min) of unknown products when co-chromatographed with standards.

	% MeOH	GA_{30}	GA_3	GA_{29} cat.	Unknown
cv. Meteor	30%	8.4	11.4	13.6	10.0, 12.4
cv. Progress No.9	30%	8.4	11.4	13.6	9.9, 12.4

Fraction 5

This fraction appeared similar from both cultivars, both fractions containing at least 4 partially resolved radioactive peaks. $[^3H]GA_1$ appeared to co-chromatograph with the earliest eluting peak of both extracts. However, the peak was so small that addition of $[^3H]GA_1$ obscured it completely and the retention times may not necessarily have been identical. If the peak did represent $[^3H]GA_1$ then the maximum amount present would be ca. 2×10^4dpm per extract (0.03% of applied label). GA_{29} catabolite has a very similar retention time to GA_1 and would probably also co-chromatograph with the peak.

TABLE 25. Fraction 5 Retention times (min) of unknown products when co-chromatographed with GA_1.

	% MeOH	GA_1	Unknown
cv. Meteor	32.5%	11.2	11.2*, 13.3, 13.7, 15.7
cv. Progress No.9	32.5%	11.2	11.2*, 12.8, 13.8, 15.8

* Obscured by $[^3H]GA_1$ standard

Fraction 6

At 40% MeOH both fractions showed a poorly resolved band of radioactivity eluting between ca. 8 min and ca. 14 min. At least 4 radioactive peaks were partially resolved from the cv. Progress No.9 fraction. In the fractions from both cultivars the peak with a retention time of 10.8 min was the largest. This was particularly so for the cv. Meteor, where the size of the peak may have masked the presence of metabolites detected in the cv. Progress No.9. fraction.

TABLE 26. Fraction 6 Retention times (min) of unknown products when co-chromatographed with standards.

	% MeOH	GA_{29} cat.	GA_{35}	Unknown
cv. Meteor	40%	6.8	9.7	10.8, 12.3
cv. Progress No.9	40%	-	-	8.5, 9.7, 10.8, 12.1

Although the cv. Progress No.9 peak, retention time 9.7 min had a similar retention time to GA_{35} it is considered unlikely that [^{3}H]GA_{35} was a product. The peak with a retention time of 9.7 min was a minor component and was poorly resolved, probably representing a heterogeneous group of products. GA_{35} is 3β- and 11β-hydroxylated. 11β-hydroxylation of GAs has not been observed previously in peas. Low levels of radioactivity precluded further analysis of the peak.

Fraction 7

Three radioactive peaks were detected from both cultivars (Table 27). In view of the previous detection of 12α-hydroxy GA_9 as a product from [^{3}H]GA_9 feeds (Frydman and MacMillan, 1975; Sponsel and MacMillan, 1977; Railton et al., 1974a,b) the possible presence of the structurally similar [^{3}H]GA_{31} was further investigated. The whole of each fraction was injected at 40% MeOH and the eluent collected. The portions containing the early eluting radioactive peak were derivatised to form methoxycoumaryl esters and co-injected with GA_{31} CE standard at 70% MeOH (R_t GA_{31} CE = 9.3 min). A single radioactive peak, with a retention time of 10.5 min was detected from both cv. Meteor and cv. Progress No.9. It was concluded that [^{3}H]GA_{31} was not present in the extracts. GA_{10}, previously identified as a product (possibly non-metabolic) from [^{3}H]GA_9 feeds (Railton et al., 1974a,b) would be expected to be contained in this fraction, but no traces were detected.

TABLE 27. Fraction 7 Retention times (min) of unknown products and standards when co-chromatographed.

	% MeOH	GA_{31}	GA_5	GA_{10}	Unknown
cv. Meteor	40%	14.7	-	19.1	14.2, 16.3, 20.0
cv. Progress No.9	40%	14.7	19.4	19.4	14.4, 17.0, 20.0

Fraction 8

Analysis of this fraction from cv. Meteor and cv. Progress No.9 at 45% MeOH showed two clearly resolved radioactive peaks of about equal size. In both extracts the early eluting peak co-chromatographed with [^{3}H]GA_{20} (Table 28). Subsequently the whole of each fraction was injected at 45% MeOH, and the [^{3}H]GA_{20}-like

TABLE 28. Fraction 8 Retention times (min) of unknown products and GA_{20} standard when co-chromatographed.

	% MeOH	GA_{20}	Unknown
cv. Meteor	45%	14.2	14.2, 17.5
cv. Progress No.9	45%	14.1	14.1, 17.2

zone collected and derivatised to form methoxycoumaryl esters. The derivatised extracts were co-chromatographed with [^{3}H]GA_{20} CE

and GA_{16} CE standards at 65% MeOH (GA_{16} co-chromatographs with GA_{20} on reverse phase HPLC). A single radioactive peak, co-chromatographing with [^{3}H]GA_{20} CE (R_t = 19.2 min) was detected from both cv. Meteor and cv. Progress No.9 fractions. The retention time of GA_{16} CE was 18.1 min.

The derivatised peak of cv. Meteor and of cv. Progress No.9 was subsequently shown to co-chromatograph with [^{3}H]GA_{20} when analysed by normal phase HPLC using a 5µm CPS-Hypersil support and a solvent of DCM:Hexane:EtOH (19:78:3). The R_t of [^{3}H]GA_{20} CE was 11.5 min.

