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TRANSLOCATION AND METABOLISM OF
GIBBERELLINS IN SEEDLINGS OF
PHASEOLUS COCCINEUS L.

A thesis submitted to the University of Glasgow for
the degree of Doctor of Philosophy

by

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September 1976

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ABBREVIATIONS

AMO 1618	2'-isopropyl-4'-trimethylammoniumchloride -
*	5'-methylphenylpiperidine-1-carboxylate
BE	benzyl ester
CCC	(2-chloroethyl)trimethylammonium chloride
Ci	curie
cpm	counts per minute
dpm	disintegrations per minute
eV	electron volt
FSD	full scale deflection
GA	gibberellin
GC-MS	gas chromatography - mass spectrometry
GLC	gas-liquid chromatography
GLRC	gas-liquid radiochromatography
GPC	gel permeation chromatography
HMW	high molecular weight
HPLC	high-performance liquid chromatography
IAA	indole-3-acetic acid
k'	capacity factor, HPLC
k _d	liquid-liquid partition coefficient
LMW	low molecular weight
MS	mass spectrometry
mol	gram-molecule
Pa	pascal
POPOP	2, -(5-phenyloxazolyl)benzene
PPO	2,5-diphenyloxazole
PVP	polyvinylpyrrolidone
R _f	relative retention , TLC
RT	retention time
THF	tetrahydrofuran

*
amu atomic mass unit iv.

TIBA	2,3,5-triiodobenzoic acid
TLC	thin-layer chromatography
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
UV	ultraviolet
W	watt

SUMMARY

The investigations reported here concern the translocation and metabolic relationships of gibberellins (GAs) in light-grown seedlings of Phaseolus coccineus L., in which GA₁, GA₄, GA₅ and GA₂₀ are known to be endogenous components (Bowen et al., 1973). These four GAs were equally effective in promoting subapical elongation of shoot explants when applied exogenously to the apical bud or stem base. The apical buds or stem bases of intact seedlings were treated with [³H] GA₁, GA₄, GA₅, GA₈, GA₉, GA₁₂ aldehyde or GA₁₄. The redistribution of radioactivity from apically applied GAs differed considerably from that observed after applications to the stem base. Apically applied GAs were retained by the apical bud and underwent relatively little metabolism in a 24h period, whereas basal application resulted in extensive redistribution of the [³H] GAs and of a high proportion of chromatographically distinct radioactive metabolites. No evidence was obtained for the export of [³H] GAs from cotyledons to the remainder of the developing seedling.

The identity of the conversion products of applied [³H] GAs was further investigated by analytical HPLC of extracts previously extensively purified by gel-permeation and charcoal adsorption chromatography and preparative-scale HPLC. Analysis of extracts by analytical HPLC permitted the identification of a number of metabolites by subsequent mass spectrometry. The apparent rates of conversion of [³H] GAs differed considerably, the most rapid disappearance being observed after feeding [³H] GA₈, whereas recoveries of [³H] GA₅ were high relative to other GAs. Each applied [³H] GA was found to give rise to a characteristic array of products, and there was thus no evidence for the accumulation of a single terminal metabolite. Mass spectrometric evidence demonstrated

conclusively that $[^3\text{H}]\text{GA}_4$ was converted to $[^3\text{H}]\text{GA}_1$, and $[^3\text{H}]\text{GA}_9$ to $[^3\text{H}]\text{GA}_{20}$, in high yields. $[^3\text{H}]\text{GA}_1$ gave rise to small amounts of a compound with chromatographic properties identical to those of GA_8 , but there was no significant accumulation of further metabolites of $[^3\text{H}]\text{GA}_{20}$. There was no observable accumulation of free GA-like metabolites after $[^3\text{H}]\text{GA}_{14}$ treatment, but complex spectra of metabolites were obtained after $[^3\text{H}]\text{GA}_{12}$ aldehyde feeds.

All of the applied GAs and each of their free GA-like metabolites were converted to acidic butanol-soluble conjugates which probably represented glucosyl ethers. In the case of $[^3\text{H}]\text{GA}_1$, $[^3\text{H}]\text{GA}_5$, and $[^3\text{H}]\text{GA}_{20}$ treatments, these conjugates represented the major metabolites recovered. In addition, $[^3\text{H}]\text{GA}_4$, $[^3\text{H}]\text{GA}_{12}$ aldehyde and $[^3\text{H}]\text{GA}_{14}$ feeds provided significant quantities of presumptive glucosyl esters of the applied GAs and their acidic ethyl acetate-soluble metabolites.

GA metabolism in P. coccineus seedlings was found to differ both quantitatively and qualitatively from that reported for Pisum sativum (Railton et al., 1974; Frydman and MacMillan, 1975), Gibberella fujikuroi (MacMillan, 1974b) and Cucurbita maxima endosperm preparations (Graebe and Hedden, 1974). However, some resemblances to the conversions of applied $[^3\text{H}]\text{GAs}$ by developing P. vulgaris seeds (Yamane et al., 1975) were noted. In P. coccineus seedlings, both biosynthetic and catabolic mechanisms appear to be active, with the result that applied GAs are readily metabolised, but the accumulation of products is less marked than in either Gibberella fujikuroi or developing seeds.

SECTION I

I N T R O D U C T I O N

INTRODUCTION

The gibberellins (GAs) are diterpenoid acids which were originally identified as secondary metabolites of the fungus Gibberella fujikuroi (Saw.) Wr. by virtue of their ability to induce pathological overgrowth in host rice seedlings (see Stowe and Yamaki, 1957). Gibberellins have subsequently been found to occur in a number of higher plant species, and a considerable body of evidence indicating that they are endogenous determinants of plant growth has been reviewed by Paleg (1965), Brian (1966) and Jones (1973). This evidence includes the ability of applied GAs to imitate environmental stimuli in evoking characteristic physiological responses, the correlation of reduced internode elongation with GA deficiency, and the variation in levels of GA-like activity with environmental conditions.

Investigations into the mode of GA action and its relationship to environmental factors have however been impeded by the related problems of isolation and identification. A large number of GAs has now been isolated and chemically characterised (Fig.1). Some of these occur in both G. fujikuroi and higher plants, while the presence of others appears to be restricted to either the fungus or higher-plant tissues. The quantities of GAs associated with higher-plant tissues, while small in comparison with those synthesised by G. fujikuroi cultures, can nevertheless readily be detected by bioassays (see Bailiss and Hill, 1971; Reeve and Crozier, 1975). However, considerable underestimation of GA levels in plant extracts often results from the distortion of the bioassay response by the many impurities present. Furthermore, characterisation by physico-chemical methods is necessary if the GA-like activity detected by bioassay is to be attributed to an identifiable GA. In order to obtain a GA-like

fraction of sufficient size and purity for this type of analysis, large quantities of plant material must be extracted and rigorously purified.

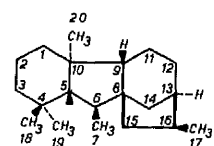
As a result of the formidable technical problems associated with GA isolation, there have been few definitive characterisations of GAs from vegetative tissue (Kawarada and Sumiki, 1959; Murofushi et al., 1966; Harada and Nitsch, 1967; Harada and Yokota, 1970; Bowen et al., 1973; Gaskin et al., 1973). Immature seed, in contrast, is considerably richer in GAs, and characterisations of seed GAs have been more numerous (see Lang, 1970; Nitsch, 1970; Takahashi, 1974).

Analytical procedures

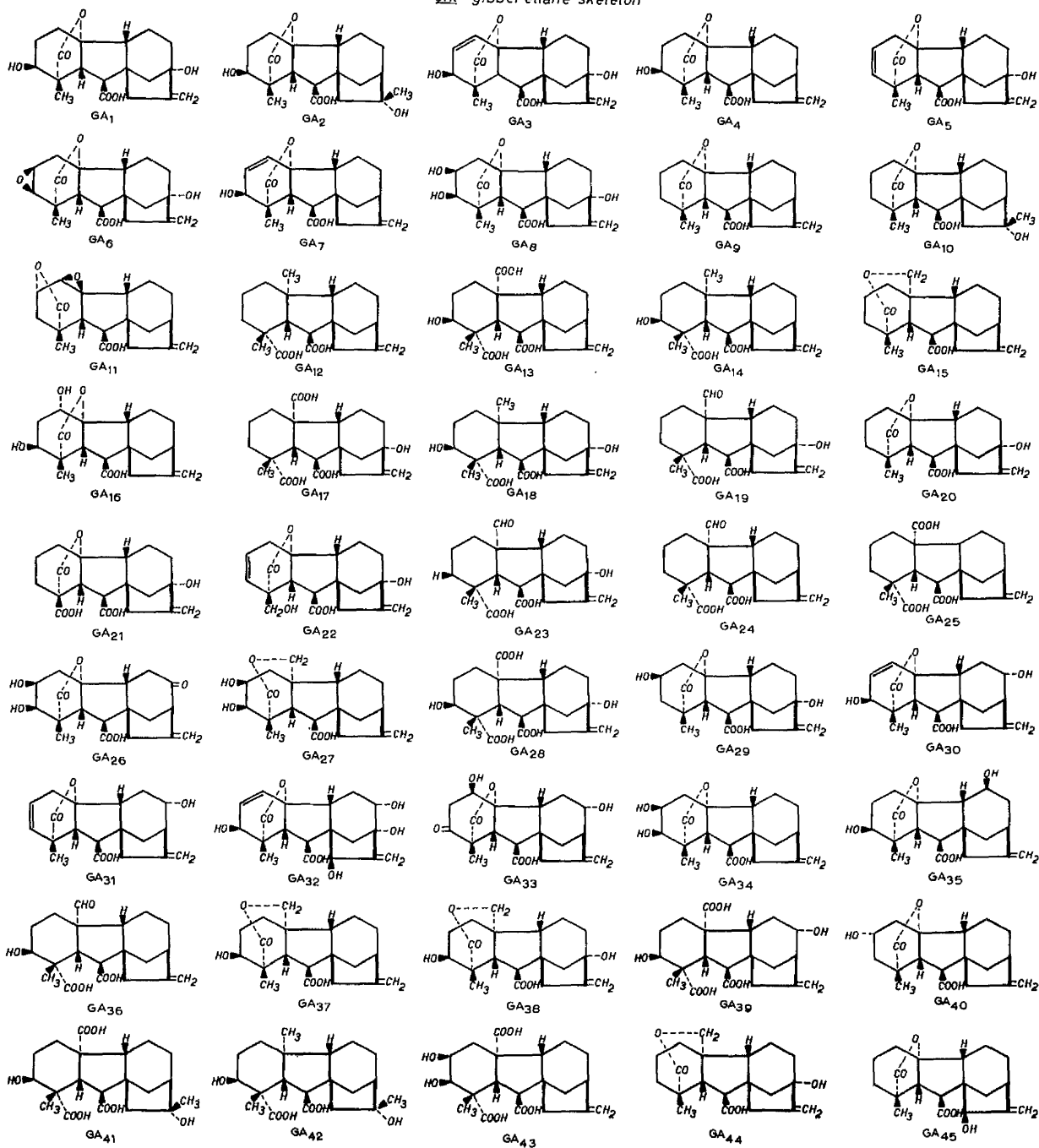
Procedures for the extraction and identification of GAs have recently been reviewed (Russell, 1975). Solvent fractionation of extracts provides an initial separation of free acidic GAs and their glucosyl derivatives, and each fraction is generally subjected to a number of subsequent purification procedures. The first of these steps is intended to separate GAs as a group from extraneous, non-GA-like material. Group separation has been attempted by partition and adsorption chromatography on various supports, including charcoal-celite mixtures (West and Phinney, 1959), silicic acid (Murofushi et al., 1966), Sephadex G-50 (Tamura et al., 1968) and G-10 (Crozier et al., 1969), polyvinylpyrrolidone (Glenn et al., 1972), and polyamide (Railton and Wareing, 1973), while a novel molecular exclusion technique based on porous polystyrene has been described by Reeve and Crozier (1976). Use has also been made of counter-current distribution procedures (Murofushi et al., 1966; Crozier et al., 1969).

FIGURE 1.

Structure of the gibberellane skeleton and
gibberellins 1-45 (from Frydman et al., 1974;
Graebe et al., 1974b; Takahashi, 1974; Bearder
et al., 1975; Reeve and Crozier, 1975).



ent-gibberellane skeleton



The analytical separation of individual GAs following group separation requires a method of high resolving power, and in this respect thin-layer chromatography has been superseded by column chromatographic techniques which, in addition to possessing superior peak capacity, generally offer a large sample capacity and can therefore accommodate relatively impure extracts. The two most effective supports in current use are Sephadex LH-20 (MacMillan and Wells, 1973), and silicic acid with a formic acid stationary phase (Powell and Tautvydas, 1967). Analysis times associated with the LH-20 system, however, are inconveniently long, while the silicic acid partition system in its original form suffers from low chromatographic efficiency and irreproducibility of retention times. In spite of these disadvantages, under favourable circumstances these procedures have been reported to purify extracts to a level compatible with GC-MS (e.g. Bowen et al., 1973) for which a minimal concentration of 5% of GA-like material is required (MacMillan, 1972).

Subsequent modifications of the silicic acid partition column have established it as the basis of a high-performance liquid chromatograph combining large sample capacity and high resolving power (Reeve et al., 1976). This procedure has been found to purify individual components to a degree compatible with conventional analytical HPLC systems. Although the sample capacity of analytical HPLC is typically low, it does offer very high chromatographic efficiency and considerable flexibility in the choice of supports, mobile and stationary phases. Furthermore, the procedure is non-destructive, sample derivatisation is not obligatory and columns can be operated at ambient temperature (Done et al., 1972). For these reasons, HPLC clearly has considerable potential in plant growth-regulator analysis, but as its development has been relatively recent (see Knox, 1974), there are few examples of its application

to endogenous plant hormones (Pool and Powell, 1974; Dekhuijzen and Gevers, 1975; Sweetser and Vatvars, 1976). Further reference will be made in this thesis to the separation of GA metabolites by sequential preparative and analytical HPLC, a combination of techniques not previously used in GA analysis, but whose applicability to analytical problems of this nature has been confirmed by results obtained in the course of this study.

The use of GLC systems, in contrast, is widespread, and as a consequence of its high peak capacity, GLC has frequently been applied to the analysis of plant extracts, although its utility is severely restricted both by low sample capacity and a requirement for sample volatilisation. Furthermore, since the response of flame-ionisation detectors is non-specific, it is often impossible to identify peaks attributable to GAs against a background of extraneous material. The combination of GLC with radioactivity monitoring (GLRC) enables radioactivity maxima to be correlated with mass peaks and with the retention times of standards, and this technique has formed the basis of a large number of metabolic studies (e.g. Patterson and Rappaport, 1974; Durley et al., 1975; Reeve et al., 1975; Yamane et al., 1975). However, as in all chromatographic methods, the coincidence of retention times provides no more than circumstantial evidence of identity, and the most valuable application of GLC may lie in its combination with mass spectrometry.

Biosynthesis

In G. fujikuroi, GAs are synthesised in relatively large amounts, and isolation and chemical identification of biosynthetic intermediates can often be carried out by GC-MS without extensive prior purification. While the biosynthetic sequence in Gibberella is consequently well established (see MacMillan, 1971; MacMillan and

Pryce, 1973; West, 1973; MacMillan, 1974a,b), information on corresponding pathways in higher plants is considerably more fragmentary, and has been obtained almost entirely from cell-free enzyme preparations of immature seeds and fruits (see Barendse, 1975). Of these, only preparations of Cucurbita maxima seeds have been shown to carry out all the reactions necessary to convert the precursor, mevalonic acid, to a C₁₉-GA (Graebe et al., 1974a,b). Detailed studies on the biosynthesis from mevalonate of (-)-kaurene, the first tetracyclic intermediate, and the successive oxidation steps by which (-)-kaurene is converted to (-)-7 β -hydroxykaurenoic acid, have been carried out using cell-free preparations of Echinocystis macrocarpa endosperm (see West, 1973). There is some chromatographic evidence for the production of GA₁₂ aldehyde and GA₁₂ from (-)-kaurenoic acid in this system (West, 1973). The levels of identifiable GAs in E. macrocarpa preparations are low (MacMillan, 1972), which is perhaps indicative of a low biosynthetic capacity, and may in turn explain the failure to detect intermediates of the later stages of the GA biosynthetic pathway in this system. In contrast, in C. maxima systems it has been possible to demonstrate these reactions by suitable adjustment of reaction conditions (Graebe and Hedden, 1974).

The sequence of reactions leading to (-)-7 β -hydroxykaurenoic acid, and the production of GA₁₂ aldehyde by contraction of the B-ring, appear to be similar in preparations of G. fujikuroi, E. macrocarpa and C. maxima (MacMillan, 1971; West, 1973; MacMillan 1974a). GA₁₂ aldehyde has been established as the immediate precursor of C₂₀-GAs in both G. fujikuroi (Cross et al., 1968) and C. maxima (Graebe et al., 1974b). However, the sequence of subsequent reactions leading from C₂₀- to C₁₉-GAs, and the identities of many intermediates, differ considerably in the two systems (Fig.2).

The mechanism by which C₂₀-GAs give rise to C₁₉-GAs in higher plants is not well established. Durley et al. (1974) obtained [³H] GA₂₃ and [³H] GA₁ as metabolites of [³H] GA₁₄ in Pisum sativum seedlings, and proposed that GA₂₃ was converted directly to GA₁ by the enzymic equivalent of a Baeyer-Villiger oxidation similar to that apparently occurring in G. fujikuroi (Birch et al., 1959; Hanson and White, 1969). However, GA₁₄, GA₂₃ and GA₁ are not known to be endogenous to Pisum. The immediate endogenous precursors of the C₁₉-gibberellin, GA₄, produced from GA₁₂-aldehyde in the C. maxima system have not to date been identified (Graebe et al., 1974b)

Transformations of C₁₉-GAs

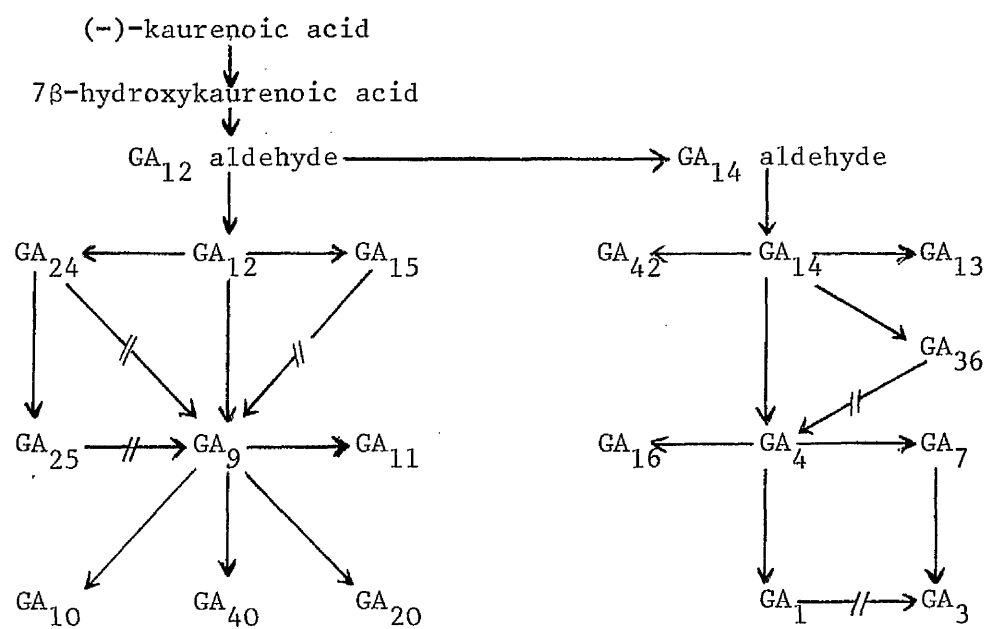
Vegetative and reproductive tissues of a number of higher-plant species have been shown to transform applied radioactive GAs (see Barendse, 1975). However, Frydman and MacMillan (1975) considered that experimental criteria providing information representative of natural transformations have in many cases not been met, and the physiological significance of many reported observations is consequently difficult to assess. In most instances, the products of applied C₁₉-GAs have been found to be either other C₁₉-GAs having a greater degree of hydroxylation than the substrate, or glucosyl derivatives of C₁₉-GAs.

Higher-plant systems appear to differ from G. fujikuroi in that hydroxylation at C-13 often occurs before the elimination of C-20, whereas in G. fujikuroi, 13-hydroxylation is the final step (MacMillan, 1974b; Takahashi, 1974). In keeping with this observation, [³H] GA₁₄ gives rise to [³H] GA₁₈ in pea seedlings (Durley et al., 1974). However, this may not be obligatory, as the non-hydroxylated C₁₉-gibberellin GA₁₉ is converted to its 13 α -hydroxy derivative, GA₂₀, in developing pea seeds (Frydman and MacMillan, 1975) and pea seedlings (Railton et al., 1974), and GA₄

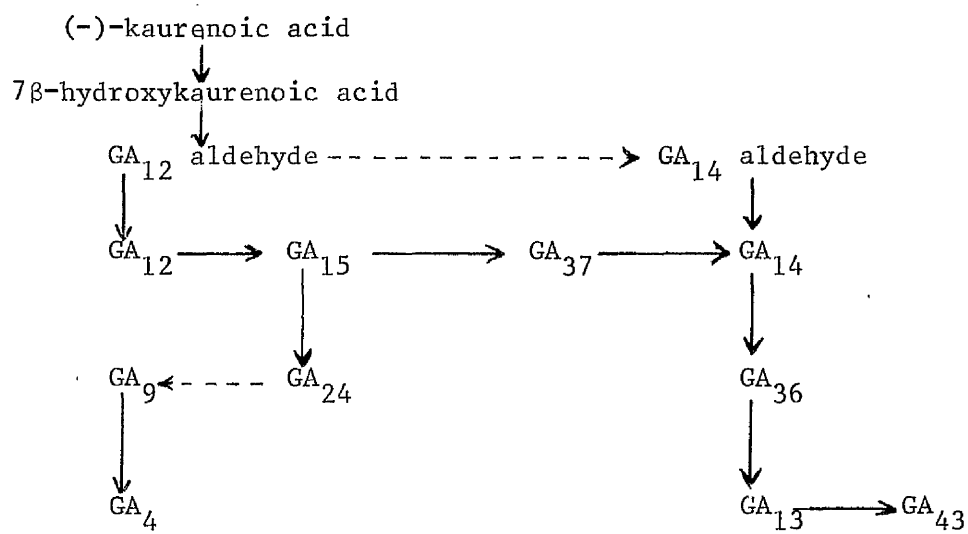
FIGURE 2

Gibberellin biosynthetic relationships in Gibberella fujikuroi mutant B1-41a. (MacMillan, 1974b) and Cucurbita maxima endosperm. (Graebe et al., 1972; 1974a,b; Graebe and Hedden, 1974).