It was therefore concluded that [^{3}H]GA_{20} was a product formed from [^{3}H]GA_9 by both cv. Meteor and cv. Progress No.9. This is consistent with the results of previous workers using both seeds and seedlings (Frydman and MacMillan, 1975; Sponsel and MacMillan, 1977; Railton *et al.*, 1974a,b). From Table 19 it is thought likely that the peak numbered 18 from the original traces (figures 22 to 26) is the one subsequently identified as [^{3}H]GA_{20}, representing up to *ca.* 6% of the applied radioactivity.

Fraction 9

Analysis of this fraction from both cultivars at 55% MeOH revealed two peaks of approximately equal size. The peaks had similar retention times. The peaks eluted before GA_{51} and GA_{34}, but after GA_{20} and GA_{16}. None of the other standards tested eluted between these GAs. The identity of the products was therefore not determined.

TABLE 29. Fraction 9 Retention times (min) of unknown products.

	% MeOH	Unknown
cv. Meteor	55%	8.9, 10.4
cv. Progress No.9	55%	8.9, 10.3

Fraction 10

This fraction from both extracts contained a high proportion of the recovered radioactivity. Both fractions were similar, each containing at least four partially resolved peaks when analysed at 55% MeOH (Table 30). The ratios of the peak heights were similar. The early eluting peaks of each extract had similar retention times to the GA_{51} standard at 55% MeOH. GA_{34} and GA_{51} co-chromatograph under these conditions. GA_{51} has previously been observed as a product from [^{3}H]GA_9 in peas (Sponsel and MacMillan, 1977).

TABLE 30. Fraction 10 Retention times (min) of unknown peaks and of GA_{51} when co-chromatographed.

	% MeOH	GA_{51}	Unknown
cv. Meteor	55%	12.1	12.0, 12.6, 13.8, 15.6
cv. Progress No.9	55%	12.6	12.3, 12.8, 14.1, 15.8

The whole of each fraction was injected at 55% MeOH and 1 min fractions collected as the radioactivity eluted. The fractions expected to contain any [^{3}H]GA_{51} present were derivatised to methoxycoumaryl esters and co-chromatographed with GA_{51} CE at 75% MeOH. Each derivatised fraction contained a single radioactive peak which had a retention time of 8.2 min (R_t GA_{51} = 9.7 min; R_t GA_{34} CE = 9.2 min). It was concluded that [^{3}H]GA_{34} and [^{3}H]GA_{51} were not present. This is surprising as GA_{51} is an endogenous constituent of peas and has been previously identified as a metabolite of [^{3}H]GA_9 in developing seed (Sponsel and MacMillan, 1977).

Fraction 11

In both fractions GA_4 and GA_7 standards co-chromatographed with a broad band of poorly resolved radioactive compounds when co-chromatographed at 60% MeOH. (R_t GA_4 = 12.4 min, R_t GA_7 = 10.3 min). These GAs have not previously been identified in Pisum either as endogenous constituents or as metabolites. It was concluded that GA_4 and GA_7 were probably absent from the extracts, but that if present the amount was less than 3×10^4 dpm per extract (0.05% of applied label).

Fraction 12

Isocratic analysis of this fraction at 65% MeOH showed a single peak, with a retention time similar to that of [^{3}H]GA_9. (R_t [^{3}H]GA_9 = 13.1 min).

Fractions 13, 14, 15

Not analysed.

TABLE 31. Summary of GA standards tested. (-) indicates that the GA was absent, a number indicates the fraction in which the GA was possibly present

GA	29	30	3	29cat	1	35	31	10	5	20	16	51	34	7	4	9
cv. Meteor	3(?)	-	-	5(?)	5(?)	-	-	-	-	8	-	-	-	-	-	12
cv. Progress No.9	3(?)	-	-	5(?)	5(?)	-	-	-	-	8	-	-	-	-	-	12

A summary of the GA standards tested is shown in Table 31. It can be seen from figure 29 that there is extremely good agreement between the actual reverse phase gradient profiles of the combined extracts (figure 28) and the profiles projected using the R_{10} values and the peak size estimates obtained from the isocratic analyses (figure 29). All fractions had profiles similar to those expected, with the exception of fraction 9. It is apparent from figure 28 that one major and two smaller peaks would be expected in this fraction. However, two equal sized peaks were detected from both cultivars. This result is consistent with the appearance of the fractions as predicted from the original analyses of the component

extracts (i.e. 1, 8.5, 25 and 48.5h) of the bulked extracts (Table 21). This table shows that peaks 20 and 22 were expected to be of equal size with a minor component inbetween. No minor component was detected in the analysis of fraction 9, but it is considered likely that the peaks detected are numbers 20 and 22.

Both the retention times and the sizes of the radioactive peaks detected from cv. Meteor and cv. Progress No.9 were almost identical, and this, coupled with the similarity of the original extracts (Table 18) suggests that $[^3H]GA_9$ is metabolised in the same way by both cultivars. $[^3H]GA_{20}$ was identified as a product, with conversions of ca. 5% of applied label, which is very similar to that found by Railton et al. (1974a,b). $[^3H]GA_{29}$ was also possibly present in fraction 3. None of the other GAs tested as standards are thought to be products.

12α-hydroxy GA_9 (dihydro GA_{31}), a major product in earlier reports, was not available for co-chromatography. However, the absence of the structurally similar GA_{31} was confirmed by co-chromatography. GA_{31} and 12α-hydroxy GA_9 differ by a single double bond in the 2,3 position, as do GA_5 and GA_{20}. From the difference in retention times of the latter pair it is anticipated that 12α-hydroxy GA_9 would have a R_{10} of ca. 43% MeOH, eluting before GA_{31}. Based on this supposition it would be expected to elute in fraction 6, where it could be represented by any of the peaks detected. Peaks 12 and 13 in the original extracts (Table 18)

are thought to be present in this fraction. No 3β-hydroxylated products were identified.

3.7.5. [^{3}H]GA$_9$ FEEDS: SUMMARY

[^{3}H]GA$_9$ is rapidly metabolised to a large number of compounds when applied to cultivars Alaska, Meteor and Progress No.9 grown in the light, and also when applied to cv. Alaska grown in the dark. The identity of most of these products remains unknown, although [^{3}H]GA$_{20}$ was identified from cultivars Meteor and Progress No.9. A range of known GAs were shown to be absent from the products of cultivars Meteor and Progress No.9. Metabolism of [^{3}H]GA$_9$ seems to be similar in these two cultivars. It was not possible to draw conclusions about the identities of products formed from cv. Alaska, as extensive breakdown occurred in the extract analysed.

3.8 $[^3H]GA_{20}$ METABOLISM

A time-course feed of $[^3H]GA_{20}$ was made in order to compare metabolism in the cultivars Alaska, Meteor and Progress No.9. Prior to the experiment the radiochemical purity of the $[^3H]GA_{20}$ was confirmed by isocratic reverse phase HPLC at 50% MeOH (R_t $[^3H]GA_{20}$: 8.7 min). (Figure 30). $[^3H]GA_{20}$ was applied to the apices of seven day old light-grown plants, of the cultivars Alaska, Meteor and Progress No.9, each plant receiving 930,000 dpm (80ng). Ten plants of each cultivar were extracted 1, 8 and 27h after treatment.

The apex of each plant was washed with 100% MeOH prior to extraction. The shoots were then excised at the cotyledonary node and extracted using the same method as described for the second $[^3H]GA_9$ time course (section 3.7.3.). The distribution of radioactivity is shown in Table 32. It is apparent that the amount of radioactivity recovered from all three cultivars decreased with time, suggesting that metabolism was occurring.

TABLE 32. $[^3H]GA_{20}$ Feed: Recovery of applied radioactivity. Figures are percentages of the applied radioactivity calculated from dpm.

	cv. Alaska			cv. Meteor			cv. Progress No.9		
	1h	8h	27h	1h	8h	27h	1h	8h	27h
Fraction: 100% MeOH rinse	48%	32%	21%	39%	19%	10%	45%	17%	14%
Toluene	0%	0%	0%	0%	0%	0%	0%	0%	0%
Aqueous	0%	1%	2%	0%	1%	3%	0%	1%	4%
EtOAc	47%	62%	63%	59%	62%	64%	46%	50%	57%
Total Recovered	95%	96%	86%	98%	82%	77%	91%	68%	75%