1. Gibberella fujikuroi



2. Cucurbita maxima



is converted to GA₁ in Pinus radiata pollen (Kamienska et al., 1976), Phaseolus vulgaris seeds (Yamane et al., 1975) and dwarf rice seedlings (Durley and Pharis, 1973).

Subsequent 2 β -hydroxylation of GA₂₀ yields GA₂₉ in pea seeds (Frydman and MacMillan, 1975) and seedlings (Railton et al., 1974), and also in Bryophyllum leaves (Durley et al., 1975). The 2 β -hydroxylation of GA₁ to GA₈ has been reported in barley aleurone layers (Nadeau et al., 1972), dwarf rice (Railton et al., 1973), dwarf pea (Stoddart et al., 1974), dwarf maize (Davies and Rappaport, 1975a) and Phaseolus coccineus seedlings (Reeve et al., 1975), and Phaseolus vulgaris seeds (Yamane et al., 1975). Hydroxylation at C-2 converts GA₄ to GA₃₄ in dwarf rice seedlings (Durley and Pharis, 1973), Douglas-fir shoots (Wample et al., 1975) and Pinus pollen (Kamienska et al., 1976). In general, 2 β -hydroxylated GAs are less active in bioassays than their non-hydroxylated analogues (Reeve and Crozier, 1974) and this reaction has been proposed as a mechanism whereby GA activity is modified (Rappaport et al., 1974; Reeve and Crozier, 1974).

In addition to its transformation to GA₂₀ by 13 α -hydroxylation, GA₉ is hydroxylated at the C-2, C-12 and C-16 positions to form 2 β -hydroxy GA₉, dihydro-GA₃₁ (12 α -hydroxy GA₉) and GA₁₀ (16 α -hydroxy GA₉) respectively (Railton et al., 1974; Frydman and MacMillan, 1975). Pea seedlings also convert [³H] GA₅ to [³H] GA₃ (Durley et al., 1973).

The physiological significance of individual GAs occurring in a plant is difficult to assess when their apparent ease of conversion is taken into account. Some evidence for functional specificity of GAs has been put forward (Michniewicz and Lang, 1962). However, the alternative view, that all GAs represent intermediates in a pathway leading to a single functional species, has received support from data of Mertz and Lutz (1973, 1975), who reported a latent period of

6-8 h before the response of pea internodes to GA₁ or GA₅ could be detected, whereas the response to GA₃ was extremely rapid. It was therefore proposed that GA₁ and GA₅ were not effective per se in this system, but required conversion to an active, GA₃-like product. In higher plants GA₃ has not been shown to generate further free GA-like metabolites (Asakawa et al., 1974b; Barendse and de Klerk, 1975), but present knowledge of the biosynthetic pathway and mode of GA action do not justify the assumption that GA₃ is a terminal or sole functional species.

In higher plants, applied radioactive GAs often give rise to products with properties similar to those of the naturally-occurring GA β -D-glucosides (glucosyl ethers) and β -D-glucosyl esters. Glycosylation appears not to occur in G. fujikuroi (MacMillan, 1974b). The radioactivity in "bound", "polar", "water-soluble", or "butanol-soluble" fractions reported by many workers is probably due to glucosyl derivatives (Barendse, 1975). Owing to technical difficulties, such as decomposition during GLC, these metabolites have seldom been characterised as rigorously as have free acidic GAs. Identification of the aglycone obtained by enzymatic or chemical hydrolysis provides an indirect means of characterisation (e.g. Frydman and MacMillan, 1975; Lorenzi et al., 1976). However, mass spectrometric reference data for the glucosyl derivatives themselves are available (Yokota et al., 1975), and positive identification of GA₃-O- β -D-glucoside as the principal metabolite of [³H]GA₃ in Phaseolus vulgaris seedlings was achieved by this method (Asakawa et al., 1974b).

Glycosylation is a common detoxication mechanism in higher plants (Parke, 1968), but the occurrence of native GA glucosyl derivatives and their facile hydrolysis in vivo suggests that interconversions between conjugated and free GAs may also influence the effective concentration of endogenous GAs (Sembdner et al., 1972,

1974; Rappaport et al., 1974). It has also been proposed that the increased water-solubility conferred by glycosylation may assist the vascular translocation of GAs (Sembdner et al., 1968).

The accumulation of relatively large amounts of GA-like substances in developing seeds and fruits has facilitated their identification (see Lang, 1970; Nitsch, 1970; Moore and Ecklund, 1975) and has stimulated attempts to correlate the events of seed ripening and germination with changes in the availability of free GAs (e.g. Barendse et al., 1968; Dale and Felipe, 1968; Sembdner et al., 1968; Dale, 1969). Maturation is accompanied by a decrease in the complement of free GAs in seeds. Thus, GA₁, GA₄, GA₅, GA₆, GA₈, GA₃₇, GA₃₈, and GA₈ glucoside occur in immature seeds of P. vulgaris (Durley et al., 1971; Hiraga et al., 1974b) but at maturity GA₁, GA₈, GA₈ glucoside, and glucosyl esters of GA₁, GA₄, GA₃₇ and GA₃₈ are found (Hiraga et al., 1974a). Similarly, the free GAs in P. coccineus seeds diminish during maturation, and only GA₈ glucoside is detectable in mature seeds (Sembdner et al., 1968). Changes in the distribution of radioactivity between acidic ethyl acetate-soluble and butanol-soluble fractions during maturation and germination of pea and P. coccineus seeds indicated that free GAs were converted to glucosides during maturation, and possibly hydrolysed and released on germination (see Lang, 1970). However, Yamane et al. (1975) have observed rapid conversion of [³H] GA₁ to [³H] GA₈ glucoside in germinating P. vulgaris seeds, and in etiolated P. vulgaris seedlings, levels of glycosylated GAs were found to exceed those of free GAs (Hiraga et al., 1974b). Thus there must be some doubt as to whether GA glucosyl derivatives in seeds do indeed represent a depot accessible by hydrolysis, and whether GAs so released are involved in the early stages of seedling growth.

Mode of action of GAs

Levels of extractable GA-like activity have frequently been correlated with environmental variables and with physiological phenomena (see Paleg, 1965; Brian, 1966; Jones, 1973). However the subcellular events responsible for changes in GA levels have less often been identified. For example, the phytochrome-controlled unrolling of etiolated grass leaves in response to illumination (Virgin, 1962), is associated with rapid increases in endogenous GA levels following illumination (Reid et al., 1968; Beevers et al., 1970; Loveys and Wareing, 1971). This increase, which appears to be confined to an etioplast-rich fraction, is itself under phytochrome control (Cooke and Saunders, 1975a,b; Evans and Smith, 1976), and is partially inhibited by AMO 1618 (Cooke et al., 1975; Cooke and Saunders, 1975a,b). However, further metabolism of existing GAs as well as biosynthesis de novo may be involved, since illumination of leaf homogenates promoted the conversion of [^3H] GA₉ to more polar compounds (Reid et al., 1972, 1974).

In dwarf and tall maize seedlings, rates of conversion of [^3H] GA₁ were similar, indicating that dwarfism in the d-5 mutant studied is not a consequence of modified GA₁ metabolism (Davies and Rappaport, 1975a,b). Dark-grown Phaseolus coccineus seedlings contained more endogenous GA-like activity per plant than did their light-grown counterparts, in which stem growth was inhibited, and also converted [^3H] GA₄ more slowly (Bown et al., 1975). Railton and Wareing (1973) found no correlation between the falling GA levels induced in Solanum andigena by short days, and the rate of disappearance of [^3H] GA₁. However, in Bryophyllum daigremontianum, the proportion of [^3H] GA₂₀ converted to 3-epi-GA₁ (3 α -hydroxy GA₂₀ or pseudo-GA₁) was greater under inductive short days than under long (Durley et al., 1975). Wample et al. (1975) associated phases of bud set, bud break

and elongation in Douglas-fir with differential rates of conversion of [^3H] GA₄.

It must, however, be recognised that conversion of C₁₉-GAs is not necessarily the only, or even the most important, mechanism controlling the effective concentration of GAs. Pool sizes may also be influenced by the rate of supply of precursors, and it has been suggested by Graebe (1968) that the production of (-)-kaurene may be an important rate limiting step in GA biosynthesis, since it marks the point of departure from the common isoprenoid pathway. Synthesis of (-)-kaurene in a number of cell-free systems from higher plants is sensitive to growth retardants (Dennis et al., 1965; Anderson and Moore, 1967; Graebe, 1968; Shechter and West, 1969; Coolbaugh et al., 1973). Maximum kaurene-synthesising activity in immature pea seeds immediately precedes maximum GA production (Coolbaugh and Moore, 1969), and elongation in pea shoots and their de-etiolation following illumination were both associated with increased incorporation of [^{14}C] -mevalonate into [^{14}C] -kaurene (Ecklund and Moore, 1974). Endogenous turnover rates, however, are difficult to estimate (Brown and Wetter, 1972), since rates of both synthesis and transformation must be determined for each intermediate. Under these circumstances, estimates of pool sizes and rates of metabolism of exogenously-applied substrate may not give an accurate indication of overall rate of endogenous GA turnover.

Although the overall pattern of GA biosynthesis has emerged in outline, the distinction between biosynthetic and functional metabolism remains obscure. Considerable attention has been devoted to the effects of GAs on aleurone metabolism and consequently the sequence of events connecting GA production to the initiation of amylolytic activity in this specialised system is relatively well characterised (see Briggs, 1973; Jones, 1973; Varner, 1974; Mann, 1975). In contrast,

the primary mode of action of GAs in elongation growth is not as well understood. Rapid response studies, which have contributed considerably to an understanding of auxin action (Davies, 1973; Evans, 1974) have been carried out for GAs in few instances (Broughton and McComb, 1971; Warner and Leopold, 1971). However, the nature of the response to applied GAs by isolated stem sections of a number of genera, including Cucumis (Katsumi et al., 1965), Avena (Montague et al., 1973) and Lactuca (Silk and Jones, 1975) suggests the existence of several experimental systems appropriate to such studies.

Some evidence for specific intracellular receptor sites has been adduced for auxins (reviewed by Cuatrecasas, 1974; Kende and Gardner, 1976). Initial attempts to associate applied GAs with subcellular components were inconclusive (Yamaki, 1964; Kende, 1967; Ginzburg and Kende, 1968; Asakawa et al., 1974a), but specific binding was inferred from the accumulation of radioactivity from [^3H] GA₁ and [^3H] GA₅, and not their biologically inactive methyl- and keto-derivatives, in elongating regions of pea epicotyls (Musgrave et al., 1969). Stoddart et al. (1974) using [^3H] GA₁ of high specific radioactivity, isolated two protein fractions from pea epicotyls, both of which had the ability to bind [^3H] GA₁, but not its metabolite, [^3H] GA₈, nor the inactive analogue, 3-epi-GA₁ (pseudo-GA₁). The successful preparation of GA affinity adsorbants has been reported by Knöfel et al. (1975) and this technique may provide a means of concentrating and identifying receptor sites and thereby locating the site of GA action in the cell.

Further progress may be facilitated by recently developed syntheses yielding radioactive GAs of high specific activity. Although the classical method of supplying radioactive substrates to G. fujikuroi cultures has the advantage of yielding products inaccessible by chemical

means (MacMillan, 1974b), the low specific radioactivity of fungal metabolites makes them unsuitable for binding studies and incompatible with the low endogenous GA levels of higher plants. The selective palladium-catalysed reduction of GA₃ originally devised by Cross et al. (1962) and Jones and McCloskey (1963) was exploited, using tritium-enriched hydrogen, to yield [1,2-³H] GA₁ from GA₃ (Pitel and Vining, 1970), and [1,2-³H] GA₄ from GA₇ (Durley and Pharis, 1973). The specific activity of [³H] GA₁ obtained from GA₃ was subsequently increased by the use of carrier-free tritium gas (Nadeau and Rappaport, 1974). Similar procedures were used by Murofushi et al. (1974) to synthesise [2,3-³H] GA₂₀ from GA₅ methyl ester, and [1-³H] GA₅ from GA₃ methyl ester, and by Yokota et al. (1976) to synthesise [1,2-³H] GA₉ from $\Delta^{2,3}$ GA₉ methyl ester. Subsequent treatment of [1-³H] GA₅ with osmium tetroxide gave [1-³H] GA₈ (Murofushi et al., 1974). An alternative route to radioactive GAs, introduction of label at the exocyclic methylene group by means of radioactive Wittig reagent, has been described by Cross et al. (1968).

Translocation

In sporophytes, GA biosynthesis is considered to be associated mainly with root apices and young leaves of the apical bud, principally on the evidence of agar-diffusion experiments (reviewed by Lang, 1970; Barendse, 1975). Root-synthesised GAs appear to be exported in the xylem sap (e.g. Carr et al., 1964; Sitton et al., 1967; Reid et al., 1969), but it has been inferred that GAs travel from the apical bud to the subapical elongating zone by a symplastic route (Lang, 1970). Diffusible GA-like activity is considered to reflect biosynthesis de novo, since levels are enhanced by exogenous sucrose or mevalonate (Phillips, 1971), and reduced by CCC (Jones and Phillips, 1967).

Diffusible GAs have been taken to represent the mobile, and therefore physiologically functional, proportion of the total extractable GA-like activity (Jones, 1968, 1973) and GA-like fractions obtained by diffusion and extraction differ considerably in the amount and chromatographic behaviour of GA-like substances present (Atsmon *et al.*, 1968; Jones, 1968; Jones and Lang, 1968; Cleland and Zeevaart, 1970; Crozier and Reid, 1972).

The discrepancy between extractable and diffusible GAs implies a degree of physical compartmentation in the tissue. GA-like activity has been associated with chloroplast- or etioplast-rich preparations (Stoddart, 1968; Evans and Smith, 1976), and chloroplasts have been shown to incorporate (-)-kaurenoic acid into GA-like substances (Stoddart, 1969). Many of the enzymes associated with terpenoid biosynthesis, including mevalonate kinase (Rogers *et al.*, 1965) are found in chloroplasts. However, enzymes ^{associated} with (-)-kaurene biosynthesis in pea cotyledons and Echinocystis macrocarpa endosperm are confined to the soluble fraction of cell-free extracts (Upper and West, 1967; Coolbaugh and Moore, 1971), as is the enzyme system converting GA₁ to GA₈ in P. vulgaris cotyledons (Patterson *et al.*, 1975). The series of oxidative reactions by which (-)-kaurene is converted to (-)-kaurenoic acid is catalysed by microsomal fractions of E. macrocarpa preparations (Murphy and West, 1969), and in pea cotyledons the further oxidation of (-)-kaurene depends on its association with a non-catalytic carrier protein (Moore *et al.*, 1972). Associations at the molecular level may therefore represent significant sequestration mechanisms (Oaks and Bidwell, 1970) in addition to physical compartmentation involving cell organelles.

It is not known to what extent compartmentation causes differentiation between the metabolism of applied and endogenous GAs. The possibility that exogenously applied substrate may fail to reach

normal metabolic compartments is indicated by data of Coolbaugh and Moore (1971), who observed that cell-free preparations of pea cotyledons consistently failed to convert exogenous (-)-kaurene, although (+)-kaurene synthesised in situ was readily further metabolised. In G. fujikuroi mutant Bl-41a, the substrate specificity of enzymes associated with later stages of GA biosynthesis is evidently low, and it was suggested that misleading results might be obtained from similar non-specific conversion in feeding experiments on higher plants (MacMillan, 1974b). However, it is of interest to note that incubation of GA₄, GA₅ and GA₇ with non-specific hydroxylating systems produced only the α -ketone derivatives (Jones et al., 1971) which have, to date, not been reported as products of experiments in vivo.

There is as yet no evidence to suggest that the mechanism of intercellular GA transport is related to the primary mode of action, as has been proposed for auxins (Hertel, 1974). Although the inhibitory effect of TIBA on lateral GA₃ movement in Helianthus stem segments (Libbert and Kentzer, 1961; Libbert and Gerdes, 1964) suggests that metabolic energy may be expended in this process, the export of GAs by young leaves of the apical bud to the subapical region (Jones and Phillips, 1964, 1966) has been viewed as a diffusion phenomenon (Lang, 1970).

Localised accumulation of GA-like activity can be correlated with geotropic and phototropic curvatures (Phillips, 1972a,b; Railton and Phillips, 1973; El-Antably and Larsen, 1974a,b; Reches et al., 1974; El-Antably, 1975a,b), and the asymmetric redistribution of radio-activity following application of [¹⁴C] GA₃ to geotropically stimulated maize seedlings led to the suggestion that the accumulation of endogenous GA-like activity associated with tropic curvature was due to

unidirectional GA transport (Webster and Wilkins, 1974). However, Phillips and Hartung (1976) were unable to correlate displacement of [^3H] GA₁ with geotropic curvature of Helianthus stem segments, and concluded that localised biosynthesis rather than translocation was responsible for observed gradients of GA-like activity. Libbert and Gerdes (1964) observed the redistribution of GA₃ during phototropic curvature of Avena coleoptile segments and proposed that localised accumulation of GAs is not causal to the response but is a consequence of differential growth rates on opposite sides of bending segments.

Attempts to characterise GA transport in isolated segments using classical donor-receiver techniques have produced conflicting results. A basipetal polarity similar to that typical of auxin transport was reported for Coleus petiole segments (Jacobs and Kaldewey, 1970; Jacobs and Pruett, 1973), and for Phaseolus coccineus roots (Hartung and Phillips, 1974), whereas no polarity of transport could be detected in segments of Zea coleoptiles (Hertel et al., 1969; Wilkins and Nash, 1974) or P. coccineus internodes (Phillips and Hartung, 1974). An additional interpretative difficulty concerns the relationship between the behaviour of exogenously applied GAs in excised tissues and that of endogenous GAs in intact plants.

In intact plants, GAs are thought to circulate in the vascular system in the same manner as other organic assimilates (Lang, 1970), since GA-like activity has been found in xylem and phloem exudates (e.g. Carr et al., 1964; Phillips and Jones, 1964; Hoad and Bowen, 1968). The interchange of radioactivity from [^{14}C] GA₃ between phloem and xylem of Salix indicates that lateral GA movement between vascular elements may also occur (Bowen and Wareing, 1969). Few direct studies of GA translocation in intact plants have however been made. Application of aphids provides the only reported means of access to the phloem

contents of intact plants (Hoad and Bowen, 1968) but has the disadvantage that insect digestive processes may affect the composition of the exudate (Eschrich, 1970). Some investigators have depended on the localisation of radioactivity from $[^{14}\text{C}]\text{GA}_3$ by chemically non-discriminating means such as autoradiography (Zweig et al., 1961; McComb, 1964), or on the growth response of stems as an indication of the arrival of applied GA_3 (Chin and Lockhart, 1965; Chailakhyan et al., 1974). The identity of the translocated compound is consequently equivocal, since applied GA_3 has been shown to be readily transformed by intact plants. When $[^3\text{H}]\text{GA}_3$ was supplied to roots and stem bases of P. vulgaris seedlings, radioactivity was found in the mature aerial parts within 48 h, a pattern consistent with xylem movement (Asakawa et al., 1974a). However, the radioactivity was associated not only with $[^3\text{H}]\text{GA}_3$, but also with 2-O- β -D-glucosides of GA_3 , iso-GA₃ and gibberellenic acid, and the β -D-glucoside of a fourth, unidentified GA-like compound (Asakawa et al., 1974b). In Pharbitis nil seedlings, $[^{14}\text{C}]\text{GA}_3$ was rapidly converted to a single glucoside having greatly reduced biological activity (Barendse and de Klerk, 1975).

Some investigators have deduced the identity of translocated species from comparisons of relative proportions of metabolites in different organs following GA treatment. After application of $[^3\text{H}]\text{GA}_{20}$ to Bryophyllum leaves, radioactivity accumulating in growing shoots was associated mainly with $[^3\text{H}]\text{GA}_{20}$ itself, whereas in the leaves the ratio of $[^3\text{H}]\text{GA}_{20}$ to metabolites was considerably lower (Durley et al., 1975). This was taken to reflect translocation of $[^3\text{H}]\text{GA}_{20}$ itself. Another experimental approach, direct investigation of xylem sap, showed that the GA-like activity detected in spring bleeding sap from two woody species (Acer platanoides and Ulmus glabra) was due to glucosides of GA_3 and GA_8 (Sembdner et al., 1968).

Since the vascular translocation of GAs appears to be non-specific, it seems likely that GA supply is regulated by metabolic events occurring outside the vascular system. As a result, the potential physiological effectiveness of mobile GAs may be considerably modified by metabolic transformations occurring at the end of the transport pathway. Qualitative and quantitative differences in GA-like substances in different parts of plants are consistent with this possibility (e.g. Radley, 1958; Reid and Crozier, 1971; Crozier and Reid, 1971, 1972; Michniewicz and Kriesel, 1972; Frydman and Wareing, 1973; Wallerstein *et al.*, 1973). More direct evidence for local conversion of translocated GAs was obtained by Crozier and Reid (1971, 1972). When the root apices of P. coccineus seedlings were removed, GA₁, the principal GA of control plants, soon disappeared from the remainder of the plant. However, a GA₁₉-like component, previously present in relatively small quantities, accumulated in apical buds, stems and the remainder of the root tissue. It was therefore proposed that root apices are normally a site of conversion of the GA₁₉-like component to GA₁, which is then exported to the aerial parts (Crozier and Reid, 1971, 1972). The removal of sites of metabolic conversion may similarly be responsible for the transitory increases in GA-like activity following excision of apical buds or roots of pea seedlings (Sebanek, 1965, 1966).

Reciprocal grafting of dwarf and tall pea seedlings (Lockhart, 1957; Lockard and Grunwald, 1970) showed that the height attained by grafted seedlings was determined by the origin of the stem and not that of the rootstock. Stems from dwarf varieties grafted on rootstocks from tall varieties reached the same final height as ungrafted dwarf plants, and it was also observed that when GA₃ was applied to the roots, it promoted stem elongation to the same degree in both cases (Lockard

and Grunwald, 1970). These authors concluded that root-synthesised GAs had no direct effect on stem elongation, but that the rate of conversion to an active form was regulated by the stem. Some evidence has been presented for the occurrence of substances specifically inhibiting the gibberellin response in light-grown dwarf peas (Köhler and Lang, 1963; Tamura et al., 1972; Komoto et al., 1973; Ikegami et al., 1974). Although some of these substances are macromolecular and possibly proteinaceous (Komoto et al., 1973), this fraction did not form a stable complex with [^3H] GA₁ (Ikegami et al., 1974) and its relationship to the [^3H] GA₁-binding protein fractions isolated from pea epicotyls by Stoddart et al. (1974) is therefore not apparent.