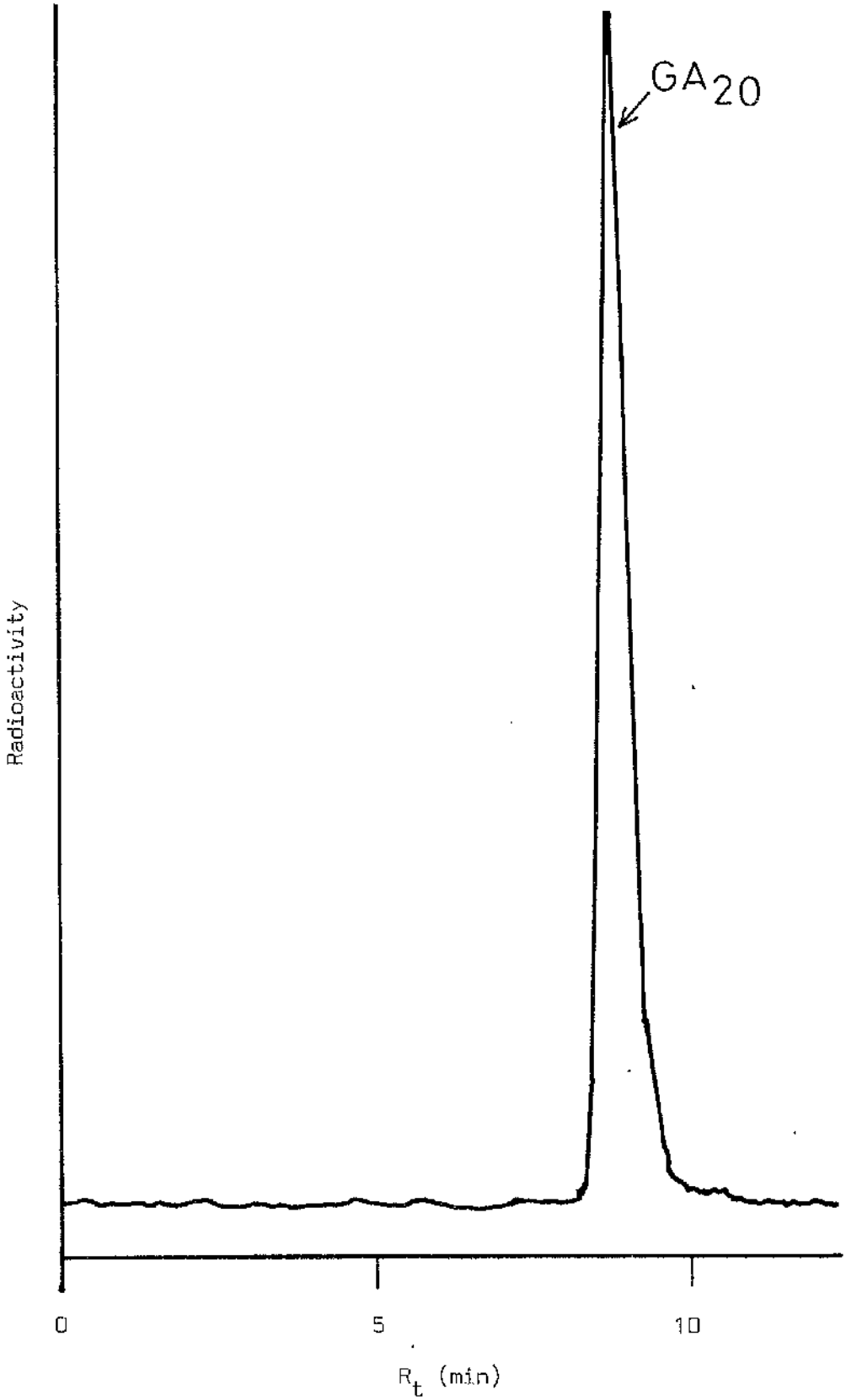


FIGURE 30. Reverse phase HPLC analysis of [^{3}H]GA_{20} immediately before application to plants. Mobile phase: 50% MeOH; isocratic; 1cm^3 min^{-1}. Detector: Radioactivity monitor, 100 cps f.s.d., 10s time constant. (No further radioactive compounds eluted during a short gradient to 100% MeOH).

Reverse phase HPLC analyses of 2% aliquots of the acidic EtOAc fractions revealed that GA_{20} was the major compound present. Analysis of the control extract, obtained after adding $[^3H]GA_{20}$ to a methanolic tissue homogenate, showed that it contained similar products to those observed in the time course extracts, with the exception of peak 2 which was not detected in the control extract. The amounts of peak 2 detected are shown in Table 33. Injection of larger aliquots of the 27h extracts of cv. Alaska, cv. Meteor, cv. Progress No.9 and of the control extract confirmed the initial analyses (figure 31). Peak sizes were quantified from peak height, as described in section 3.7.3.

TABLE 33. $[^3H]GA_{20}$ Feeds: Amount of Peak 2. Figures represent percentages of applied dpm. (limit of detection ca. 2% of applied label).

	1h	8h	27h
Alaska	-	-	4%
Meteor	-	4%	6%
Progress No.9	-	3%	6%

The identity of the putative metabolite, peak 2, was not established. Peak 2 from all three cultivars co-chromatographed on co-injection. GA_1 and GA_8 were shown to be absent by co-chromatography with the 27h extracts. GA_{29} and GA_{29} catabolite would also appear to be absent, based on the retention times

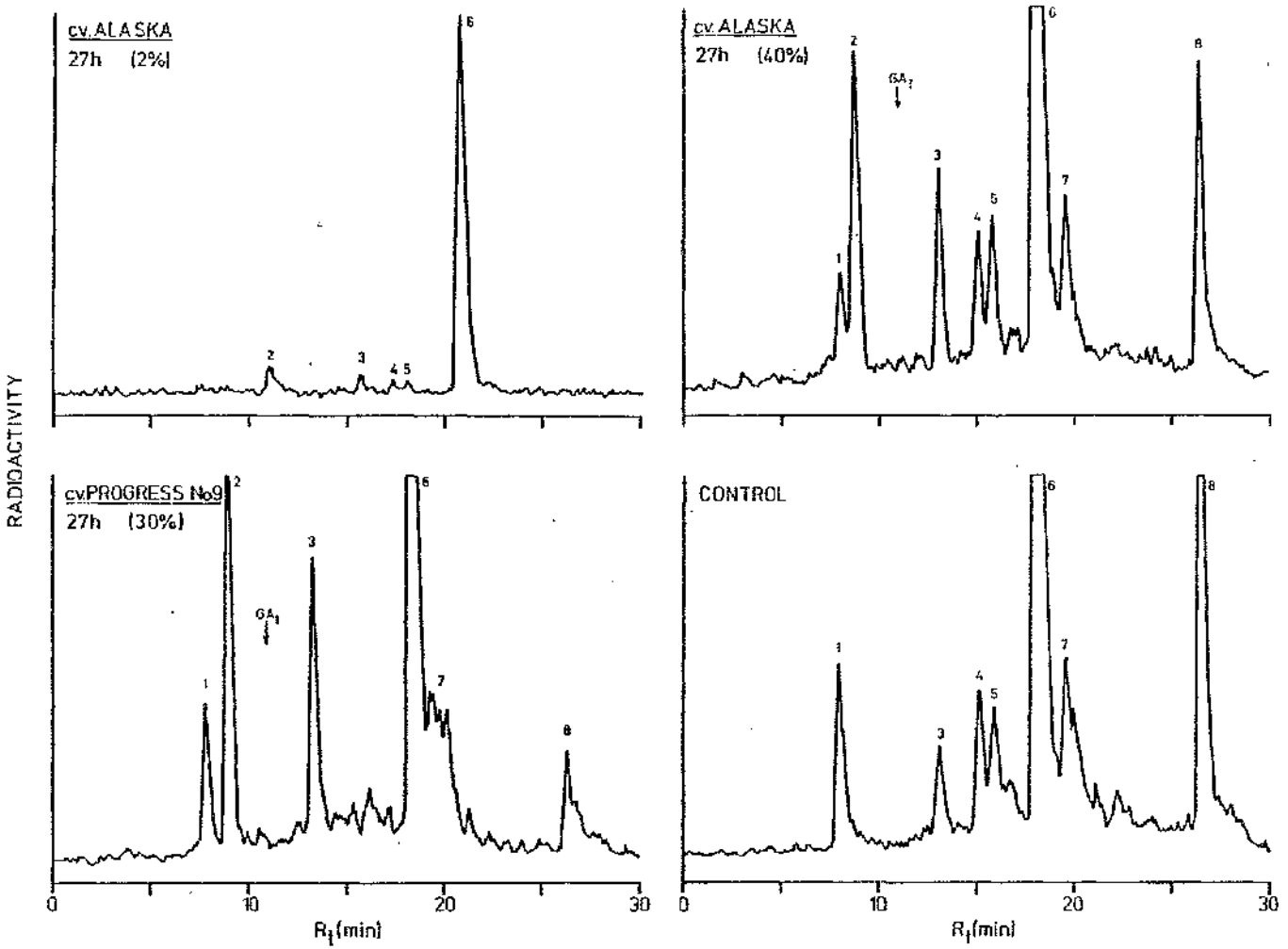


FIGURE 31. Reverse phase HPLC analyses of products of [^{3}H]GA_{20}. Traces shown are typical of those obtained. Percentages represent the proportions of the acidic EtOAc fractions injected. The elution position of GA_7 after co-chromatography is indicated. Mobile phase: 25-60% MeOH, 0-20 min; 60-100% MeOH, 21.5-23.5 min; 1cm^3 min^{-1}. Detector: Radioactivity monitor, 30 cps f.s.d., 10s time constant.

presented in section 3.2. The detection limit of the analyses carried out was ca. 2% of the applied radioactivity for the 1h and 8h extracts, and ca. 0.2% for the 27h extracts.

The identities of the breakdown products were not established. Rood et al. (1982) observed several breakdown products of [^{3}H]GA_{20}. One of these was less polar than GA_{20} on reverse phase HPLC analysis and was identified as C/D ring-re-arranged GA_{20}. It is possible that peak 9 is C/D ring-re-arranged GA_{20}, which was also observed as a product of [^{3}H]GA_{20} by Durley et al. (1975).

DISCUSSION

4.1.1. [^{3}H]GA FEEDS: INTRODUCTION

It is difficult to determine to what extent the products of the feeds of labelled GAs reflect the normal pattern of metabolism of the endogenous GAs. Frydman and MacMillan (1975) suggested the following criteria for conducting feeds to help ensure that the metabolism of the applied substrate reflects the metabolism of the native GA; (i) the exogenously applied GA and its metabolites are endogenous compounds, (ii) the GA is applied at a level calculated to be equivalent to the endogenous level, (iii) the GA is fed when its endogenous level is maximal, and (iv) the incubation period corresponds to the time interval between the maximal endogenous levels of the substrate and the product. In practice these criteria are somewhat difficult to fulfill for seedlings since: a) few accurate estimates of the distribution and size of the endogenous GA pools in seedling tissues are available, and b) the penetration and distribution of the applied label are difficult to control and will tend to break sub-cellular compartmentation.

Once it has been concluded that a conversion probably represents an endogenous step it is still necessary to consider the limitations of the data. The amount of product detected represents a pool size, and generally provides no indication of the rate of turnover. Rates of turnover are very difficult to estimate (see discussion of Bown et al., 1975). The amount of product that accumulates is usually expressed as a percentage of the label fed, which gives no indication of the actual mass

of product formed. There are therefore a number of limitations that should be borne in mind when attempting to interpret the conversion of labelled exogenous GAs in terms of endogenous GA metabolism.

4.1.2. [^{3}H]GA_{12} ALDEHYDE FEEDS

Studies with cell-free systems have shown that GA_{12} aldehyde is an intermediate in the _in vitro_ formation of GAs in developing pea seeds (Ropers _et al._, 1978; Kamiya and Graebe, 1983). GA_{12} aldehyde has not, however, been detected as an endogenous constituent of _Pisum_. In the present study [^{3}H]GA_{12} aldehyde was found to breakdown to some extent during storage and during extraction (figure 19). Other workers have reported auto-oxidation of GA_{12} aldehyde to GA_{12} and other products (Graebe _et al._, 1972; Evans and Hanson, 1975), but this does not always seem to be a problem (e.g. Graebe _et al._, 1974). Nash (1976) reported feeds of [^{3}H]GA_{12} aldehyde to _Phaseolus coccineus_ seedlings. Low levels of a range of more polar products were reported in the acidic EtOAc fractions, but only [^{3}H]GA_{12} was observed in addition to [^{3}H]GA_{12} aldehyde in the control extraction.

In the present study small amounts of a range of radioactive products more polar than GA_{12} aldehyde were observed in the acidic EtOAc fraction,but it was not possible to distinguish metabolites from the breakdown products seen in the control extractions. The low levels of metabolic products observed may be due to; i) poor penetration of the substrate to the

active site, ii) rapid further metabolism of products or iii) a limited capacity to metabolise [^{3}H]GA_{12} aldehyde. Metabolism leading to immediate loss of label does not seem likely since the ^{3}H label was attached to carbon-17 and metabolic conversionsinvolving changes at the C-17 position on the GA skeleton appear to be rare.

Further studies are required in order to clarify GA_{12} aldehyde metabolism in pea seedlings. If breakdown and/or the lack of accumulation of metabolites continued to be a problem then GA_{12} or GA_{53} could possibly be used as alternative substrates. A further possibility in view of the results of Kamiya and Graebe (1983) would be to develop a cell-free system from seedlings to investigate C_{20}-GA metabolism, although this is likely to be difficult.