It has been pointed out that the estimation of GA levels in extracts can contribute little further to an understanding of dynamic gibberellin relationships in developing plants (MacMillan, 1974a; Bown et al., 1975). The technical problems associated with the isolation and assay of GAs have been outlined above, but in addition to these, interpretative difficulties arise from the available information on GA metabolism which indicates differing rates of turnover of a number of related intermediates. Analysis of extracts can clearly yield little direct information on transient intermediates or on the relationship of individual components. Although inferences made on structural grounds may indeed be relevant to subsequent investigations, more rigorous, established approaches, such as those utilising tracers, enzymes or mutant organisms having blocked biosynthetic pathways, are required in order to characterise the kinetic changes associated with GA metabolism.

The investigations reported in this thesis represent an attempt to establish the metabolic relationships of GAs in seedling tissue of a higher plant as a necessary preliminary to future studies of GA-

mediated growth. Seedlings of scarlet runner bean, Phaseolus coccineus L., were considered to be suitable experimental material, since this species has been the subject of a considerable body of GA research. The effects of applied GA₃ and GA₄ on internode elongation in P. coccineus have been investigated (Crozier et al., 1973; Bown et al., 1975), and the rates of elongation of etiolated and light-grown seedlings have been correlated with endogenous GA levels and with the rate of utilisation of [³H] GA₄ (Bown et al., 1975). More significantly, P. coccineus is one of a small number of species in which GAs native to vegetative tissue have been characterised (Crozier et al., 1971; Bowen et al., 1973). The principal endogenous GAs of vegetative tissue were found to be GA₁, GA₄, GA₅ and GA₂₀, and these also occur amongst the GAs characterised in immature seed of this species by Durley et al. (1971), namely GA₁, GA₄, GA₅, GA₆, GA₈, GA₁₇, GA₁₉, GA₂₀ and GA₈ glucoside. The four seedling GAs exemplify different oxidation states and could therefore be biogenetically related. While on structural grounds GA₄, GA₅ or GA₂₀ could each represent a precursor of GA₁, GA₄ is the most likely candidate on the evidence of conversions in other higher-plant systems already described. However, GA₁ could not be identified as a product of [³H] GA₄ metabolism in P. coccineus seedlings grown under red light (Reeve et al., 1975).

By analogy with G. fujikuroi and C. maxima (see Fig. 2) the four hydroxylated C₁₉-GAs occurring in P. coccineus seedlings could be products of a pathway on which GA₁₂ aldehyde, GA₁₄ and GA₉ are intermediates. These compounds are of particular interest because they represent respectively the first gibberellane intermediate, the first 3 β -hydroxylated GA in the Gibberella and Cucurbita systems, and a non-hydroxylated C₁₉-GA which would be expected to generate a number of hydroxylated products. A striking difference between the biosynthetic

pathways proposed for G. fujikuroi and C. maxima is apparent in the relationship between GAs derived directly from GA₁₂ aldehyde and those arising from its 3 β -hydroxy-derivative, GA₁₄ aldehyde (Fig. 2). In Gibberella, there appear to be no interconversions between members of the GA₁₂ aldehyde and GA₁₄ aldehyde "families", whereas in C. maxima these are biosynthetically related in a complex manner. The metabolism of GA₉ in the two systems also differs. In G. fujikuroi mutant Bl-41a, GA₉ is converted to GA₁₀, GA₁₁, GA₂₀ and GA₄₀ (MacMillan, 1974b). In C. maxima, GA₉ has not yet been established as an endogenous component but exogenous GA₉ is efficiently converted to GA₄ (Graebe and Hedden, 1974).

While GA₉, GA₁₂ aldehyde and GA₁₄ have not been shown to occur in P. coccineus, their presence is not necessarily excluded since several minor, unidentified GA-like components of low polarity were found in seedling extracts (see Crozier and Reid, 1971; Crozier et al., 1973; Bown et al., 1975). In view of the differences between the fungal and C. maxima pathways already described, the products of applied GA₉, GA₁₂ aldehyde and GA₁₄ formed by P. coccineus seedlings and the metabolic relationships between the four characterised GAs are clearly of considerable interest, and these form the subject of the investigations reported here.

SECTION II

M A T E R I A L S A N D M E T H O D S

MATERIALS AND METHODS

1. Plant material
2. Growth experiments
3. Radioactive compounds
 - 3.1 Application to plant material
 - 3.2 Localisation and determination of radioactivity
4. Extraction, purification and identification procedures
 - 4.1 Solvent extraction and partition
 - 4.2 Thin-layer chromatography
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 - 4.5 Enzymatic hydrolysis
 - 4.6 High-performance liquid chromatography
 - 4.6.1 Preparative system
 - 4.6.2 Analytical systems
 - 4.7 Mass spectrometry

MATERIALS AND METHODS

1. Plant material

Seeds of scarlet runner bean (Phaseolus coccineus L. cv. Prizewinner), supplied by Thomas Dagg and Sons, Glasgow, or Charles Sharpe, Sleaford, were soaked in aerated running tap water for 12h, drained, and allowed to germinate at 25°C for 72h between sheets of dry filter paper. The testas were then removed and uniformly germinated seeds sown in moist vermiculite in 50 cm x 30 cm x 8 cm seed trays. The trays were transferred to an environmental chamber maintained at 25°C and providing a 16h photoperiod of combined fluorescent and incandescent light (Atlas warm-white, 75/85W; Phillips K, 150W; combined intensity 85W.m⁻² at plant level). Seedling ages quoted in days are reckoned from the day of sowing.

2. Growth experiments

Four days after sowing, uniform shoots were severed 4 cm below the apical bud and rapidly transferred to individual 5 x 50 mm vials each containing 1 ml distilled water. Each shoot explant received 1 µg of GA₁, GA₄, GA₅ or GA₂₀ in 5 µl 80% aqueous ethanol. The solution was either added to the water in the vial, which was completely taken up within 12h and subsequently replaced with water only, or injected into the apical bud by means of a microlitre syringe. Each treatment was replicated 10 times. An India-ink mark was placed 10 mm below the apical bud, and after 72h the increase in length of this region was measured using vernier calipers. Statistical differences between treatments were determined by variance-ratio and t tests.

3. Radioactive compounds

Radioactive compounds used are listed in Table 1. [³H] GA₁ (30 Ci.mmol⁻¹) was obtained from New England Nuclear, Boston,

Massachusetts, and [^{14}C] sucrose from the Radiochemical Centre, Amersham, England; sources of other compounds are stated under Acknowledgments. The original specific activities were modified in some experiments by the addition of the corresponding carrier GA (for sources see Acknowledgments). Stocks were stored in ethyl acetate at -20°C and periodically examined for radiochemical purity by TLC or HPLC.

3.1 Application to plant material

Each seedling received 1 μl or 5 μl of an 80% ethanolic solution containing between 0.05 and 0.5 μCi of radioactivity. The solution was injected 1 mm below the surface of various parts of the seedling (apical bud, stem, hypocotyl, cotyledons or root) by means of a microlitre syringe. Plant material was frozen and stored at -20°C immediately after each experiment.

Table 1
Radioactive compounds

<u>Compound</u>	<u>Specific activity</u>	<u>Preparation</u>
$[1,2\text{-}^3\text{H}]\text{GA}_1$	0.42 Ci.mmol $^{-1}$	Pitel and Vining, 1970
	30.0 Ci.mmol $^{-1}$	Nadeau and Rappaport, 1974
$[1,2\text{-}^3\text{H}]\text{GA}_4$	1.87 Ci.mmol $^{-1}$	Cross <u>et al.</u> , 1962
$[1\text{-}^3\text{H}]\text{GA}_5$	5.30 Ci.mmol $^{-1}$	Murofushi <u>et al.</u> , 1974
$[1\text{-}^3\text{H}]\text{GA}_8$	0.43 Ci.mmol $^{-1}$	"
$[2,3\text{-}^3\text{H}]\text{GA}_{20}$	3.30 Ci.mmol $^{-1}$	"
$[2,3\text{-}^3\text{H}]\text{GA}_9$	46.0 Ci.mmol $^{-1}$	Yokota <u>et al.</u> , 1976
$[17,17'\text{-}^3\text{H}]\text{GA}_9$	17.5 mCi.mmol $^{-1}$	Cross <u>et al.</u> , 1968
$[17,17'\text{-}^3\text{H}]\text{GA}_{12}$ aldehyde	25.0 mCi.mmol $^{-1}$	"
$[17,17'\text{-}^3\text{H}]\text{GA}_{14}$	61.0 mCi.mmol $^{-1}$	"
$[\text{U}\text{-}^{14}\text{C}]\text{sucrose}$	10.0 mCi.mmol $^{-1}$	from $^{14}\text{CO}_2$

3.2 Localisation and determination of radioactivity

Frozen plant material and other samples unsuitable for direct scintillation counting were prepared for radioassay by combustion in a liquid-scintillation sample oxidiser (Intertechnique model IN 4101). Scintillant formulations were as follows: tritium-containing samples, 18 ml toluene containing 70% v/v dioxane, 0.5% w/v PPO and 0.03% POPOP; ^{14}C -containing samples, 15 ml toluene containing 33% v/v 2-phenethylamine, 22% v/v methanol, 5% v/v water and 0.7% w/v PPO.

Samples not requiring oxidation were assayed in toluene containing 0.5% w/v PPO and 0.03% w/v POPOP. Radioactivity determinations were made using either a Packard 3380 scintillation spectrometer with automatic external standardisation and quench correction facility, or an ICN-Tracerlab Corumatic 200 with efficiency correction made by reference to a series of quenched standards (Rogers and Moran, 1966). Recorded counts were corrected for background radiation and are expressed as disintegrations per minute (dpm).

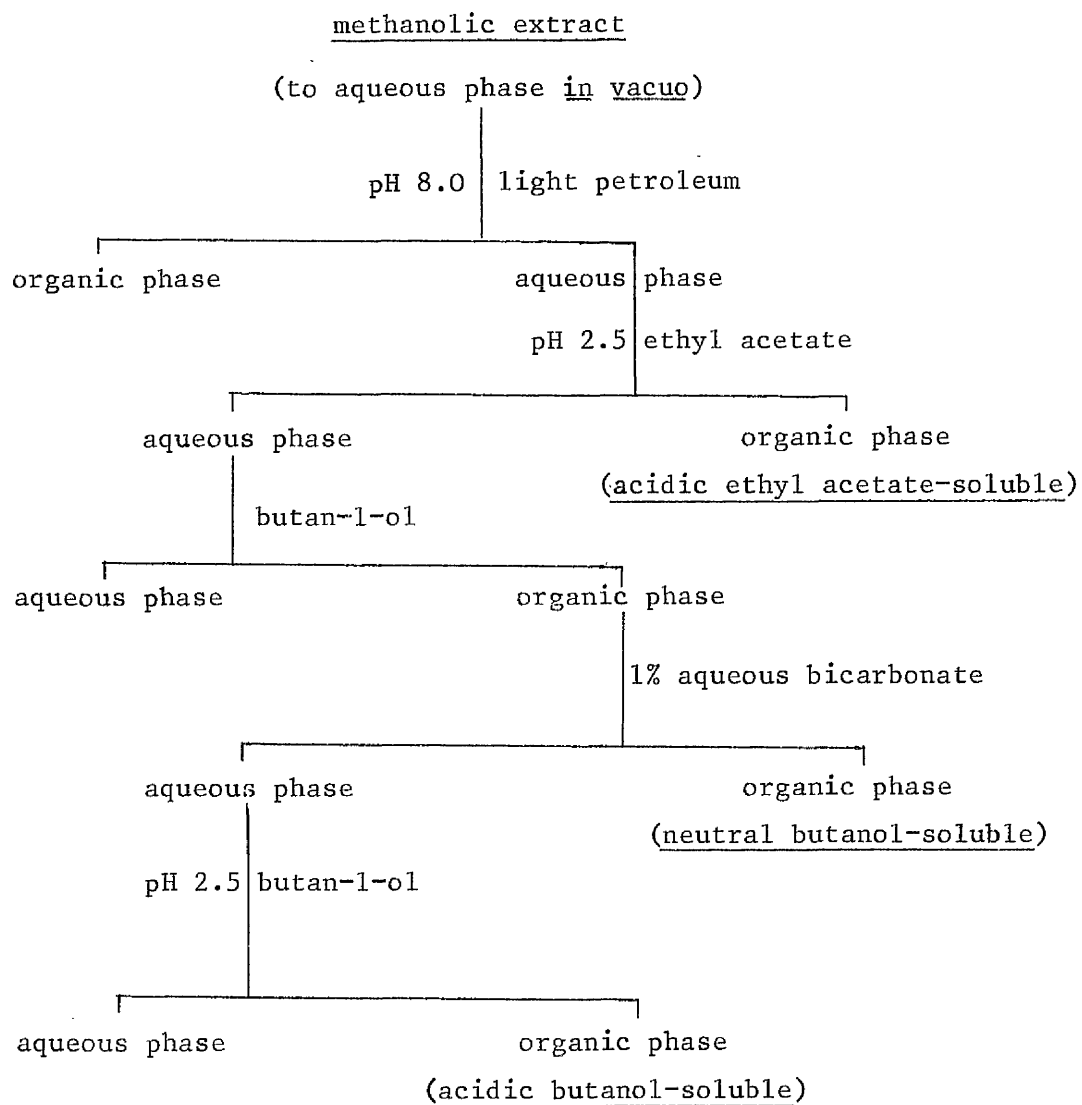
4. Extraction, purification and identification procedures

4.1 Solvent extraction and partition

Plant material was homogenised for 20s in a Waring blender in cold 95% methanol (5 ml.g^{-1} fresh weight), the extract was filtered and the residue washed twice with fresh methanol. The combined methanolic extracts were reduced to the aqueous phase in vacuo at 35°C , resuspended in an equal volume of 0.5M phosphate buffer, pH 8.0, slurried with 5% w/v PVP, and filtered. The filtrate was extracted twice with equal volumes of light petroleum (boiling range $60^{\circ} - 80^{\circ}\text{C}$) and further partitioned to yield the acidic ethyl acetate - soluble, neutral butanol-soluble and acidic butanol-soluble fractions (Fig. 3).

FIGURE 3

Solvent fractionation procedure



4.2 Thin-layer chromatography

Samples dissolved in 200 μ l methanol-ethyl acetate (1:1 v/v) were strip-loaded on pre-coated chromatography sheets (Eastman Kodak silica gel G, 0.25 mm layer). The chromatograms were developed for a distance of 15 cm in benzene - butan-1-ol - acetic acid, 80:20:5 v/v/v, dried in still air and divided into 20 half-R_f zones. Silica gel scraped from each zone was eluted for 3h in 1 ml 95% methanol in a scintillation vial before addition of scintillant.

4.3 Gel permeation chromatography

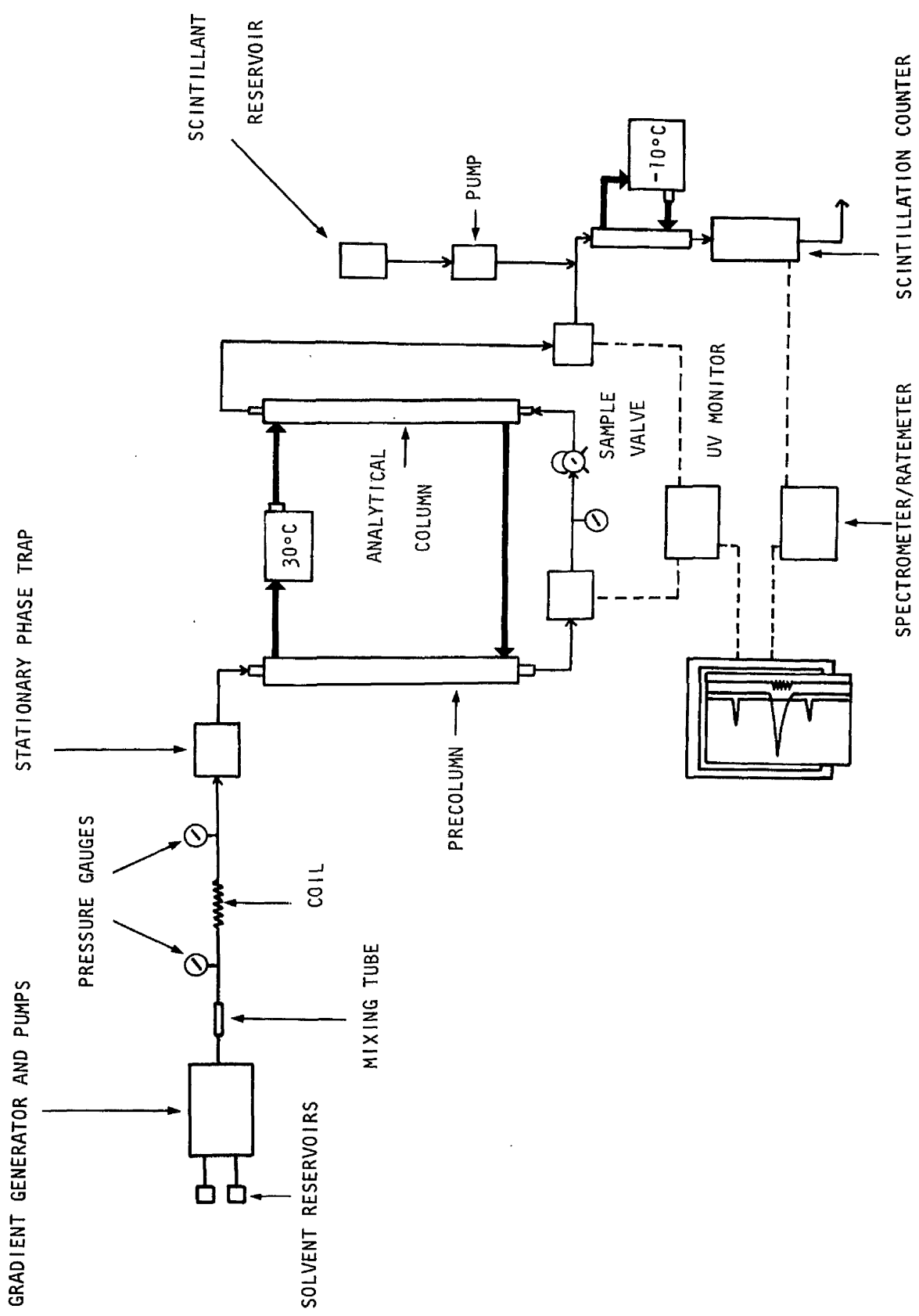
Crude acidic ethyl acetate-soluble extracts were redissolved in freshly redistilled tetrahydrofuran (THF) and introduced into a gel permeation chromatograph (Reeve and Crozier, 1976), which consisted of two 25 mm x 100 cm columns of porous polystyrene beads (BioBeads SX-4, BioRad Laboratories, Richmond, California; exclusion volume, 350 ml, flow rate, 3 ml.min⁻¹). The samples were eluted with THF, successive 10 ml eluate fractions were monitored for radioactivity by liquid-scintillation counting of suitable aliquots, and fractions associated with radioactivity maxima were pooled and dried in vacuo at 35°C.

4.4 Charcoal adsorption chromatography

A 20 x 300^{mm}_A glass column with fritted base was packed with 1:2 charcoal-celite mixture (Darco G-60 charcoal, Atlas Chemical Industries, Wilmington, Delaware; Celite 545, Hopkin and Williamson, Chadwell Heath, Essex). Acidic ethyl acetate-soluble fractions were applied to 20 mm-diameter filter paper discs, the discs were air-dried and placed on the charcoal-celite bed, and radioactivity was eluted from the column in successive 100 ml volumes of acetone - water, 1:4; acetone - water, 4:1; and 100% acetone. Eluates were reduced to the aqueous phase in vacuo at 30°C, adjusted to pH 2.5 with 2M H₂SO₄, and the radioactivity recovered in ethyl acetate. Butanol-soluble extracts were applied to the

FIGURE 4

Preparative liquid chromatograph with UV monitor
and radioactivity detector (adapted from Reeve et al.,
1976).



column dissolved in minimum volumes of water, buffer salts were eluted with 50 ml water, and radioactivity subsequently eluted with successive 50 ml volumes of methanol - water, 3:2; 100% methanol; and acetone - methanol, 1:4. Solvents were removed and the aqueous phase reduced to a small volume in vacuo at 25°C.

4.5 Enzymatic hydrolysis

Samples were dissolved in 0.2M acetate buffer, pH 4.4, containing 0.1% w/v Sigma cellulase (crude β -glucosidase, E.C. 3.2.1.4, from Aspergillus niger). The ratio of enzyme to sample was approximately 10:1 by weight. The mixture was incubated at 37°C for between 18 and 24h on a reciprocating shaker, then adjusted to pH 2.5 by addition of 2M H₂SO₄ and the hydrolysis products recovered in ethyl acetate. The efficiency of hydrolysis estimated by TLC exceeded 90%.

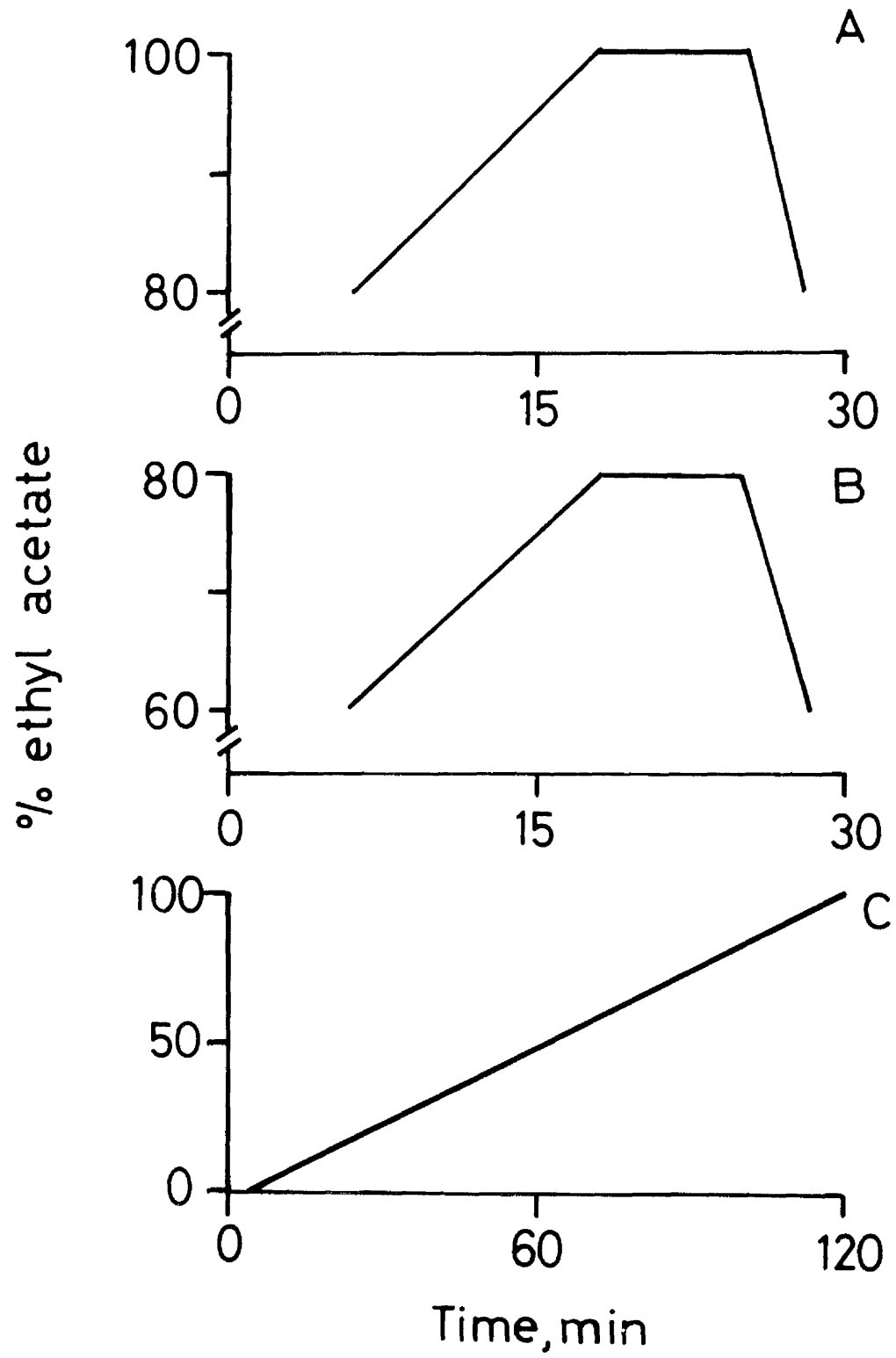
4.6 High-performance liquid chromatography

4.6.1 Preparative system

The preparative HPLC system with on-line radioactivity detector has been fully described elsewhere (Reeve et al., 1976), and is shown in outline in Fig. 4. Acidic ethyl acetate-soluble samples were eluted with increasing percentages of ethyl acetate in hexane from a silicic acid partition column (Partisil 10 or Partisil 20, Reeve Angel Scientific Co., Maidstone, Kent; bed dimensions, 10 x 450 mm; void volume, 28.5 ml; flow rate, 5.0 ml. min⁻¹; stationary phase, 40% formic acid). The mobile phase gradients depicted in Fig. 5 were generated by an ISCO model 382 Dialagrad gradient former. The column effluent was monitored for absorbance at 254 nm by an ISCO model UA-5 absorbance monitor, and combined with scintillant (30% v/v toluene, 20% w/v naphthalene, 1.2% w/v PPO, 0.06% w/v POPOP in dioxane). The combined effluent was shielded from light, cooled to -5°C and passed through a spiral glass flow cell fitted to a manual scintillation counter with

FIGURE 5

Eluent gradients for preparative HPLC system



spectrometer-ratemeter (ICN Pharmaceuticals, Hersham, Surrey).

Absorbance at 254 nm and radioactive counting rate were transcribed by a dual-pen flatbed recorder, and radioactivity maxima were automatically integrated.

The system was used in both preparative and analytical modes. The high speed of analysis permitted rapid initial investigation of suitable aliquots of extracts, radioactive components being located and quantified by the on-stream radioactivity monitor and associated peak integrator. Radioactive components were then isolated from the bulk of the extract by collecting successive 2.5 ml eluate fractions directly from the column outlet without addition of scintillant. The location of radioactivity zones was confirmed by liquid-scintillation counting of suitable aliquots, and fractions associated with radioactivity maxima were pooled, dehydrated over anhydrous sodium sulphate and dried under a nitrogen stream.

4.6.2 Analytical systems

Samples were allowed to react for 2h at 70°C with 50% v/v dimethylformamide dibenzylacetal in dry dioxane (dimethylformamide dimethylacetal was obtained from Pierce Chemical Co., Rockford, Illinois, and converted by reaction with benzyl alcohol). The resulting conversion of GAs to their benzyl esters, which was 80 - 90% efficient, facilitated analysis on silicic acid adsorption columns eluted with non-acidic solvent systems, and also permitted detection of the GA benzyl esters by absorbance changes at 254 nm. The presumptive GA benzyl esters in derivatised samples were separated from excess derivatising reagent and residual starting material by preparative HPLC as already described. A suitable aliquot of each derivatised sample was then combined with appropriate GA benzyl ester standards and chromatographed in analytical system 1 (Partisil 10, bed dimension

4.6 x 500 mm; mobile phase, 3% or 8% THF in dichloromethane half-saturated with water; flow rate, 1.6 ml.min⁻¹). The eluate was monitored for radioactivity either by collecting successive 0.8 ml fractions for liquid-scintillation counting, or by an on-stream radioactivity detector, and the location of radioactivity maxima was compared with the retention times of the UV-absorbing standards. Where sufficient material to permit characterisation by mass spectrometry was available, the bulk of the sample was then chromatographed in the same system, and the UV-absorbing zone with retention time corresponding to that of the radioactivity maximum was collected for further purification in system 2 (Partisil 10, bed dimensions 4.6 x 250 mm; mobile phase, 100% diethyl ether, water-saturated; flow rate, 1.6 ml.min⁻¹). In both systems, samples dissolved in 2 - 10 µl of mobile phase were injected on-column through a PTFE-rubber septum. The pressure associated with a flow rate of 1.6 ml.min⁻¹ was 600 psi (4.1 x 10⁶ Pa) for the 500 mm column, and 300 psi (2.0 x 10⁶ Pa) for the 25 mm column, maintained by a constant pressure pump (Applied Research Ltd., Luton, Beds.). The column eluates were passed through an ISCO model UA-5 absorbance monitor with a 5 mm path length flow cell. Recovery of radioactivity from the silicic acid absorbance columns was 60 - 65%.

4.7 Mass spectrometry

Samples were taken up in glass capillaries and analysed using the direct inlet system of an AEI MS 30 mass spectrometer operated at an ionising potential of 70 eV and a source temperature of 230°C. Spectra were recorded at a scan speed of 30s per decade and compared with those of authentic GA benzyl esters run under the same conditions within 30 min of the sample.

SECTION III

E X P E R I M E N T A L

EXPERIMENTAL

1. Effect of GA_1 , GA_4 , GA_5 and GA_{20} on shoot elongation
2. Redistribution of radioactivity from $[^3H]GA_1$, $[^3H]GA_4$, $[^3H]GA_5$ and $[^3H]GA_{20}$ in shoots
3. Redistribution of radioactivity from $[^3H]GA_1$, $[^3H]GA_4$, $[^3H]GA_5$ and $[^3H]GA_{20}$ in germinating seeds
4. Translocation of applied $[^3H]GAs$ in intact seedlings
 - 4.1 Redistribution of radioactivity from $[^3H]GAs$ and $[^{14}C]$ sucrose
 - 4.2 Chromatographic analysis of bleeding sap.
 - 4.3 Chromatographic distribution of radioactivity in individual seedling parts
 - 4.4 Export and metabolism of $[^3H]GA_4$ by individual seedling parts
5. Metabolism of $[^3H]GAs$ by intact seedlings
 - 5.1 $[^3H]GA_1$ feeds
 - 5.2 $[^3H]GA_4$ feeds
 - 5.3 $[^3H]GA_5$ feeds
 - 5.4 $[^3H]GA_8$ feeds
 - 5.5 $[^3H]GA_9$ feeds
 - 5.6 $[^3H]GA_{12}$ aldehyde feeds
 - 5.7 $[^3H]GA_{14}$ feeds
 - 5.8 $[^3H]GA_{20}$ feeds

EXPERIMENTAL

1. Effect of GA₁, GA₄, GA₅ and GA₂₀ on shoot elongation

GA₁, GA₄, GA₅ and GA₂₀, the principal endogenous GAs of P. coccineus seedlings (Bowen et al., 1973), exhibit different degrees of biological activity in a number of bioassay systems (Reeve and Crozier, 1975). In order to compare their relative effectiveness in promoting shoot elongation in P. coccineus, the apical buds or cut basal ends of shoot explants were treated with 1 µg of each GA. The subapical elongation of the explants after 72h is summarised in Table 2. Elongation was significantly promoted in all treatments (P = 0.01), but there was no significant difference between treatments with the four GAs, nor between apical and basal treatments.

TABLE 2

Subapical elongation in mm of shoot explants, 72h after apical or basal treatment with 1 µg of GA₁, GA₄, GA₅ or GA₂₀ (mean increase in length of 10 replicates per treatment, ± 95% confidence limits). Water-treated controls, 3.7 ± 0.64 mm.

	GA ₁	GA ₄	GA ₅	GA ₂₀
apically applied	6.5 ± 1.40	7.5 ± 0.68	5.7 ± 0.71	6.0 ± 0.83
basally applied	7.4 ± 0.87	7.2 ± 0.89	7.2 ± 0.64	6.4 ± 0.73

2. Redistribution of radioactivity from [³H]GA₁, [³H]GA₄, [³H]GA₅ and [³H]GA₂₀ in shoots.

Shoot elongation results from the activity of a subapical meristem (Sachs, 1965), which may be supplied with GAs diffusing basipetally from the apical bud (see Lang, 1970). After application of [³H]GA₁ or [³H]GA₅ to the basal cut surface of pea epicotyl explants,

TABLE 3a

Percentage of applied radioactivity recovered from shoot explants 24h after apical (A) or basal (B) treatments with 0.1 μ Ci per explant of [3 H]GA₁, [3 H]GA₄, [3 H]GA₅ or [3 H]GA₂₀ (specific activity 0.13 Ci.mmol⁻¹). Mean percentage of two replicates.

	[3 H]GA ₁		[3 H]GA ₄		[3 H]GA ₅		[3 H]GA ₂₀	
	A	B	A	B	A	B	A	B
primary leaf pair	1.7	2.7	1.2	0.4	10.0	3.5	5.7	3.3
trifoliolate leaf bud	76.7	0.1	25.9	0.1	85.2	0.4	39.3	1.0
subapical 0 - 10 mm	0.1	0.1	0.3	0.1	0.2	0.2	0.2	0.4
subapical 11 - 20 mm	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.2
subapical 21 - 30 mm	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.4

TABLE 3b

Percentage of applied radioactivity recovered from basal agar receiver blocks, 24h after apical treatment with 0.1 μ Ci per explant of [3 H]GAs as in Table 3a. Mean percentage of two replicates.

	[3 H]GA ₁	[3 H]GA ₄	[3 H]GA ₅	[3 H]GA ₂₀
explant length(mm)				
2	2.5	0.2	1.4	2.5
5	2.1	0.4	2.6	3.9
10	1.2	0.3	0.1	1.1
15	0.2	0.2	0.1	0.1
20	-	-	0.1	0.2

radioactivity accumulated in the subapical region (Musgrave et al., 1969). In order to determine whether the observed GA-promoted elongation of P. coccineus shoot explants was associated with localised subapical GA accumulation, the apical buds or basal cut ends of explants were supplied with equivalent amounts of [^3H]GA₁, [^3H]GA₄, [^3H]GA₅ or [^3H]GA₂₀ and the distribution of radioactivity investigated after 24h.

There was no consistent indication of preferential accumulation of radioactivity in the region immediately subtending the apical bud (Table 3a), and the amount of radioactivity in this region was approximately similar after apical or basal treatment with any of the four GAs. Thus the equivalence in growth response to apical and basal treatments with these GAs (Table 2) may reflect the presence of similar GA concentrations in the subapical region. In contrast to the lack of accumulation of radioactivity in the subapical region, however, up to 10% of the recovered radioactivity was found to be associated with the primary leaf pair 24h after either apical or basal treatment.

The amount of radioactivity lost from explants as a result of basipetal transport was estimated by applying agar receivers to the cut surfaces of apically treated explants. As can be seen from Table 3b, the amount of radioactivity recoverable from the agar receivers at 24h decreased with increasing explant length, but even when the explants were severed as little as 2 mm below the apical bud, the radioactivity found in the receivers represented no more than 2.5% of the applied dose.

3. Redistribution of radioactivity from [^3H]GA₁, [^3H]GA₄, [^3H]GA₅ and [^3H]GA₂₀ in germinating seeds.

Developing seeds may derive GAs from hydrolysis of glucosyl derivatives associated with the cotyledons (see Lang, 1970). In order to establish whether a route exists for GA transport between cotyledons and embryo of germinating P. coccineus seeds, [^3H]GAs were injected into either the distal region of one cotyledon or the embryonic axis of

of previously imbibed seeds. The axis, or a suitable area of the cotyledonary surface, was exposed by removing part of the testa, and 1 μ l of ethanolic solution containing 0.05 μ Ci of radioactivity was injected. Treated seeds were maintained at 25°C in individual 4-cm petri dishes lined with moist filter paper. At 24h intervals, the embryo and cotyledons were separated and processed in a liquid-scintillation sample oxidiser.

Approximately 50% of the applied radioactivity could be recovered from seeds by 24h, but no further loss of radioactivity subsequently took place. The distribution of radioactivity between embryo and cotyledons is therefore expressed as a percentage of the total recovered (Table 4). Over 90% of the recovered radioactivity was found in the cotyledons within 24h of application to the axis, and this proportion remained relatively constant up to 96h, although there was some evidence of a movement of radioactivity back into the embryo by 120h, particularly in [3 H]GA₁ and [3 H]GA₄ treatments.

In contrast, the earliest indication of radioactivity reaching the embryo after cotyledonary applications was at 72h. However, only 5% of the total radioactivity was associated with the embryos at this time and there was no evidence of further accumulation. Thus the predominant direction of [3 H]GA movement in P. coccineus seeds during the first 96h of germination appears to be from embryo to cotyledons. When the cotyledons of 4-day-old seedlings were treated with [3 H]GA₄, the export of radioactivity to the remainder of the seedling during the succeeding 4 days was small (Table 5), and thus the possibility that export from the cotyledons might increase with increasing seedling age was not confirmed.

TABLE 4

Percentage distribution of radioactivity in germinating seeds treated with [^3H]GA₁, [^3H]GA₄, [^3H]GA₅ or [^3H]GA₂₀ (specific activity 0.13 Ci.mmol⁻¹). E, embryo; C, cotyledons. Mean percentage of two replicates.

Time (h)		24	48	72	96	120
<u>Axis treated</u>						
[^3H]GA ₁	E	1.4	0.8	0.9	0.4	35.8
	C	98.5	99.1	99.0	99.5	64.1
[^3H]GA ₄	E	4.0	1.4	21.2	0.4	9.3
	C	95.9	98.5	78.7	99.5	90.6
[^3H]GA ₅	E	6.9	5.2	6.8	2.3	2.9
	C	93.0	94.7	93.1	97.6	97.0
[^3H]GA ₂₀	E	9.9	1.5	3.8	3.8	1.5
	C	91.0	98.4	96.2	96.1	98.4
<u>Cotyledons treated</u>						
[^3H]GA ₁	E	0	0	0.1	1.1	0.1
	C	100	100	99.8	98.9	99.9
[^3H]GA ₄	E	0	0	0.5	0.9	0.8
	C	100	99.8	99.4	99.1	99.2
[^3H]GA ₅	E	0	0	4.9	0.4	0.3
	C	100	100	95.0	99.0	99.7
[^3H]GA ₂₀	E	0	0	4.5	0.3	0.1
	C	100	100	95.4	99.7	99.9

TABLE 5

Percentage distribution of radioactivity in seedlings treated at four days with [^3H] GA₄ (0.1 μCi , specific activity 1.87 Ci.mmol⁻¹, applied to each cotyledon). Mean percentage of four replicates.

	apical bud	stem	cotyledons	hypocotyl	roots
<u>Time (h)</u>					
4	0.1	0.3	98.9	0.3	0.5
24	0.3	1.4	97.7	0.2	0.2
48	0.1	1.4	97.3	1.0	0.2
72	0.1	1.2	97.1	1.2	0.2

4. Translocation of applied [^3H] GAs in intact seedlings

4.1 Redistribution of radioactivity from [^3H] GAs and [^{14}C] sucrose

Seedling GAs are thought to be synthesised both by roots and by young leaves of the apical bud (see Lang, 1970), and isolated shoot apices export detectable amount of GA-like substances into agar receivers (Jones and Phillips, 1964, 1966). In view of the limited basipetal movement of [^3H] GAs indicated by the redistribution of radioactivity in shoot explants (Table 3a) it was of interest to determine whether GAs are transported basipetally as well as acropetally in intact seedlings.

Tritiated GAs or [^{14}C] sucrose were injected into the apical bud or hypocotyl 5 mm below the cotyledonary node of 4-day-old seedlings. For practical reasons, the hypocotyl, a region anatomically transitional between root and shoot (Compton, 1912) was selected as the point of injection rather than the primary root itself. The [^{14}C] sucrose treatment was included in order to establish the behaviour after injection of a substance whose normal translocation pattern is relatively well known.

After 24h, the distribution of radioactivity from each [^3H] GA resembled that of the corresponding [^{14}C] sucrose treatment (Table 6). However, in the case of each compound tested there were considerable differences between apical and hypocotyl (basal) treatments. After apical treatment, approximately 90% of the recovered radioactivity was located in the apical bud, and basipetal export appeared to have been negligible, since radioactivity in the remainder of the seedling was close to the background value. In contrast, after injection into the hypocotyl, although a significant proportion of the recovered radioactivity was associated with this region at 24h, up to 75% of the total recovered radioactivity was distributed between the apical bud, stem, cotyledons and roots. Redistribution in both upward and downward

directions had therefore taken place.

4.2 Chromatographic analysis of bleeding sap

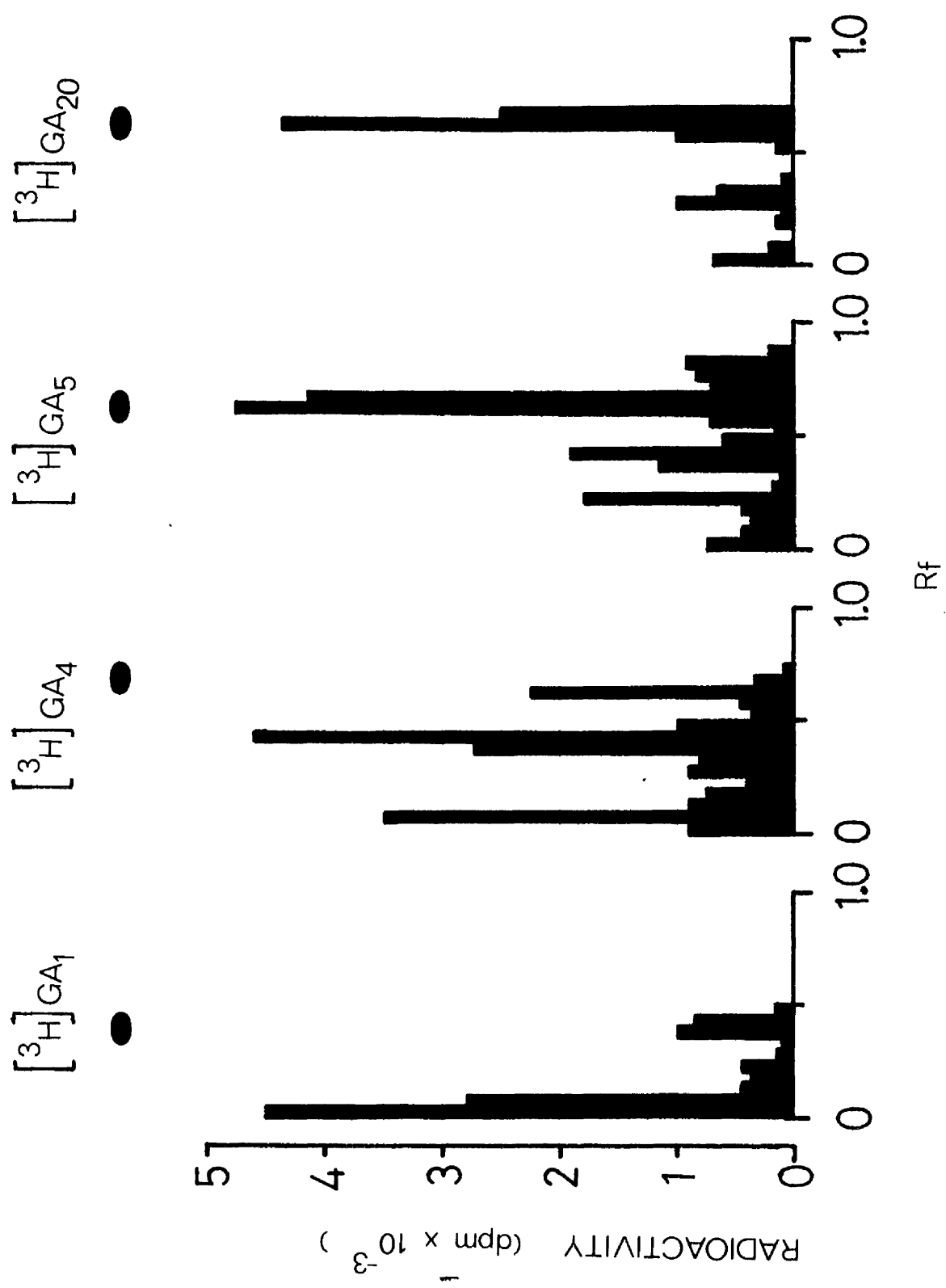
Since substances occurring in the xylem are considered to be transport forms (see Bollard, 1960; Pate, 1975), the presence of GA-like substances in bleeding sap is thought to provide evidence for the role of roots as exporters of GAs (e.g. Carr et al., 1964; Sitton et al., 1967), and also for the transport function of GA glucosides (Sembdner et al., 1968). In order to investigate the radioactive components moving upwards in P. coccineus stems after application of [^3H]GAs to the hypocotyls, an attempt was made to obtain bleeding sap as described by Gordon et al. (1974). Although this failed to yield sufficient radioactivity for analysis, probably as a result of the low root pressure of the seedlings, up to 50 μl of sap per seedling could be obtained by subjecting the decapitated plants to approximately 7×10^5 Pa pressure according to the method of Scholander et al. (1965). Four hours after application of 2.0 μCi of [^3H]GA per plant, between 0.1% and 0.2% of the applied radioactivity could be recovered in the exudate. When this was analysed by thin-layer chromatography it was apparent that the bleeding sap contained a number of radioactive compounds in addition to the original [^3H]GAs (Fig. 6). The low recoveries, however, precluded further investigation of these compounds.

4.3 Chromatographic distribution of radioactivity in individual seedling parts

The chromatographic profiles obtained from bleeding sap after [^3H]GA treatment indicated the upward transport of radioactive compounds other than the applied GAs. The chromatographic distribution of radioactivity in individual seedling parts after basal treatment was therefore investigated in order to obtain more information on the translocation of metabolites.