4.1.3. [^{3}H]GA_{14} FEEDS

In the present study 14 metabolites of [^{3}H]GA_{14} were detected, one of which was identified as [^{3}H]GA_{18}. The other products were not identified, although HPLC and GC-MS analysis showed some of them to be distinct from a number of GAs (see section 3.5.3.), including those identified by Durley *et al.* (1974a,b) as products of [^{3}H]GA_{14} metabolism. These authors have reported the only previous feeds of a C_{20}-GA to pea seedlings. They observed conversion of [^{3}H]GA_{14}, by young etiolated seedlings, cv. Meteor, to labelled GA_1, GA_8, GA_{18}, GA_{23}, GA_{28}, GA_{38} and GA_{42}. No other radioactive products were detected.

The conversion of GA_{14} to GA_{18} involves the 13-hydroxylation of a C-20-methyl GA. 13-Hydroxylation of C_{20}-GAs by a cell-free system from Pisum seeds was reported by Kamiya and Graebe (1983). GA_{12} and GA_{12} aldehyde seemed to be the preferred substrates. Although GA_{14} has not been detected as an endogenous constituent of peas, the above studies, and the isolation of 13-hydroxylated GAs from peas, are consistent with the occurrence of an early 13-hydroxylation pathway of GA biosynthesis in seeds and seedlings of Pisum.

4.1.4. $[^3H]GA_{20}$ FEEDS

GA_{20} is an endogenous component of pea shoots (Kirkwood, 1979; Davies et al., 1982; Ingram et al., 1983,1984). Ca. 80ng of $[^3H]GA_{20}$ was applied to each seedling in the present study. This is similar to the endogenous amounts of GA_{20} determined in the apical regions of shoots by Ingram et al. (1984), although much higher than the radioimmunoassay estimates reported in the present study (figure 12). It is possible that this apparent discrepancy arises because young seedling shoots contain smaller amounts of GA_{20} than the apical regions of older shoots.

In the present study only one of the products formed from the $[^3H]GA_{20}$ was of possible metabolic origin. This product was present in tall and dwarf cultivars in very low amounts. It was not identified, but co-chromatography showed it to be distinct from GA_1 and GA_8. Consideration of HPLC retention times also shows that it is unlikely to be either GA_{29} or GA_{29} catabolite.

Several possible reasons can be suggested to explain the low amount of product detected: i) the penetration of the [^{3}H]GA_{20} to the active site was poor; ii) the shoots had only a low capacity to metabolise [^{3}H]GA_{20}; iii) the products were rapidly further metabolised; and/or iv) the label was immediately lost when the [^{3}H]GA_{20} was metabolised. In all three cultivars the recovery of the applied label decreased as the duration of the feed increased, suggesting that metabolism was occurring. The low amount of product detected is probably the result of rapid turnover, since only limited loss of label would be expected following conversion to GA_1, GA_8, GA_{29} or GA_{29} catabolite. These were the only products which Ingram et al. (1984) detected as metabolites from labelled GA_{20} which had been fed to several lines of pea seedlings. The lines used had different stem length genotypes, and the authors compared metabolism of the applied label in Le and le plants.

Ingram et al. (1984) and Railton et al. (1974c) reported small amounts of GA_{29} formed by pea seedlings from labelled GA_{20}. Ingram et al. (1984) also observed small amounts of GA_1, GA_8 and GA_{29} catabolite as metabolites of GA_{20}, and suggested that a low conversion of GA_{20} to GA_1 was limiting growth in peas dwarfed as a result of the homozygous presence of the le gene. The low amounts of metabolites detected from [^{3}H]GA_{20} fed to seedlings contrast with the results of feeds to seeds, where much larger amounts of products have been detected. (Railton et al. 1974c; Sponsel and MacMillan, 1975).

Despite an extensive search, in which [^{3}H] pool sizes as low as 0.2% of the applied substrate would have been detected, [^{3}H]GA_1 was not observed as a metabolite of [^{3}H]GA_{20} in tall or dwarf seedlings. This contrasts with the results of Ingram et al. (1984), who observed conversion of exogenous GA_{20} to GA_1 and GA_8 in tall seedlings, although only very low levels of these products were detected. For example, the amount of GA_1 that was detected was always less than 0.3% of the applied GA_{20}. Ingram et al. (1984) observed dilution of the applied ^{13}C label in the GA_1 pool detected, and considered this to indicate that the observed conversion of GA_{20} to GA_1 reflected the normal pathway of synthesis of endogenous GA_1. However, this conclusion is open to doubt as similar dilution would also have occurred if the endogenous GA_1 was being formed from a precursor other than GA_{20}. The conversion of GA_{20} to GA_1 involves 3β-hydroxylation. The only other report of 3β-hydroxylation in peas is that of Durley et al. (1973) who observed low incorporation of [^{3}H]GA_5 into [^{3}H]GA_3 in the dwarf cultivar Meteor.

4.1.5. [^{3}H]GA_9 FEEDS

GA_9 is not known to be an endogenous constituent of pea seedlings, although it has been observed in extracts from developing seed (Frydman et al., 1974). There is bioassay evidence for the occurrence of GA_9 in shoots (Railton and Reid, 1974a; Proebsting et al., 1978) although the radioimmunoassay analysis, reported in section 2.4, failed to detect GA_9 in

extracts of shoots or seedlings of cultivars Alaska, Meteor and Progress No.9 (limit of detection ca. 100-500pg seedling $^{-1}$).

The metabolites of GA_9 observed in the present investigation were much more numerous than detected in previous investigations using peas (Frydman and MacMillan, 1975; Sponsel and MacMillan, 1977; Proebsting et al., 1978; Proebsting and Heftman, 1980; Railton et al., 1974a,b; Railton, 1974; Railton and Reid, 1974b). Breakdown products were observed in the control extract, but these were not thought to be making a significant contribution to the radiolabelled compounds observed in the time course extracts. $[^3H]GA_{10}$, identified as a possible non-metabolic product by Railton et al. (1974a,b) was not detected.