FIGURE 6

Thin-layer radiochromatograms of crude bleeding sap obtained 4h after treatment of hypocotyls with [^3H] GA₁, [^3H] GA₄, [^3H] GA₅ or [^3H] GA₂₀ (2.0 μCi , 10 seedlings per treatment). Solvent system: benzene - butanol - acetic acid, 80:20:5v/v/v. Marker spots indicate Rf of authentic [^3H] GAs.



For purposes of comparison, extracts of apically treated seedlings were also obtained. Because basipetal export from apical buds was too small to provide workable levels of radioactivity in the remainder of the plants, the distribution of radioactivity was examined only in the apical buds themselves. In these, approximately 80% of the recovered acidic ethyl acetate-soluble radioactivity was located at the Rf of the original [^3H]GA, the remaining 20% being associated with more polar compounds (Fig. 7).

The more extensive redistribution of basally applied [^3H]GAs, however, was allied with a greater degree of conversion than that found after apical treatments (Fig. 8). All seedling parts, with the exception of stems in some treatments, contained at least one radioactive compound chromatographically distinct from the applied [^3H]GA. These metabolites differed considerably in polarity and relative abundance between treatments and in different organs. The amount of unconverted [^3H]GA, as estimated from thin-layer chromatograms, also varied, and it was noted that [^3H]GA₅ was more slowly converted than any of the other GAs.

4.4 Export and metabolism of [^3H] GA₄ by individual seedling parts.

The chromatographic distribution of radioactivity throughout seedlings after basal application of [^3H]GAs indicated that a number of radioactive components were to be found in each organ after 24h. In order to determine whether these had been imported or locally synthesised, plants were divided into apical buds, stem, cotyledons, hypocotyl and roots. Each section was injected with 0.2 μCi [^3H]GA₄ (0.1 μCi per cotyledon) and sets of five similar sections (ten cotyledons) were incubated for 24h at 25°C in 50 ml 0.1M Tris-HCl buffer, pH 7.4, containing 0.1% sucrose. In a control experiment, sets of five intact plants received 0.2 μCi [^3H]GA₄ per plant, injected into either the apical bud, stem, cotyledons, hypocotyl or roots. Treated plants

TABLE 6

Percentage distribution of radioactivity in intact seedlings, 24h after application of [^3H] GAs or [^{14}C]sucrose (0.1 μCi per plant, specific activity 10 $\text{mCi}\cdot\text{mmol}^{-1}$) to apical buds (A) or hypocotyls (B). Mean percentage of two replicates.

		apical bud	stem	cotyledons	hypocotyl	roots
[^3H]GA ₁	A	95.7	0.5	2.4	0.3	0.8
	B	12.1	55.4	11.7	18.1	2.5
[^3H]GA ₄	A	96.3	0.3	2.5	0.2	0.5
	B	12.1	53.7	8.7	10.1	15.3
[^3H]GA ₅	A	96.8	0.8	1.4	0.2	0.6
	B	13.3	43.0	20.1	17.8	3.1
[^3H]GA ₂₀	A	96.3	0.7	0.3	2.1	0.5
	B	27.2	34.0	9.2	25.4	3.3
[^3H]GA ₉ ^a	A	92.6	1.8	0.2	0.1	0.1
	B	14.3	8.1	43.4	27.9	6.2
[^3H]GA ₁₂ ald.	A	99.2	0.1	0.2	0.1	0.4
	B	5.5	22.5	30.3	28.7	12.9
[^3H]GA ₁₄	A	98.9	0.2	0.2	0.1	0.5
	B	7.2	25.8	3.4	58.2	5.3
[^{14}C]sucrose	A	99.1	0.9	0	0	0
	B	12.0	19.9	29.2	4.7	34.1

^aSimilar results were obtained from [$2,3\text{-}^3\text{H}$]GA₉ and [$17,17'\text{-}^3\text{H}$]GA₉

FIGURE 7

Thin-layer radiochromatograms of acidic ethyl acetate-soluble extracts of apical buds, 24h. after [^3H] GA treatment (0.3 μCi , 5 seedlings per treatment, specific activity 0.13 Ci. mmol^{-1}). Solvent system: benzene - butan-1-ol - acetic acid, 80:20:5 v/v/v. Marker spots indicate Rf of authentic [^3H] GAs.

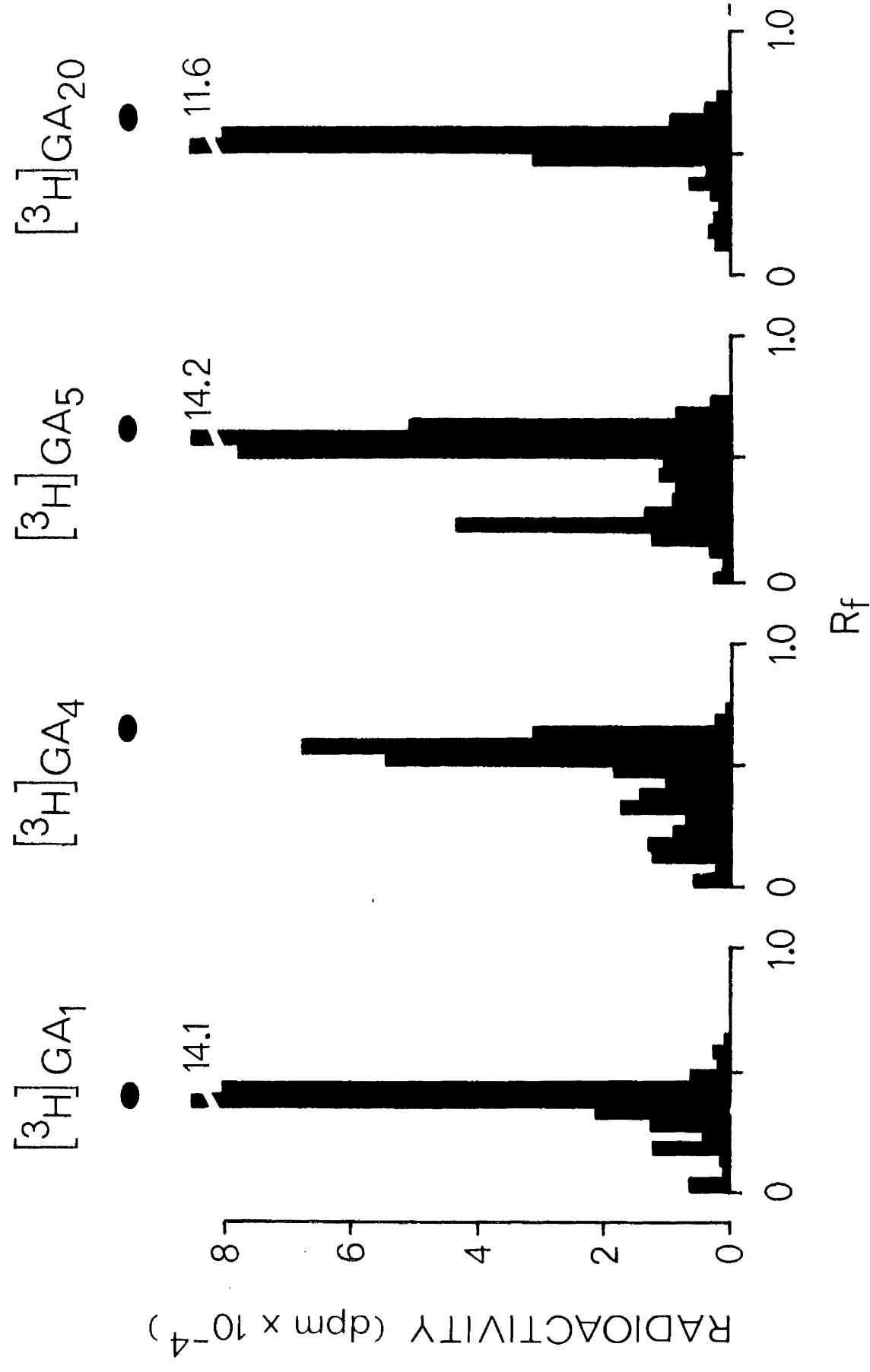
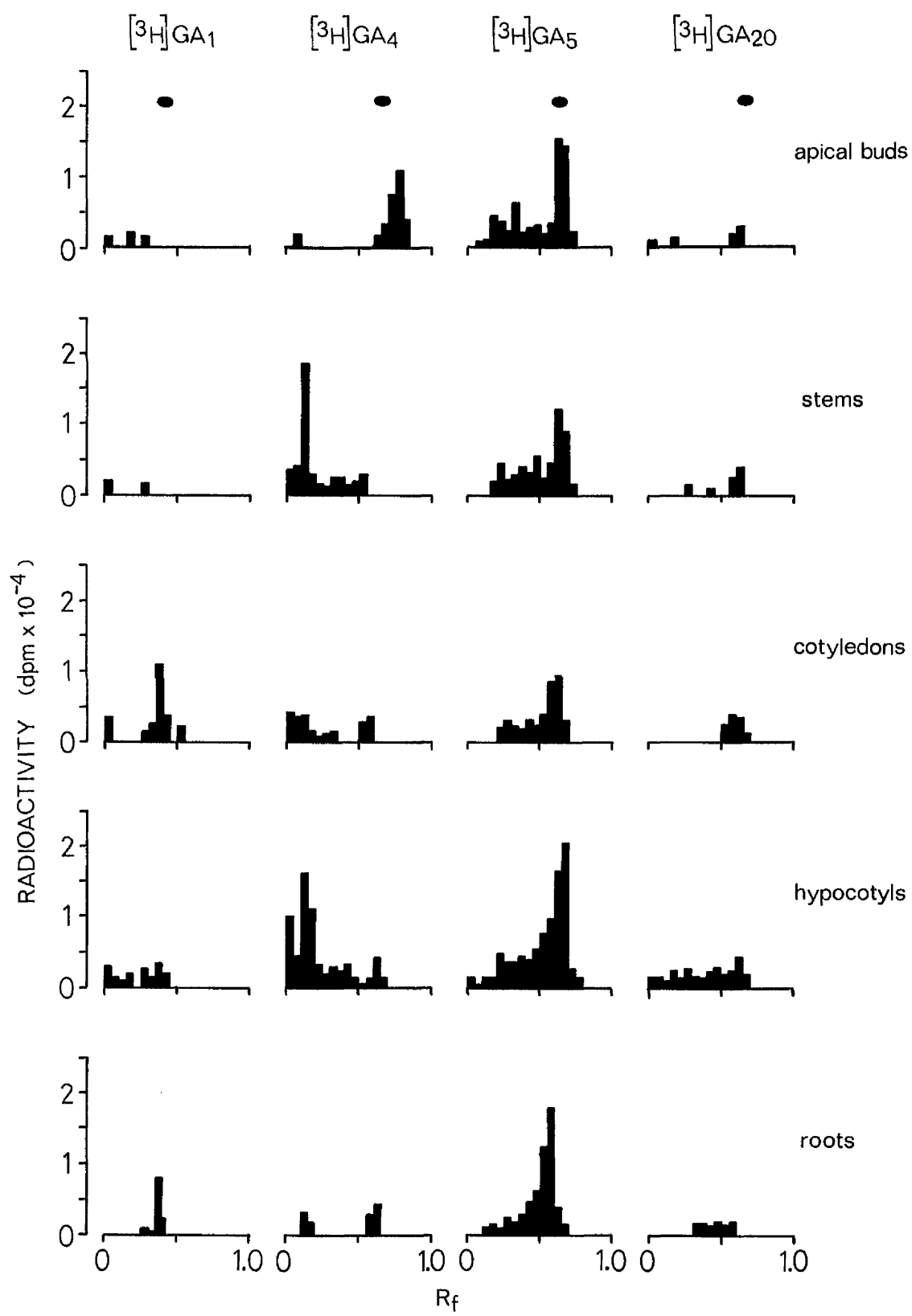


FIGURE 8

Thin-layer radiochromatograms of acidic ethyl acetate-soluble extracts of apical buds, stems, cotyledons, hypocotyls and roots, 24h after basal [^3H]GA treatment (0.3 μCi , 5 seedlings per treatment, specific activity 0.13 Ci.mmol^{-1}). Solvent system: benzene - butan-1-ol - acetic acid, 80:20:5 v/v/v. Marker spots indicate R_f of authentic [^3H]GAs.



were maintained at 25°C for 24h, and treated organs were then separated from the remainder of the plant. Acidic ethyl acetate-soluble extracts of the treated and untreated parts, and of the isolated organs and their incubation solutions, were obtained and examined by TLC (Fig. 9).

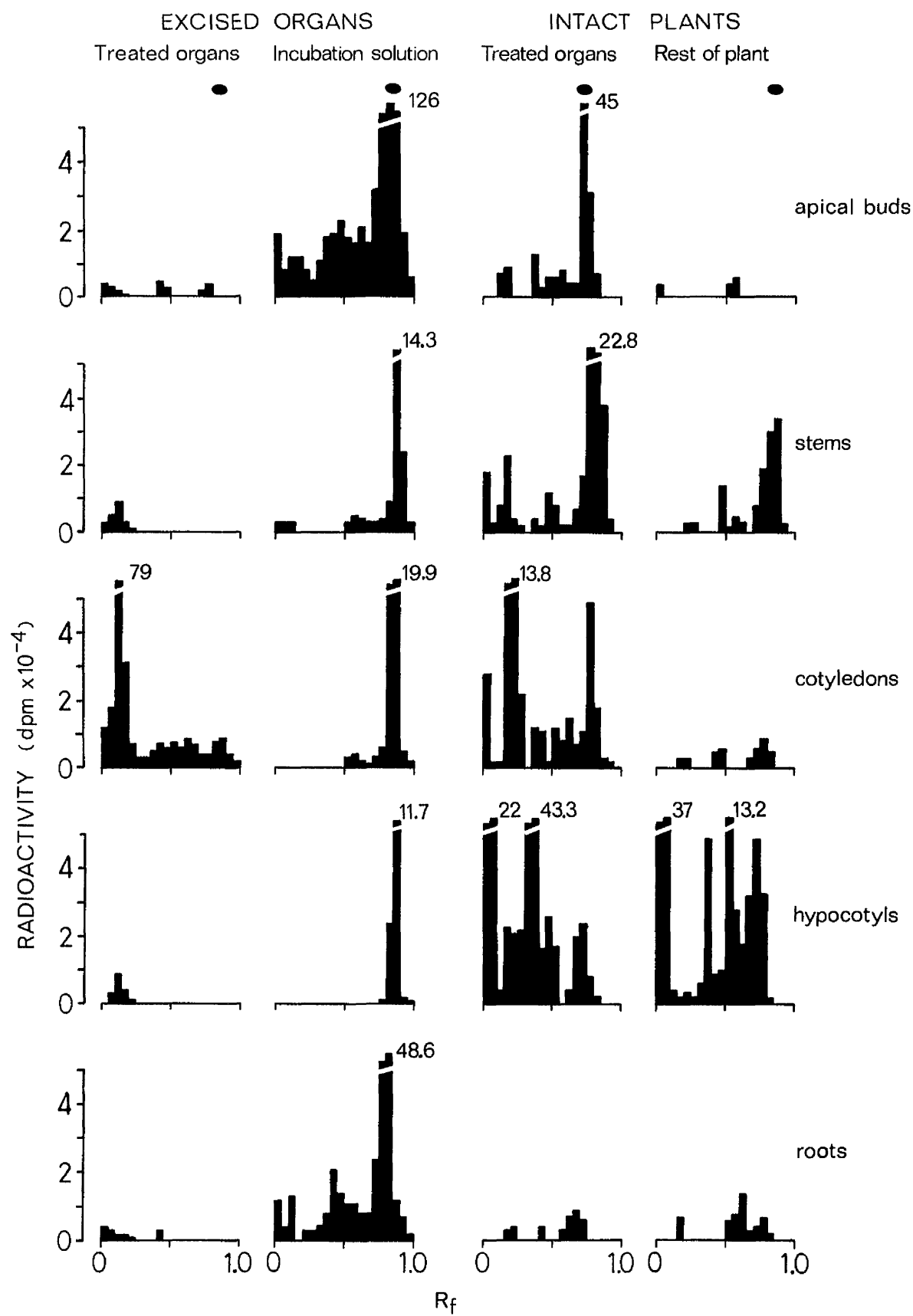
The radioactivity lost from the isolated sections to the incubation medium was associated almost entirely with [^3H]GA₄, and represented a large proportion of the applied dose, considerably more than was exported to the remainder of the plant by the corresponding attached organs in the control experiment. The major radioactive component found in isolated sections, however, was more polar than [^3H]GA₄, and was also found in the incubation solution after treatment of isolated apical buds and roots. In intact seedlings, in contrast, the polar compound was a minor component, except after application of [^3H]GA₄ to cotyledons or hypocotyls. A more complex pattern of metabolites was obtained from intact plants than from isolated organs, from which it appeared that exchange of metabolites between different parts of the plant, as well as localised conversions, determined the spectrum of products obtained.

5. Metabolism of [^3H]GAs by intact seedlings

After basal application of [^3H]GAs, a considerably smaller percentage of the applied radioactivity could be recovered at 24h than after apical treatment (Table 7), and in the case of [^3H]GA₁, [^3H]GA₄, [^3H]GA₅ and [^3H]GA₂₀, thin-layer chromatography (figs. 7,8) suggested that the conversion of the applied [^3H]GA to other acidic ethyl acetate-soluble products was more rapid after basal than after apical treatments. Since it therefore appeared that more rapid metabolism of applied [^3H]GAs resulted from basal than from apical application, plants were supplied basally in all subsequent experiments intended to establish the identity of the metabolites of tritiated GA₁, GA₄, GA₅, GA₈, GA₉, GA₁₂ aldehyde, GA₁₄ and GA₂₀. The effects of time and of dose size on the metabolism of each GA were investigated in advance of large-scale feeds from which

FIGURE 9

Thin-layer radiochromatograms of acidic ethyl acetate-soluble extracts of excised organs and their incubation solutions, and of corresponding treated and untreated parts of intact plants, 24h after application of [^3H]GA $_4$ (0.2 μCi per treatment, specific activity 1.87 Ci.mmol $^{-1}$). Solvent system: benzene-butan-1-ol - acetic acid, 80:20:5 v/v/v. Marker spots indicate Rf of [^3H]GA $_4$.



a yield of metabolites sufficiently high for characterisation by mass spectrometry could be obtained.

The sequence of purification procedures used in the large-scale experiments, together with the sample capacity and reduction in sample dry weight associated with each step, are summarised in Table 8. In some cases, where the viscosity of crude acidic ethyl acetate-soluble extracts prevented their introduction into the gel permeation chromatograph, an additional purification step, filtration of the crude extract in acetone solution over a bed of charcoal-celite mixture, was included.

The gel permeation system (Reeve and Crozier, 1976) provided the greatest quantitative reduction in sample dry weight, but was also of considerable analytical value in determining the distribution of radioactivity in extracts in relation to molecular weight. The retention times of authentic [^3H] GAs were found to be reproducible and to increase with decreasing molecular weight. The greater proportion of the radioactivity in acidic ethyl acetate-soluble extracts was confined to an elution zone delimited by GA₈ (m.wt. 364) and GA₉ (m.wt. 316). However, in some cases a second, distinct zone of radioactivity eluted considerably in advance of the first, indicating the presence of radioactive compounds of molecular weight greater than 364 amu. Unlike the free [^3H] GAs, the high molecular weight (HMW) fraction showed extremely high retention times ($k' > 10$) in the preparative HPLC system, and in the TLC solvent system used its R_f was typically less than 0.2, in contrast to the free GAs tested, which were considerably less polar. It was considered likely that this fraction contained neutral GA glucosyl esters, normally obtained from the crude acidic ethyl acetate-soluble extract by partition at pH 7.0 (Hiraga *et al.*, 1972; Takahashi, 1974). After treatment with β -glucosidase, the behaviour of the HMW fraction corresponded to that of free acidic GAs,

and a similar change in the thin-layer chromatographic characteristics of a sample of authentic GA_4 glucosyl ester could be brought about by enzymatic hydrolysis under the same conditions.

The acidic and neutral butanol-soluble fractions obtained in large-scale experiments also yielded free acidic GAs on enzymatic hydrolysis. On the basis of partition characteristics (see Takahashi, 1974) these fractions are expected to contain GA glucosyl ethers (also referred to as GA glucosides in the literature) and polar glucosyl esters respectively. However, in small-scale experiments, the amount of radioactivity remaining in the buffer phase after the initial extraction with ethyl acetate at pH 2.5 was usually too small to permit further fractionation and hydrolysis, and no further attempt was therefore made to identify the radioactive components of the crude aqueous fraction in these initial experiments.

5.1 $[^3\text{H}]\text{GA}_1$ feeds

5.1.1 Effect of time

Twenty seedlings were treated with $9.0 \mu\text{Ci } [^3\text{H}]\text{GA}_1$ (1.0×10^6 dpm per seedling, specific activity 30 Ci.mmol^{-1}) and sets of five seedlings were harvested 0.5, 2, 8 and 24h after treatment. Most of the recovered radioactivity was associated with the acidic ethyl acetate-soluble fraction (Table 9), and gel permeation chromatography showed that this was associated with compounds in the elution range of free GAs. According to the time of extraction, up to three radioactive components (A, B and C) could be resolved by preparative HPLC (Fig. 10). Quantitative estimates of the radioactivity in A and B are given in Table 10. On the basis of retention times, A appeared to be $[^3\text{H}]\text{GA}_1$, while B corresponded to $[^3\text{H}]\text{GA}_8$. The minor component C, which did not correspond to any available $[^3\text{H}]\text{GA}$ standard, failed to appear in subsequent experiments and may therefore be a transient intermediate, or, more probably, an artefact of the chromatographic procedure.

TABLE 7

Percentage recovery of non-volatile radioactivity in methanolic extracts, 24h after application of [^3H]GAs to apical buds (A) or hypocotyls (B). Specific activities adjusted to provide a uniform dose of 5 μg per 5 seedlings.

	A	B
[^3H]GA ₁	46.6	6.7
[^3H]GA ₄	28.7	4.7
[^3H]GA ₅	54.1	30.8
[^3H]GA ₂₀	38.7	5.3
[^3H]GA ₉	44.8	22.9
[^3H]GA ₁₂ aldehyde	71.4	45.4
[^3H]GA ₁₄	48.9	34.0

TABLE 8

Sequence of purification procedures, sample capacity and reduction in sample dry weight obtained at each step.

	sample capacity	sample dry weight ^a
methanolic tissue extract		
acidic ethyl acetate-soluble fraction		700 mg
gel permeation chromatography	1 g	300 mg
charcoal adsorption chromatography	500 mg	60 mg
preparative HPLC	100 mg	300 µg
analytical HPLC	0.5 mg	10 µg
mass spectrometry	0.5 - 1 µg minimum required for satisfactory MS	
^a Data obtained from extract of 200 seedlings, dry weight approximately 300 g.		

TABLE 9

[^3H]GA₁ feeds. Variation with time of radioactivity
(dpm $\times 10^{-6}$) in solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	42.6	2.11	0.01	-
2	42.5	2.10	0.01	0.01
8	13.5	0.67	0.05	-
24	6.4	0.31	-	-

TABLE 10

[^3H]GA₁ feeds. Variation with time of acidic ethyl
acetate-soluble metabolites (radioactivity, dpm $\times 10^{-6}$,
estimated from HPLC analysis, Fig. 10). Zone A, pre-
sumptive GA₁; Zone B, presumptive GA₈.

Time (h)	Radioactivity zone	
	A	B
0.5	1.94	-
2	1.44	0.16
8	0.48	0.05
24	0.12	-

5.1.2 Effect of dose size

The specific activity of [^3H]GA₁ (30 Ci.mmol⁻¹) was
adjusted by addition of carrier GA₁ (Table 11). The treat-
ments summarised in Table 11 were harvested after 18h. Most of
the recovered radioactivity was again associated with the acidic

TABLE 11

[³H]GA₁ feeds. Adjustment of specific activity.

Treatment	Seedlings	Dose per treatment		μg per seedling ^a
		[³ H]GA ₁ (μCi)	GA ₁ (μg)	
1	5	2.5	25	5
2	5	2.5	5	1
3	5	2.5	0	-

^a Mass associated with 0.5 μCi [³H]GA₁ disregarded.

TABLE 12

[³H]GA₁ feeds. Variation with increasing applied mass of radioactivity (dpm x 10⁻⁶) in various solvent fractions.

Treatment	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
1	17.3	0.92	0.01	-
2	6.4	0.32	0.01	-
3	6.6	0.34	0.01	-

TABLE 13

[³H]GA₁ feeds. Variation with increasing applied mass of acidic ethyl acetate-soluble metabolites (radioactivity, dpm x 10⁻⁶, estimates from HPLC analysis Fig. 11). Zone A, presumptive GA₁; zone B, presumptive GA₈.

Treatment	Radioactivity zone	
	A	B
1	0.88	0.03
2	0.28	0.01
3	0.33	-

ethyl acetate-soluble fraction (Table 12). No radioactivity could be detected in the HMW region of GPC eluates, and the acidic ethyl acetate-soluble fraction appeared to contain only the two radioactive components A and B (Fig. 11). The level of radioactivity associated with B was approximately similar for all three treatments (Table 13), although on the basis of the specific activity of the applied [^3H]GA₁ it was assumed that this represented the conversion of increasing mass in treatments 1 and 2. No qualitative changes in the spectrum of [^3H]GA₁ metabolites resulted from increasing the applied mass.

5.1.3 Identity of [^3H]GA₁ metabolites

Two hundred seedlings were treated with 5.0 mg GA₁ containing 15.0 μCi [^3H]GA₁, and harvested after 18h. Radioactivity was associated with the light petroleum fraction (32.0×10^3 dpm, discarded), the acidic ethyl acetate-soluble fraction (4.50×10^6 dpm), the acidic butanol-soluble (1.25×10^6 dpm) and neutral butanol-soluble fractions (0.48×10^6 dpm), representing a recovery of 15.8% of the applied radioactivity.

Acidic ethyl acetate-soluble fraction

Acidic ethyl acetate-soluble components eluted from the GPC column in the LMW elution range only (Fig. 12). The pooled fractions 11 - 20 (4.20×10^6 dpm) were chromatographed in the preparative HPLC system (Fig. 13). Only one zone of radioactivity (A, 3.07×10^6 dpm) could be detected and this had the same retention time as [^3H]GA₁. The benzyl ester of A co-chromatographed with authentic [^3H]GA₁ benzyl ester in the preparative HPLC system ($k' = 1.42$ in ethyl acetate - hexane, 55:45), and also in the analytical HPLC

FIGURE 10

[³H]GA₁ feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/25 aliquots), 0.5, 2, 8 and 24h after treatment.

Five seedlings, 1.75 µCi per treatment.

Column: 10 x 450 mm. Partisil 20

Stationary phase: 40% 0.5M formate

Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)

Flow rate: 5.0 ml.min⁻¹

Detector: radioactivity monitor, FSD = 600 cpm.

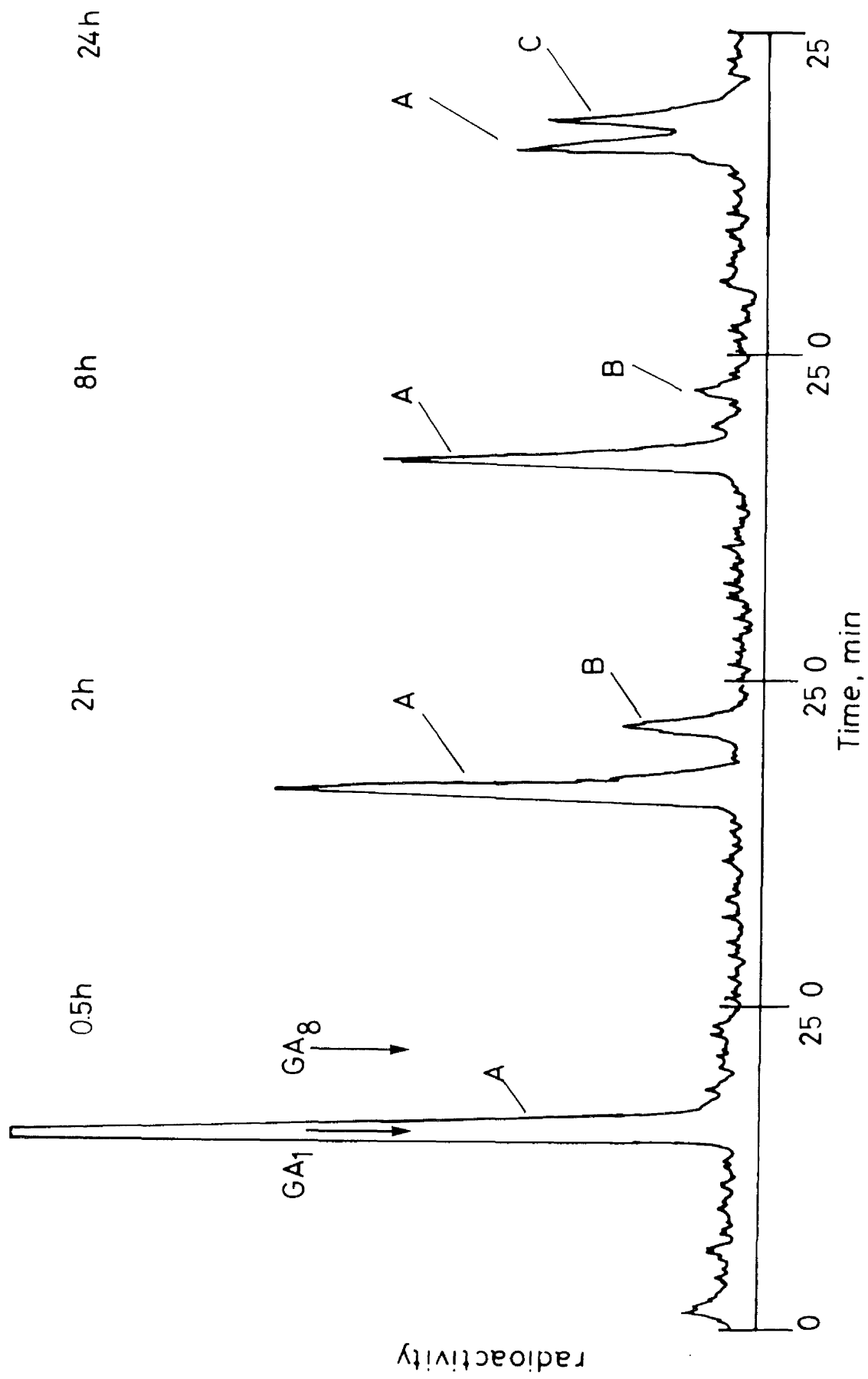
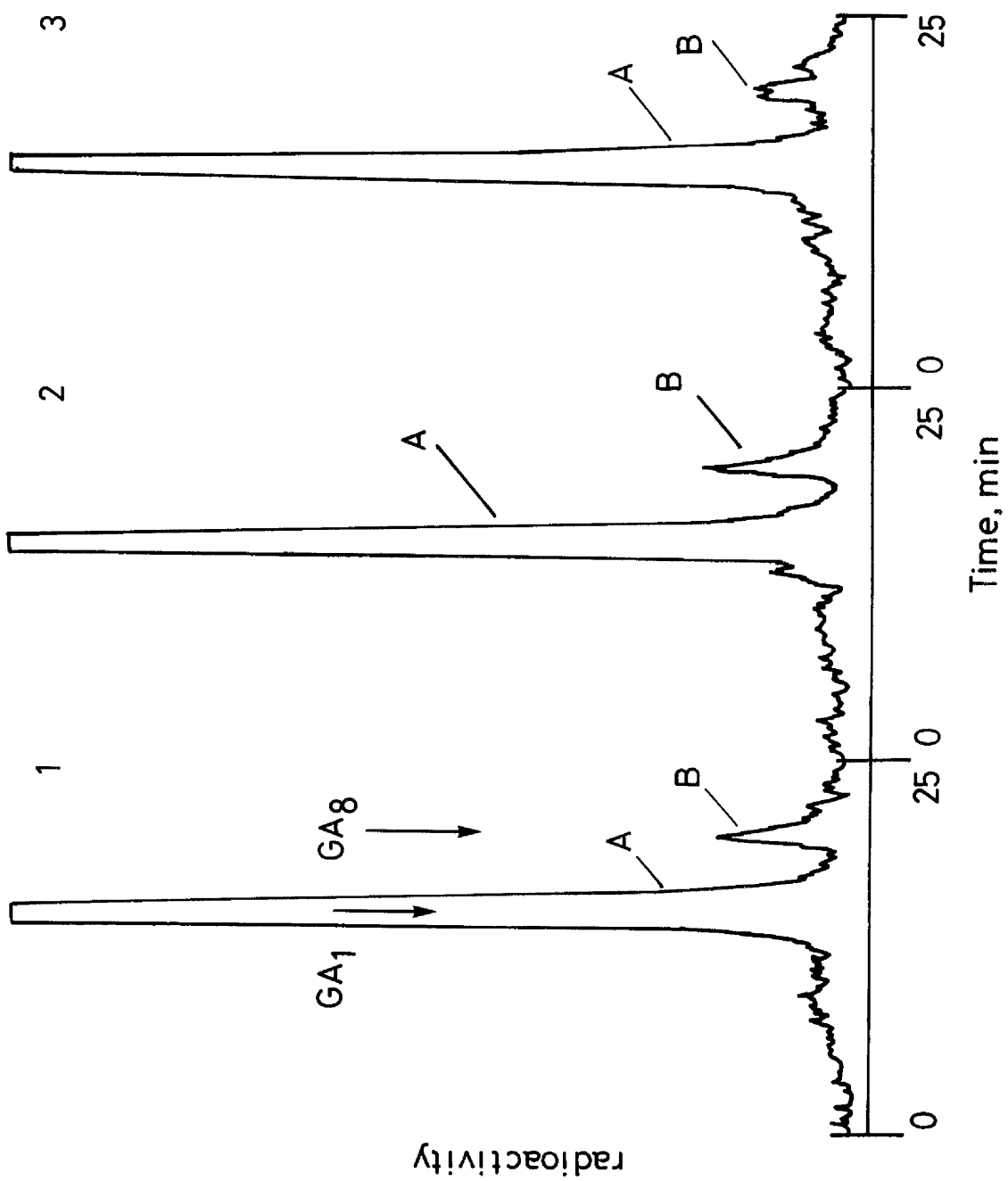


FIGURE 11

[³H]GA₁ feeds.. HPLC (preparative system) of acidic ethyl acetate-soluble fractions, 18h after treatment (5 seedlings 2.5 μCi per treatment).
Specific activities: 1, 34.2 μCi. mol⁻¹; 2, 171.0 μCi. μmol⁻¹; 3, 30 mCi. mmol⁻¹ (see Table 11). One-tenth aliquots.
Column: 10 x 450 mm. Partisil 10
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)
Flow rate: 5.0 ml. min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.



system 1 (Fig. 14). The GA₈-like zone found in the acidic ethyl acetate-soluble fractions obtained from time-course and varying-dose experiments could not be detected in the large-scale extract.

Acidic butanol-soluble fraction

The enzyme-hydrolysed acidic butanol-soluble fraction (1.05×10^6 dpm) could be resolved into two radioactive components by preparative HPLC (Fig. 13). A (0.82×10^6 dpm) had the same retention time as [³H]GA₁, and B (54.4×10^3 dpm) corresponded to [³H]GA₈. The benzyl ester of A co-chromatographed with authentic [³H]GA₁ benzyl ester in the preparative HPLC system ($k' = 1.42$ in ethyl acetate - hexane, 55:45). Analytical HPLC (system 1) resolved A into several UV-absorbing components one of which had a retention time corresponding to that of GA₁ benzyl ester (Fig. 14). This component contained approximately 90% of the recovered radioactivity, and after further purification in system 2, a mass spectrum identical to that of authentic GA₁ benzyl ester was obtained (Fig. 15). An extraneous ion at m/e 207 originates from the dimethyl silicone rubber GC-MS interface. On the basis of its partition characteristics, A is therefore taken to be the aglycone of GA₁ glucosyl ether.

Considerable difficulty was encountered in purifying the esterification products of GA₈-like fractions obtained in this and other experiments. These fractions invariably gave rise to a mixture of radioactive compounds on esterification, presumably as a result of isomerisation of GA₈, and the yields obtained in each case were consequently too low for further investigation. However, the presence of a GA₈-like compound as a product of enzymatic hydrolysis suggests that GA₈ glucosyl ether was perhaps present in the acidic butanol-soluble fraction.

FIGURE 12

[³H]GA₁ feed. Gel permeation chromatogram of acidic ethyl acetate-soluble fraction (1/500 aliquots), 18h after treatment (200 seedlings, 15.0 μ Ci).

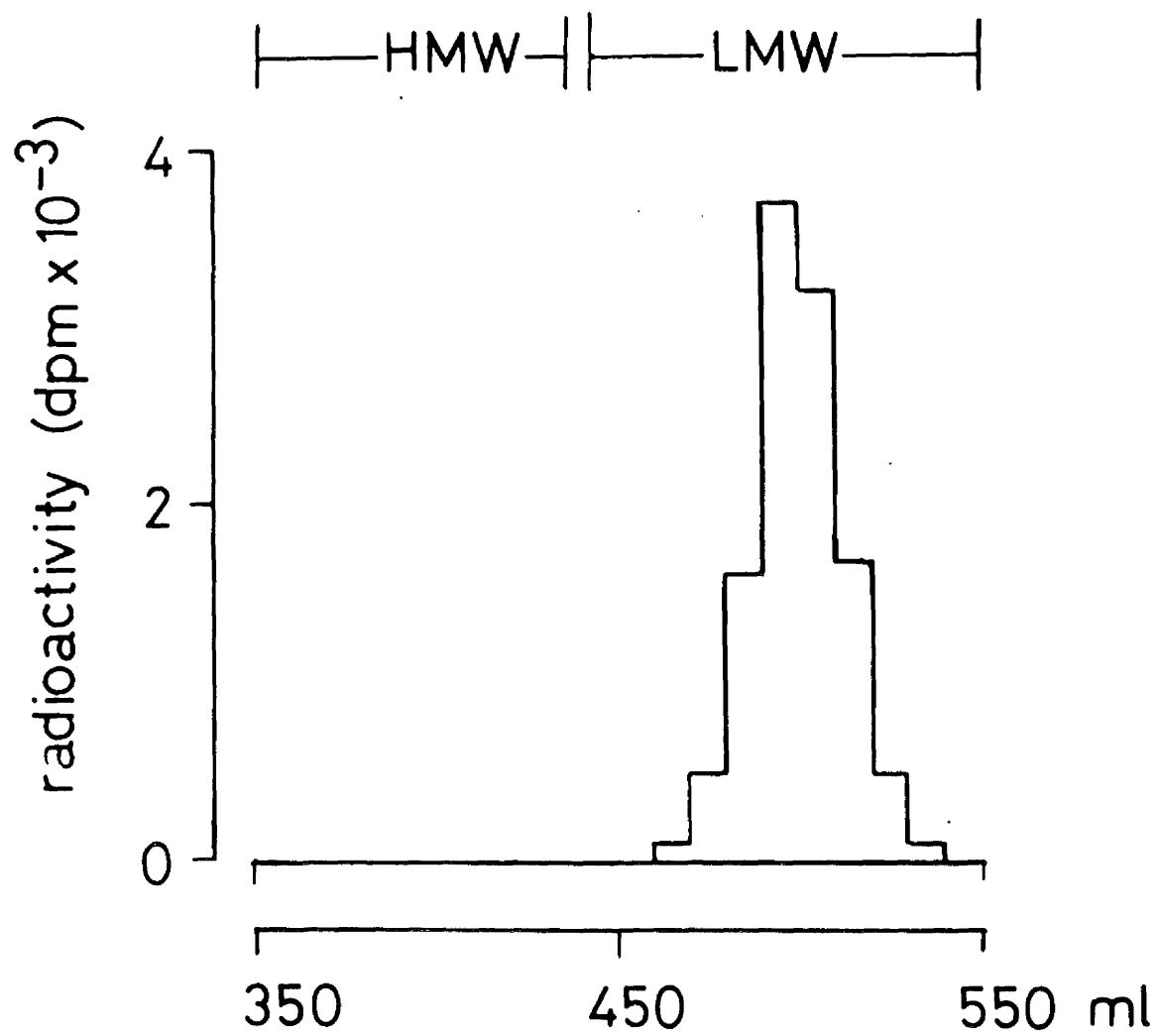


FIGURE 13

[³H]GA₁ feeds. HPLC (preparative system) of
(1) acidic ethyl acetate-soluble fraction (1/100
aliquot), and (2) enzyme-hydrolysed acidic butanol
fraction (1/10 aliquot), 18h after treatment
(200 seedlings, 15.0 µCi).
Column: 10 x 450 mm. Partisil 10
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)
Flow rate: 5.0 ml. min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.

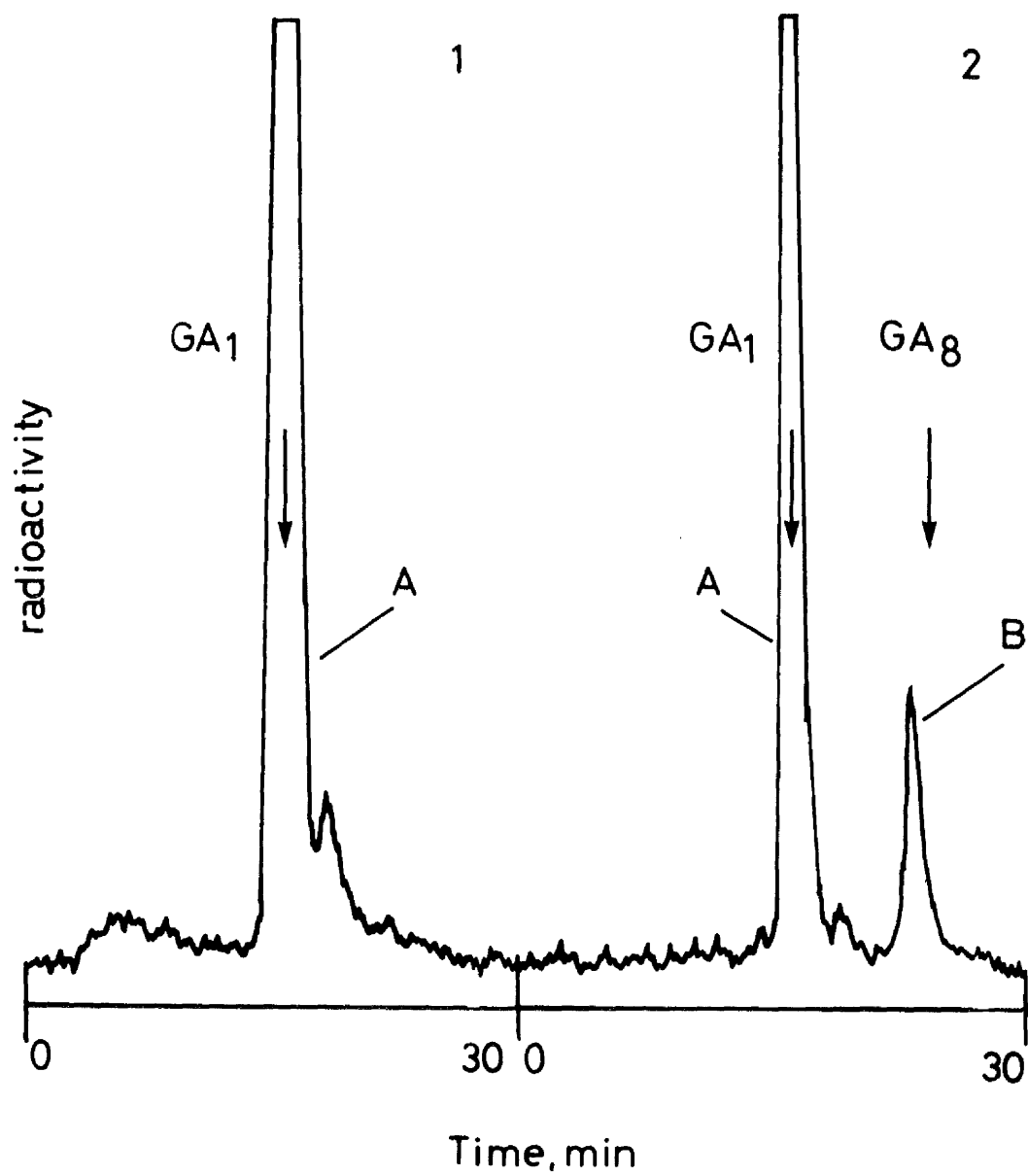


FIGURE 14

[³H]GA₁ feeds. Analytical HPLC of (1) zone A from acidic ethyl acetate-soluble fraction, and (2) zone A from hydrolysed acidic butanol-soluble fraction.
Column: 4.6 x 500 mm. Partisil 10
Mobile phase: tetrahydrofuran - dichloromethane, 8:92
Detector: UV monitor.