The metabolism of $[^3H]GA_9$ seemed to be identical in two dwarf cultivars (cv. Meteor and cv. Progress No.9), and similar in the tall cultivar Alaska, although differences between the products observed from tall and from dwarf cultivars cannot be ruled out. $[^3H]GA_9$ metabolism was also similar in light- and dark-grown plants of the cv. Alaska, although specific differences between products could also exist here.

The only metabolic products arising from $[^3H]GA_9$ that were identified in the present study were $[^3H]GA_{20}$ and possibly $[^3H]GA_{29}$, although it was possible to exclude the presence of a number of other GAs (Table 31). Kamiya and Graebe (1983) have suggested that the conversion of exogenous GA_9 to GA_{20} in peas is an artifact and does not represent the pathway for the formation of endogenous GA_{20}. GA_{51}, identified as a metabolite

of GA_9 in pea seed by Frydman and MacMillan (1975) and by Sponsel and MacMillan (1977) was not detected. The presence of [^{3}H]-12α-hydroxy GA_9, also previously reported as a product of exogenous GA_9 (Railton et al., 1974a,b; Frydman and MacMillan, 1975; Sponsel and MacMillan, 1977), could not be excluded as no standard was available for co-chromatography.

In the present study there was a rapid accumulation of polar products, indicated by an increase in the amount of radioactivity associated with the residual aqueous phase (Table 22). Frydman and MacMillan (1975) and Sponsel and MacMillan (1977) detected quite large amounts of conjugated products. In the present study no thorough analysis of conjugated products was carried out, but analysis by SEC indicated that acidic conjugates did not account for a significant proportion of the radioactivity in the acidic EtOAc fraction, at least of cv. Alaska.

Dilution of the labelled substrate with unlabelled GA_9 in the present study did not significantly affect the metabolite profile, although the amount of substrate applied varied over three orders of magnitude. This suggests that the tissue had a high capacity to metabolise exogenous GA_9. Frydman and MacMillan (1975) found that exogenous GA_9 was efficiently metabolised in developing seed.

In the present study a much larger number of products from [^{3}H]GA_9 were detected than reported in previous investigations of Pisum. This is possibly a consequence of the use of HPLC. Re-evaluation of the results of some of the earlier

experiments is required in view of the present results, as tentative identifications of Pisum GA_9 metabolites based on TLC data (Railton, 1974; Railton and Reid, 1974b) are clearly misleading. The silica gel partition column used by Proebsting et al. (1978), and by Proebsting and Heftman (1980), to analyse [^{3}H]GA_9 metabolites from the line G2, gave better resolution than TLC but the present results indicate that the resolution was inadequate to distinguish the range of potential products.

4.2. ANALYSIS OF THE ENDOGENOUS GAs OF PISUM

The identification of GA_{20} and GA_{29} as endogenous GAs of cv. Alaska seedlings is consistent with the results of previous studies of the endogenous GAs of peas, in which both these GAs have been detected in extracts of seeds and of vegetative tissues (Table 2). Bioassay and immunoassay evidence also indicated the presence of a GA_1-like component in cv. Alaska (see figure 9). However, GC-MS analysis failed to detect GA_1 and it is suggested that a novel GA may be responsible for the observed activity.

If a novel GA is responsible for the activity of fraction 42 in figure 9 then a further important question is whether this compound is biologically active in the pea. Fraction 42 was not tested in the dwarf pea bioassay. However, it is considered likely that the compound responsible for the assay activity seen would also be biologically active in peas. This is because:

1. From cross reactivity studies most GAs that are active in both the barley aleurone and lettuce hypocotyl bioassays are also active in the dwarf pea bioassay (see Reeve and Crozier, 1975).

2. In previous experiments where a GA_1-like biologically active component has been isolated from young seedlings (e.g. Kende and Lang, 1964) the compound has been biologically active when applied to dwarf peas.

The detection of a GA_1-like compound is consistent with the results of earlier workers, who have reported detecting a GA_1-like component from seeds (Komoda et al., 1968; Potts and Reid, 1983; Ingram and Browning, 1979), young seedlings (e.g. Kende and Lang, 1964; Jones and Lang, 1968; Jones, 1968), and young apical tissue (Proebsting et al., 1978; Potts et al., 1982a) of peas. The identity of the GA(s) causing this biological activity has been the subject of considerable speculation and discussion as, despite extensive investigations, GA_1 had not until recently (Ingram et al., 1984) been identified in extracts from peas. In spite of this recent identification there is some evidence, besides the present work, against GA_1 being entirely responsible for the GA_1-like bioassay activity observed:

1. GA_1 has not been identified from the GA_1-like peak of bioactivity (designated GA_E) which has been obtained consistently when extracts of seeds and of apical tissues of the line G2 have been bioassayed in the lettuce hypocotyl bioassay (Proebsting et al., 1978; Ingram and Browning, 1979; Davies et al., 1982).

It has been suggested that GA_E is GA_{19} (Ingram and Browning, 1979; Davies et al., 1982). However, GA_{19} has low activity in the lettuce hypocotyl bioassay (Crozier et al., 1970) and it was necessary to postulate either the presence of synergists or varietal differences in the lettuce cultivars to account for the biological activity seen (Davies et al., 1982).