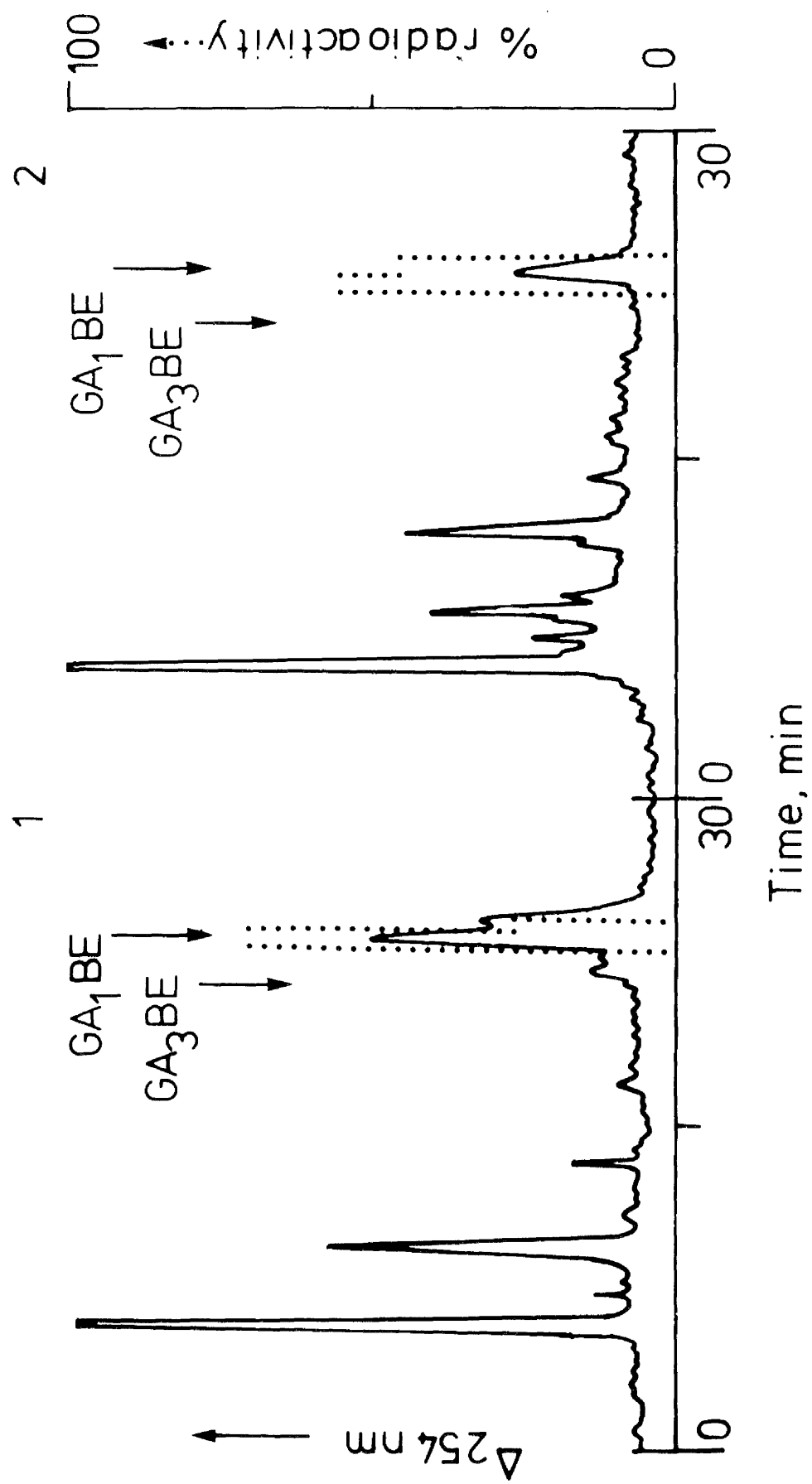
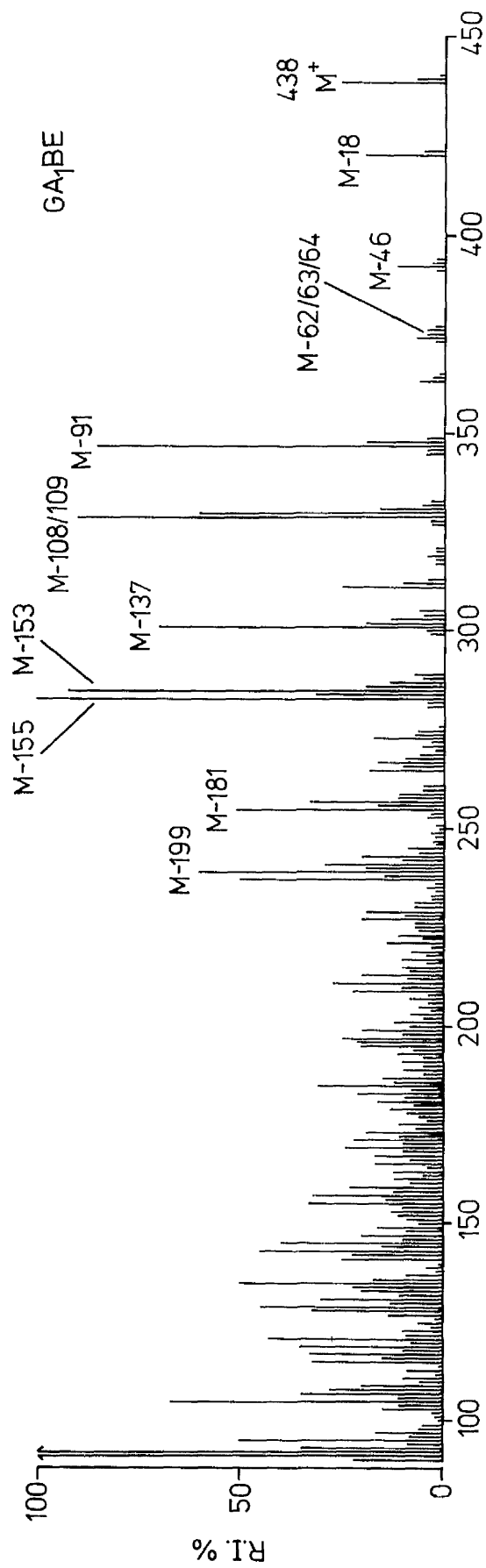


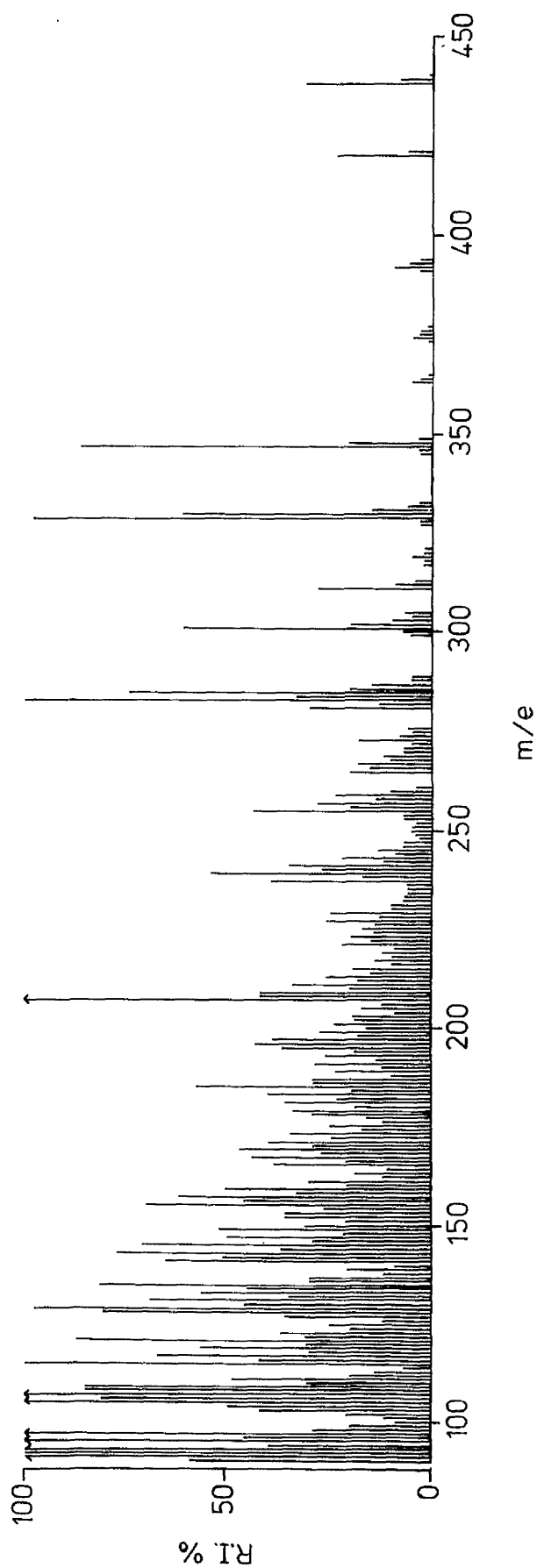
FIGURE 15

[³H]GA₁ feeds. Mass spectra of (1) authentic GA₁ benzyl ester, and (2) purified zone A from hydrolysed acidic butanol-soluble fraction.

1



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5.2 ^3H GA₄ feeds

5.2.1 Effect of time

Twenty seedlings were treated with 8.8 μCi [^3H]GA₄ (0.9×10^6 dpm per seedling, specific activity $1.87 \text{ Ci.mmol}^{-1}$), and sets of five seedlings harvested 0.5, 2, 8 and 24h after treatment. Levels of radioactivity associated with solvent fractions are summarised in Table 14. In contrast to [^3H]GA₁ feeds, a considerable proportion of the acidic ethyl acetate-soluble radioactivity eluted from the GPC column in the HMW region, and this proportion increased with time (Table 15). The overall decrease in acidic ethyl acetate-soluble radioactivity with time was accounted for by a concomitant decrease in the LMW fraction. The LMW and enzyme-hydrolysed HMW fractions were investigated by HPLC (Fig. 16) and in each case the two radioactivity maxima (A and B) which were resolved had similar retention times to GA₄ and GA₁ respectively. B increased with time in both the LMW and HMW fractions, apparently at the expense of A (Table 16).

TABLE 14

[^3H]GA₄ feeds. Variation with time of radioactivity (dpm $\times 10^{-6}$) in various solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	53.6	2.65	0.08	-
2	54.8	2.75	0.01	-
8	44.2	2.19	0.01	-
24	32.2	1.50	0.11	-

TABLE 15

[³H]GA₄ feeds. Variation with time of HMW and LMW fractions of acidic ethyl acetate-soluble extracts. Estimates (dpm x 10⁻⁶) based on radioactivity maxima in GPC eluates

Time (h)	HMW fraction	LMW fraction
0.5	0.04	2.29
2	0.38	1.23
8	0.61	1.57
24	0.54	1.11

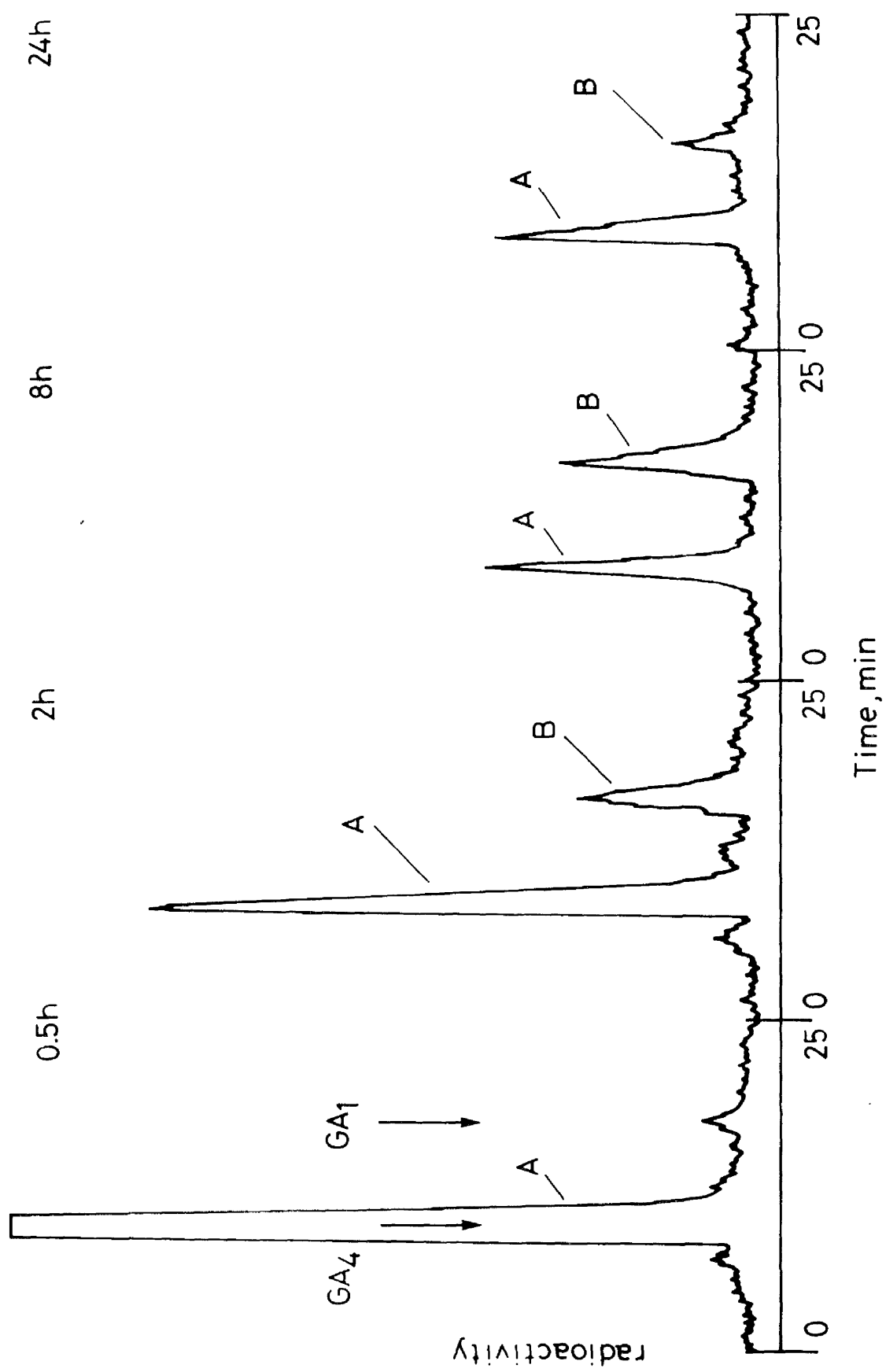
TABLE 16

[³H]GA₄ feeds. Variation with time of acidic ethyl acetate-soluble metabolites (radioactivity, dpm x 10⁻⁶, estimated from HPLC analysis, Fig. 16). Zone A, presumptive GA₄; Zone B, presumptive GA₁)

Time (h)	HMW fraction		LMW fraction	
	A	B	A	B
0.5	-	-	2.15	0.04
2	0.09	0.11	0.61	0.20
8	0.07	0.32	0.45	0.20
24	0.08	0.33	0.50	0.25

FIGURE 16

[³H]GA₄ feeds.. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/50 aliquots), 0.5, 2, 8 and 24h after treatment (five seedlings, 2.2 µCi per treatment).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate..
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)
Flow rate: 5.0 ml. min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.



5.2.2 Effect of dose size

The specific activity of [^3H]GA₄ (1.87 Ci.mmol⁻¹) was adjusted as in Table 17. Each treatment was harvested at 18h and the distribution of radioactivity between solvent fractions is shown in Table 18.

TABLE 17

[^3H]GA₄ feeds. Adjustment of specific activity

Treatment	Seedlings	Dose per treatment		μg per seedling
		[^3H] GA ₄ (μCi)	GA ₄ (μg)	
1	5	2.5	25	5.1
2	5	2.5	5	1.1
3	5	2.5	0	0.1

TABLE 18

[^3H]GA₄ feeds. Variation with increasing applied mass of radioactivity (dpm x 10⁻⁶) in various solvent fractions.

Treatment	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
1	28.8	1.49	0.02	-
2	29.3	1.42	0.10	0.02
3	20.4	0.96	0.11	-

Although the amounts of radioactivity in the LMW acidic ethyl acetate-soluble fraction increased with applied mass, this trend was reversed in the HMW fraction (Table 19) where radioactivity yields slightly decreased with decreasing specific activity.

FIGURE 17

[³H]GA₄ feeds. . HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/50 aliquots), 18h after treatment (five seedlings, 2.5 μCi per treatment). Specific activities: 1, 33.0 μCi. mol⁻¹; 2, 147.0 μCi. μmol⁻¹; 3, 1.87 mCi. μmol⁻¹ (See Table 17).

Column: 10 x 450 mm Partisil 20

Stationary phase: 40% 0.5M formate

Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)

Flow rate: 5.0 ml. min⁻¹

Detector: radioactivity monitor, FSD = 600 cpm.

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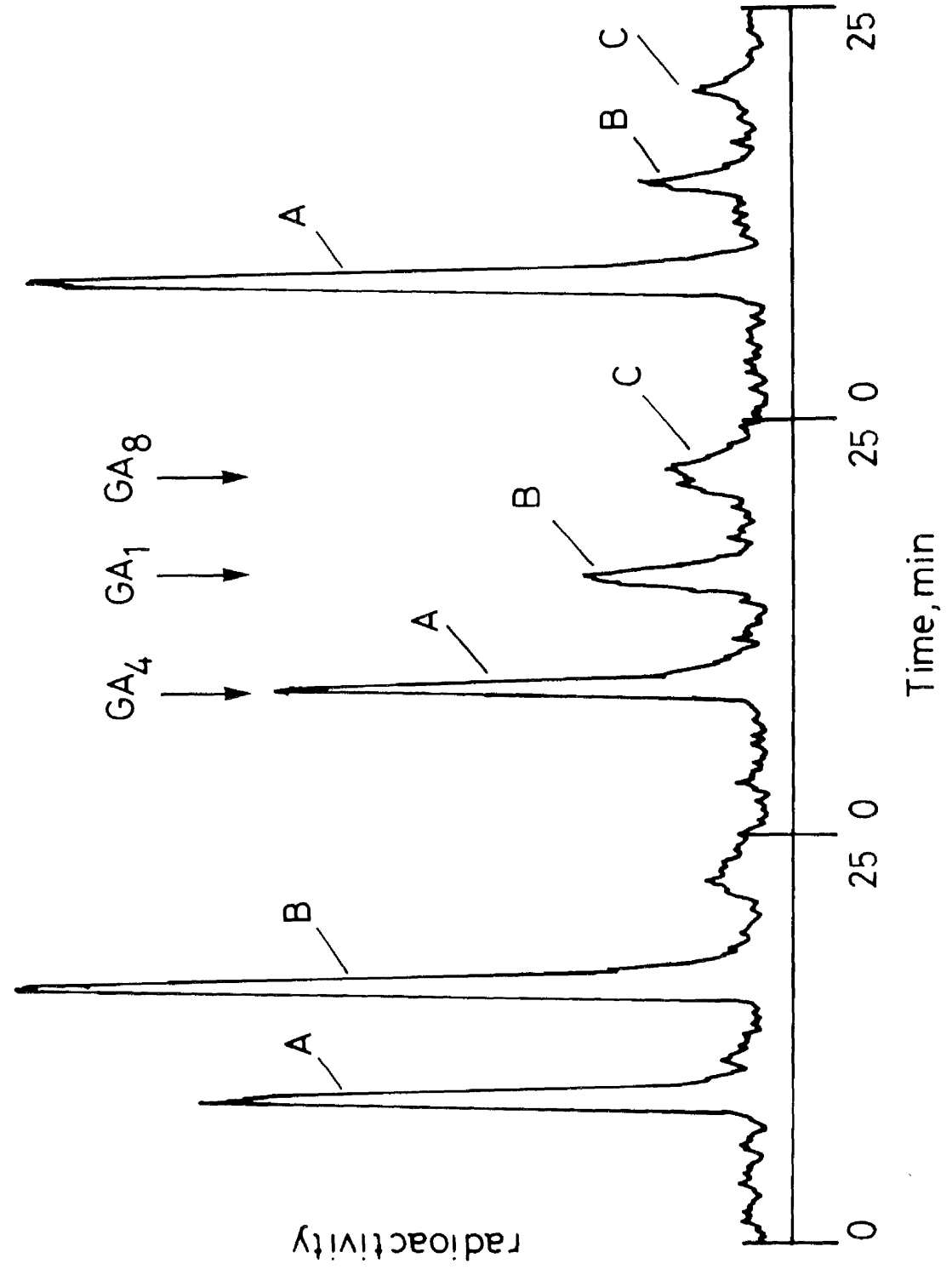


TABLE 19

[^3H]GA₄ feeds. Variation with applied mass of HMW and LMW fractions of acidic ethyl acetate-soluble extracts. Estimates (dpm $\times 10^{-6}$) based on radioactivity maxima in GPC eluates.

Treatment	HMW fraction	LMW fraction
1	0.28	1.42
2	0.32	1.08
3	0.35	0.58

On HPLC analysis of the LMW and enzyme-hydrolysed HMW fractions, two radioactive zones (A and B), corresponding to those observed in previous experiments, were resolved (Fig. 17). In addition, a third, more polar component C, with retention time corresponding to GA₈, appeared in LMW fractions. Quantitative estimates of the radioactivity associated with these metabolites are given in Table 20. Although until B and C had been positively identified it was not possible to relate dose size to metabolite mass, on the basis of the specific activity of the applied [^3H]GA₄ and its rate of disappearance it appeared that seedlings readily metabolised GA₄ at all levels supplied and produced qualitatively similar products in each case. However, the amount of B obtained in the LMW fraction increased more markedly with applied mass than did B obtained by hydrolysis from the HMW fraction.

5.2.3 Identity of [^3H]GA₄ metabolites

Two hundred seedlings were treated with 1.0 mg GA₄ containing 50 μCi [^3H]GA₄, and harvested after 24h. Radioactivity was

associated with the light petroleum fraction (0.16×10^6 dpm, discarded), the acidic ethyl acetate-soluble (36.56×10^6 dpm), acidic butanol-soluble (2.77×10^6 dpm) and neutral butanol-soluble fractions (0.12×10^6 dpm, discarded), representing a recovery of 39.1% of the applied dose.