2. GA_1 has not been identified in seeds of cv. Progress No.9, despite thorough investigations (see Sponsel, 1980a). Bioassays were not employed in these investigations, so the presence of GA_1-like biological activity in seeds of cv. Progress No.9 is speculative, although such a component has been detected in seeds of other cultivars (Reinhard and Konopka, 1967; Potts and Reid, 1983) and in young seedlings of cv. Progress No.9 (section 2.4, and also Kende and Lang, 1964; Kende, 1967; Jones and Lang, 1968; Jones, 1968). GA_1 has not been found in young seedlings of cv. Progress No.9 (Kirkwood, 1979).

3. Reinhard and Konopka (1967) isolated GA_1-like and GA_5-like components from peas, but the GA_1-like biological activity was separable from authentic GA_1 by TLC. The unknown GA, designated GA_x, had lower activity in the dwarf pea and d_1 maize bioassays than in the d_5 maize bioassay.

Ingram et al. (1984) concluded that GA_1 is the major GA controlling stem elongation in peas. The present work provided no evidence to support this hypothesis since GA_1 was not detected in extracts from tall seedlings either as an endogenous constituent or as a metabolite of $[^3H]GA_{20}$. If the suggested novel GA is able

to produce marked stem elongation in le dwarfs then re-examination of the hypothesis of Ingram et al. (1984) would be necessary.

To conclude that GA_1 is the only GA controlling stem elongation in a species requires the demonstration that this is the only endogenous GA present in normal plants that is able to promote marked stem elongation in mutants dwarfed as a result of a block in the GA biosynthetic pathway immediately prior to the formation of GA_1. In peas this involves applying the GA to le dwarfs, which appear incapable of converting GA_{20} to GA_1 (Ingram et al., 1984). In the absence of a complete knowledge of the GAs that are endogenous constituents of peas it is necessary to demonstrate that only one peak of biological activity, with a retention time identical to GA_1, is observed when extracts of tall peas are chromatographed and the fractions bioassayed using the le mutant. The chromatographic system employed must be able to separate GA_1 from other biologically active GAs. In practice it would be necessary to purify the extract using several successive high resolution chromatographic steps of different selectivity, demonstrating that after each only a single peak of GA_1-like biological activity was present.

In the present study radioimmunoassay analysis showed shoots of both tall and dwarf seedlings to contain similar levels of a GA_1-like compound, possibly the putative/novel GA. If GA_1 were the causative compound the results could still be consistent with the hypothesis that dwarfism in le plants (i.e. cv. Meteor and cv. Progress No.9) is due to the production of inadequate amounts

of GA_1 (Ingram et al., 1984). This is because morphological measurements suggest that dwarfism due to the le gene may not be expressed in young seedlings, such as those used to provide tissue for the radioimmunoassay analysis, to the same degree as in older seedlings (Reid et al., 1983).

Radioimmunoassay analysis also suggested that light-grown shoots contained higher levels of GA_1-like immunoreactive GAs than dark-grown shoots. Kohler (1970) detected higher levels of biologically active GAs in extracts of light-grown peas than in extracts of dark-grown ones. These results do not necessarily represent good evidence against the involvement of GAs in the light inhibition of stem growth since the pool sizes determined may not reflect the actual amount of endogenous GA available to stimulate growth.

The results obtained contrast with those of Bown et al. (1975) who found higher levels of GA-like biological activity in dark-grown seedlings of Phaseolus coccineus compared to light-grown seedlings. It is difficult to draw conclusions about the involvement of a growth regulator in a physiological process from estimates of pool sizes, unless it is known that the pool size monitored is responsible for determining growth. Changes in rates of GA turnover do not necessarily cause changes in the pool sizes observed. Zeevaart (1971) provided evidence that LD greatly increased GA turnover in Spinacea oleracea, although this was not obvious from bioassay comparisons of the endogenous GA pools of LD and SD plants.

In Pisum seedlings it is possible that GAs accumulate in light-grown tissue because conversion to the active GA is reduced. This would suggest that the point of control by light is late in the biosynthetic pathway. Some evidence to support this suggestion was provided by Kende and Lang (1964). These authors extracted GA_1-like and GA_5-like components from peas and bioassayed them on light and dark grown peas. The GA_1-like component was equally active when applied to either light- or AMO-1618 dwarfed dark-grown peas, but the GA_5-like component appeared less active when applied to light-grown peas than when applied to AMO-1618 dwarfed/dark-grown peas. GA_1 and GA_5 standards showed similar behaviour.

4.3. CONCLUSIONS

The major conclusions of the work presented in this thesis are therefore:

1. There appears to be a novel, possibly biologically active, GA_1-like compound in seedlings of cv. Alaska, a tall pea.

2. Light grown shoots of pea seedlings have higher levels of GA_1-like immunoreactive GAs than dark-grown seedlings, but this observation does not rule out the possibility that GAs mediate the light-induced inhibition of stem growth.

3. Pea seedlings are able to 13-hydroxylate $[^3H]GA_{14}$, a C-20-methyl GA and $[^3H]GA_9$, a C_{19}-GA.

4. Exogenous GA_9 is rapidly metabolised by pea seedlings

to a large number of products. The pattern of metabolism is identical in the dwarf cultivars Meteor and Progress No.9, and similar in the tall cultivar Alaska, although differences in specific products may exist between this cultivar and the two dwarf cultivars. Metabolism of [^{3}H]GA_9 appears similar, but not necessarily identical, in light- and dark-grown seedlings of cv. Alaska.

5. The results provided no evidence in support of the hypothesis that GA_1 is the only active GA controlling stem elongation in peas, since GA_1 was not detected in extracts from tall seedlings either as an endogenous constituent or as a metabolite of [^{3}H]GA_{20}.

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