TABLE 20

$[^3\text{H}]\text{GA}_4$ feeds. Variation according to applied mass of acidic ethyl acetate-soluble metabolites (radioactivity, $\text{dpm} \times 10^{-6}$, estimated from HPLC analysis, Fig. 17).*

Treatment	HMW fraction		LMW fraction		
	A	B	A	B	C
1	0.16	0.19	0.47	0.87	0.05
2	0.08	0.21	0.72	0.36	-
3	0.09	0.20	0.49	0.04	0.02

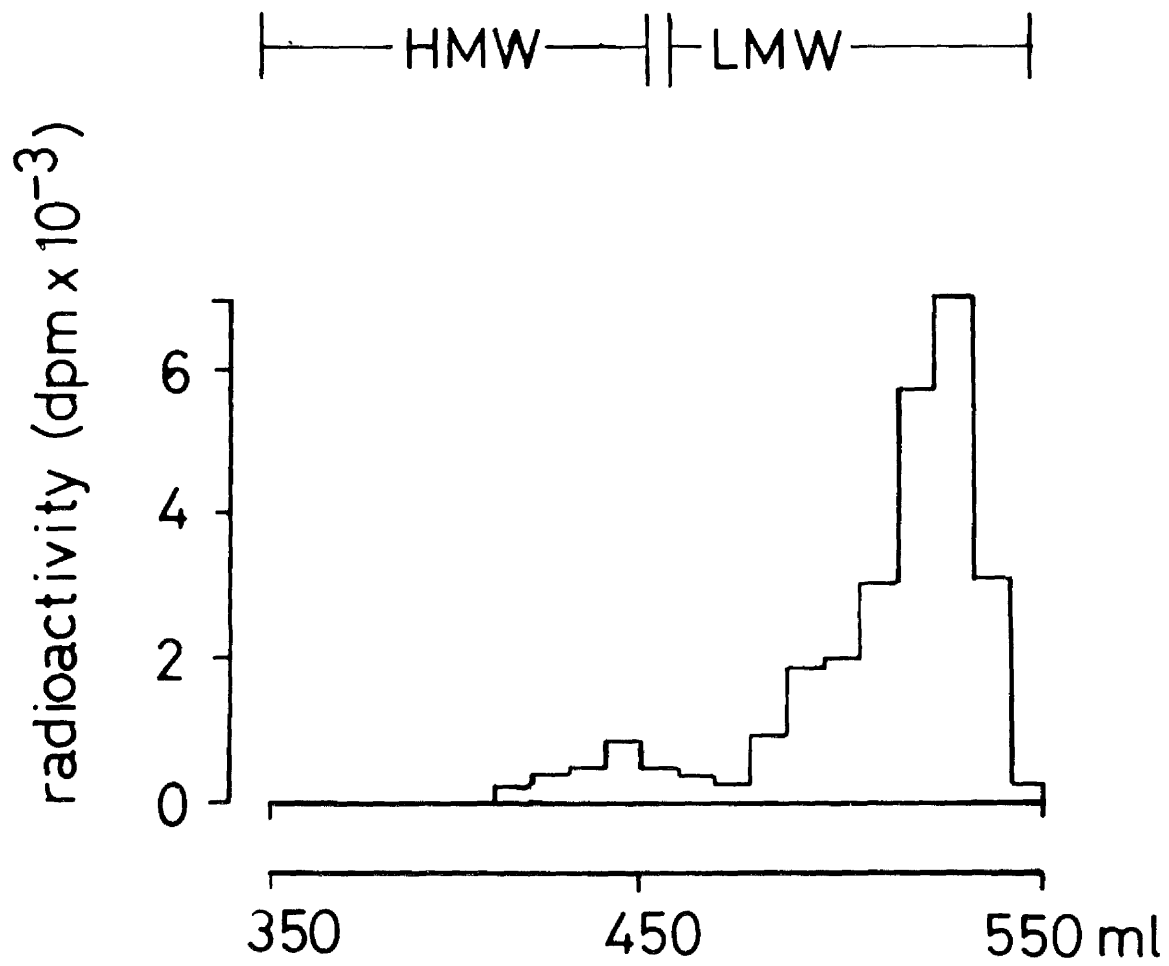
* Zone A, presumptive GA_4 ; Zone B, presumptive GA_1 ; Zone C, presumptive GA_8 .

Acidic ethyl acetate-soluble fraction

This fraction was resolved by GPC to provide HMW and LMW components (Fig. 18): HMW, fractions 7 - 12, 2.46×10^6 dpm; LMW, fractions 13 - 21, 27.27×10^6 dpm. One-twentieth of the HMW fraction was further purified by preparative TLC on silica gel (solvent system, chloroform - methanol, 3:1 v/v) and was recovered as one zone of radioactivity at R_f 0.2 - 0.3. The eluted radioactive zone (0.09×10^6 dpm) was redissolved in 25 μl 80% ethanol and 5 μl of the ethanolic solution applied to each of 5 seedlings, which were harvested and extracted after 24h. All of

FIGURE 18

[³H]GA₄ feeds. Gel permeation chromatogram
of acidic ethyl acetate-soluble fraction (1/1000
aliquots), 24h after treatment (200 seedlings,
50.0 µCi).



the recovered radioactivity was associated with the acidic ethylacetate-soluble fraction. When examined by HPLC, this fraction was found to contain a single radioactive component with retention time corresponding to that of GA₁ (Fig. 19), and TLC showed that radioactivity was no longer associated with the zone at Rf 0.2 - 0.3. The remainder of the HMW fraction was hydrolysed with β -glucosidase and yielded 1.35×10^6 dpm of acidic ethyl acetate-soluble radioactivity, from which three radioactivity maxima could be resolved by preparative HPLC (Fig. 20). The retention times of A (0.88×10^6 dpm) and B (0.01×10^6 dpm) corresponded to those of GA₄ and GA₁ respectively, but D (0.09×10^6 dpm) which was intermediate in polarity between A and B, could not be related to any available standard. A and D derivatised and A was found to contain one radioactivity maximum co-chromatographing with GA₄ benzyl ester in the preparative HPLC system ($k' = 1.52$ in ethyl acetate - hexane, 35:65). The radioactivity maximum co-chromatographed with GA₄ benzyl ester in analytical HPLC systems 1 and 2 (Fig. 21). Since A was derived from the HMW acidic ethyl acetate fraction, it is taken to be the glucosyl ester of GA₄ or a closely similar GA.

D appeared to contain a single radioactivity maximum ($k' = 0.42$ in ethyl acetate - hexane, 65:35), but when re-examined by preparative HPLC under different solvent conditions, two radioactive components (D₁, D₂) were resolved ($k'_1 = 1.63$, $k'_2 = 2.31$ in ethyl acetate - hexane, 35:65) which again could not be related to available GA benzyl ester standards.

The amount of B obtained by hydrolysis was not adequate for further analysis and thus its apparent identity with GA₁ could not be confirmed. However, on the basis of the known partition

characteristics of GA₁ glucosyl ester and GA₁ glucosyl ether, it seems unlikely that B could be derived from either of these compounds, since the conjugate from which B originated was soluble in ethyl acetate at pH 2.5. In contrast, GA₁ glucosyl ester partitions into butan-1-ol at neutral pH and GA₁ glucosyl ether is found in the acidic butanol-soluble fraction (see Takahashi, 1974).

The LMW fraction of the acidic ethyl acetate-soluble extract yielded three zones of radioactivity when fractionated in the preparative HPLC system (Fig. 20). These zones (A, 5.97×10^6 dpm; B, 1.90×10^6 dpm; C, 1.0×10^6 dpm) had retention times corresponding to GA₄, GA₁ and GA₈ respectively. Zones A and B were treated with derivatising reagent and purified by preparative HPLC. Zone A contained a single radioactivity maximum co-chromatographing with [³H]GA₄ benzyl ester ($k' = 0.78$ in ethyl acetate - hexane, 60:40). In analytical HPLC system 1, the radioactivity associated with A co-chromatographed with authentic GA₄ benzyl ester (Fig. 21). After further purification in system 2, A provided a mass spectrum identical to that of GA₄ benzyl ester (Fig. 22). An extraneous ion at m/e 149 represents phthalate impurity from the HPLC septum. Zone B contained one radioactivity maximum co-chromatographing with GA₁ benzyl ester in the preparative HPLC system ($k' = 0.50$ in ethyl acetate - hexane, 55:45), and also in analytical system 1 (Fig. 21). The UV-absorbing peak corresponding to the radioactivity maximum was further purified in system 2, after which a mass spectrum identical to that of GA₁ benzyl ester was obtained (see Fig. 15).

Acidic butanol-soluble fraction

The acidic butanol-soluble fraction was hydrolysed with β -glucosidase and 0.41×10^6 dpm recovered in ethyl acetate from the hydrolysate. Three zones of radioactivity were resolved in the

preparative HPLC system (Fig. 20): A, 30.0×10^3 dpm; B, 0.18×10^6 dpm; C, 0.16×10^6 dpm). These had retention times corresponding to GA₄, GA₁, and GA₈ respectively. A and B were derivatised, and A was found to differ from GA₄ benzyl ester (GA₄ benzyl ester, $k' = 0.63$; A, $k' = 2.47$ in ethyl acetate-hexane, 45:55). However, B co-chromatographed with authentic GA₁ benzyl ester in the preparative HPLC system ($k' = 1.42$ in ethyl acetate - hexane, 45:55), and B may therefore be the aglycone of GA₁ glucosyl ester.

5.3 [³H]GA₅ feeds

5.3.1 Effect of time

Twenty seedlings were treated with $10.0 \mu\text{Ci}$ [³H]GA₅ (1.1×10^6 dpm per seedling, specific activity 5.3 Ci.mmol^{-1}), and sets of five seedlings were harvested at 0.5, 2, 8 and 24h. On extraction, it was found that the levels of radioactivity in the light petroleum and crude aqueous fractions did not exceed background values. Although radioactivity in the acidic ethyl acetate-soluble fraction declined with time, recoveries were high relative to those obtained in experiments with other GAs (Table 21). GPC of the acidic ethyl acetate-soluble fraction showed that no detectable radioactivity was associated with the HMW region of eluates, and when the LMW fraction was investigated, no zones of radioactivity other than that corresponding to GA₅ itself could be detected (Fig. 23).

5.3.2 Effect of dose size

Sets of five plants were treated with [³H]GA₅ at three different specific activities (Table 22) and harvested at 18h. As in the time-course experiments, recoveries of radioactivity were high and little of the recovered radioactivity occurred in the

light petroleum or crude aqueous fractions (Table 23). HPLC analysis again showed that no radioactive components chromatographically distinct from GA₅ were present (Fig. 24). The crude aqueous fractions from the three treatments were pooled and the acidic butanol-soluble fraction (40.1×10^3 dpm) obtained. After enzymatic hydrolysis, two radioactive zones could be resolved by HPLC (Fig. 25). A (30.31×10^3 dpm) had the same retention time as GA₅, and B (2.00×10^3 dpm) corresponded to GA₈. Further characterisation of A and B was not attempted in view of the low yields obtained.

TABLE 21

[³H]GA₅ feeds. Variation with time of radioactivity (dpm $\times 10^{-6}$) in various solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	84.1	4.62	-	-
2	80.2	4.41	-	-
8	58.1	3.19	-	-
24	52.3	2.82	0.02	-

TABLE 22

[³H]GA₅ feeds. Adjustment of specific activity.

Treatment	Seedlings	Dose per treatment		µg per seedling
		[³ H]GA ₅ (µCi)	GA ₅ (µg)	
1	5	3.02	25	5
2	5	3.02	5	1
3	5	3.02	0	0

FIGURE 19

[³H]GA₄ feed. HPLC (preparative system) of HMW
acidic ethyl acetate-soluble fraction, (1) enzyme-
hydrolysed, (2) after feeding to seedlings.
Original feed, 200 seedlings, 50.0 µCi; back feed,
five seedlings, 0.04 µCi.
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm

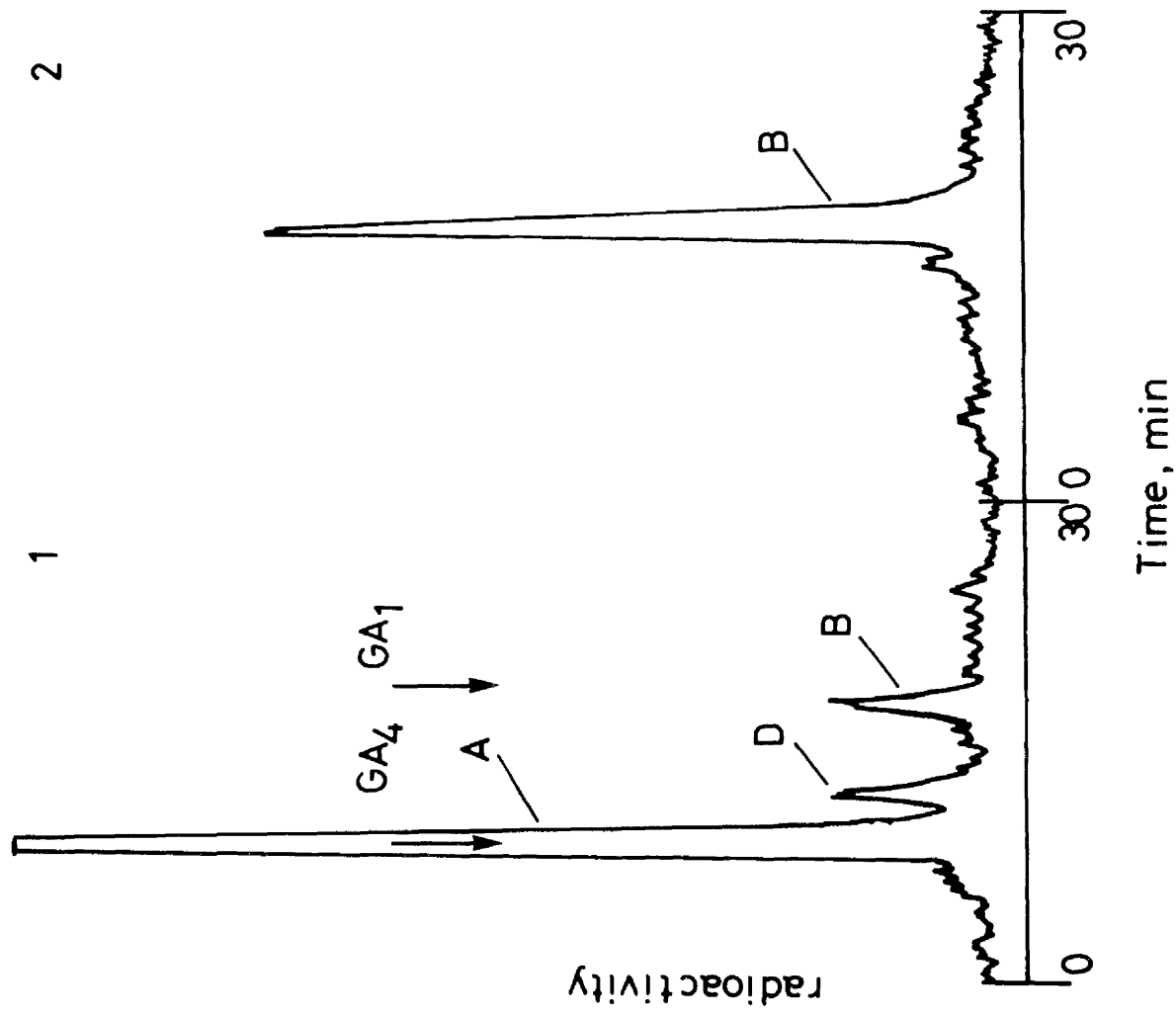


FIGURE 20

[³H]GA₄ feed. HPLC (preparative system) of (1) acidic ethyl acetate-soluble LMW fraction (1/500 aliquot); (2) hydrolysed acidic butanol-soluble fraction (1/5 aliquot), 24h after treatment (200 seedlings, 50.0 µCi).
Column: 10 x 450 mm Partisil 10
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min
Detector: radioactivity monitor, FSD = 600 cpm

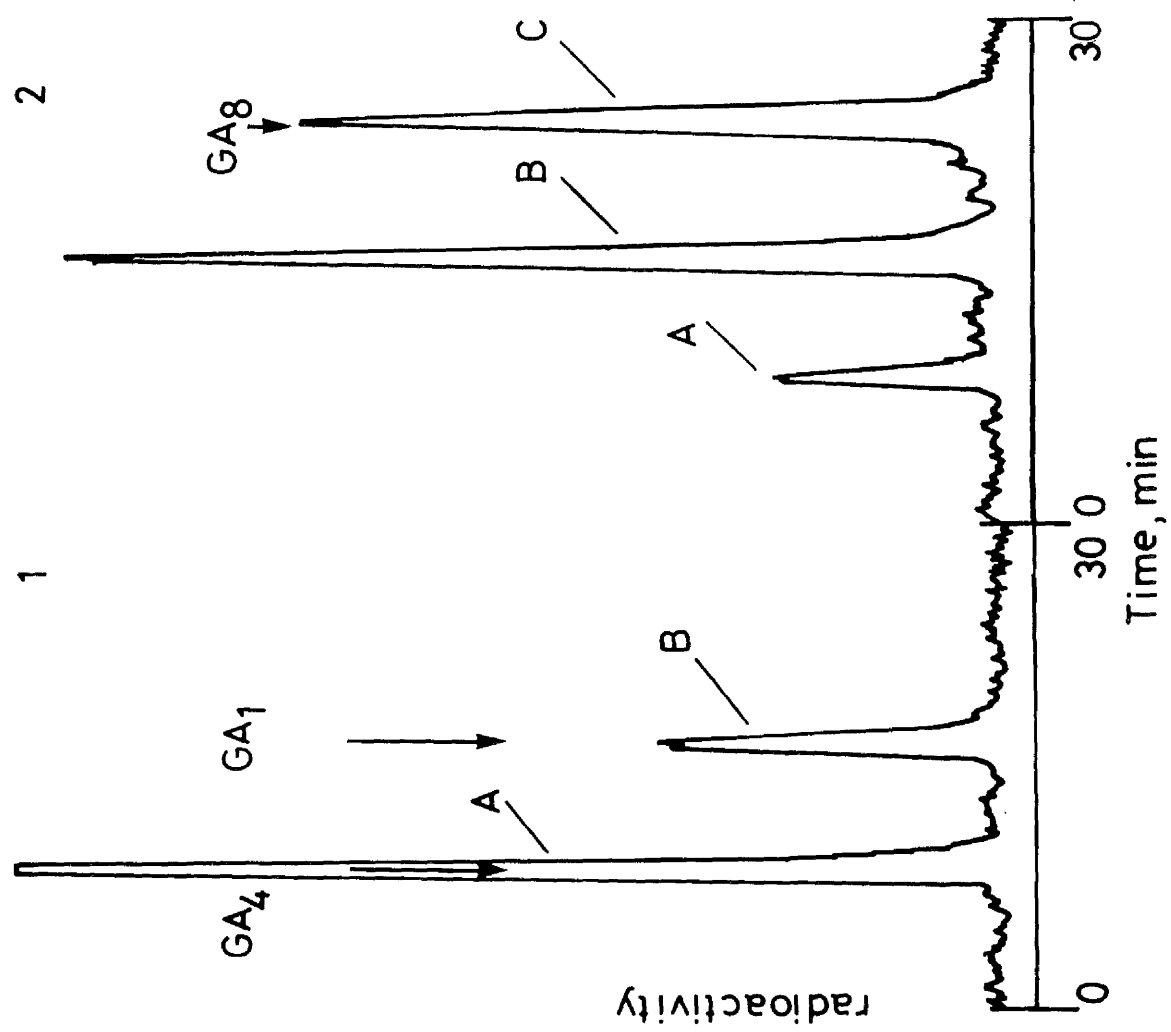


FIGURE 21

[³H]GA₄ feeds. Analytical HPLC of (1) zone A from hydrolysed HMW acidic ethyl acetate-soluble fraction; (2) zone A, and (3) zone B from LMW acidic ethyl acetate-soluble fraction.

Column: 4.6 x 500 mm Partisil 10

Mobile phase:

- (1) 1% dimethyl sulphoxide in hexane - dichloromethane, 1:1
- (2) 8% tetrahydrofuran in dichloromethane
- (3) as (2)

Detector: UV monitor

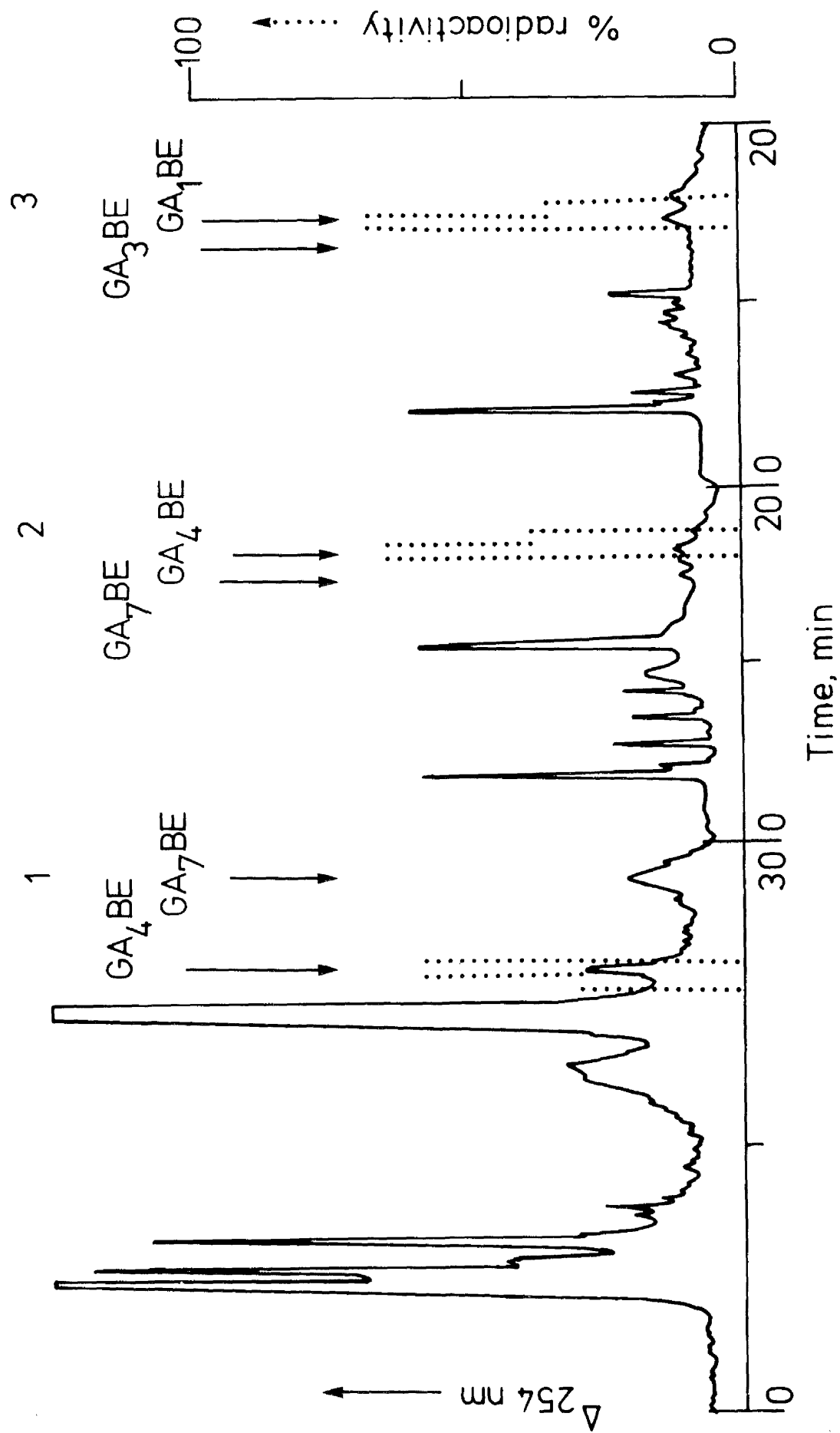
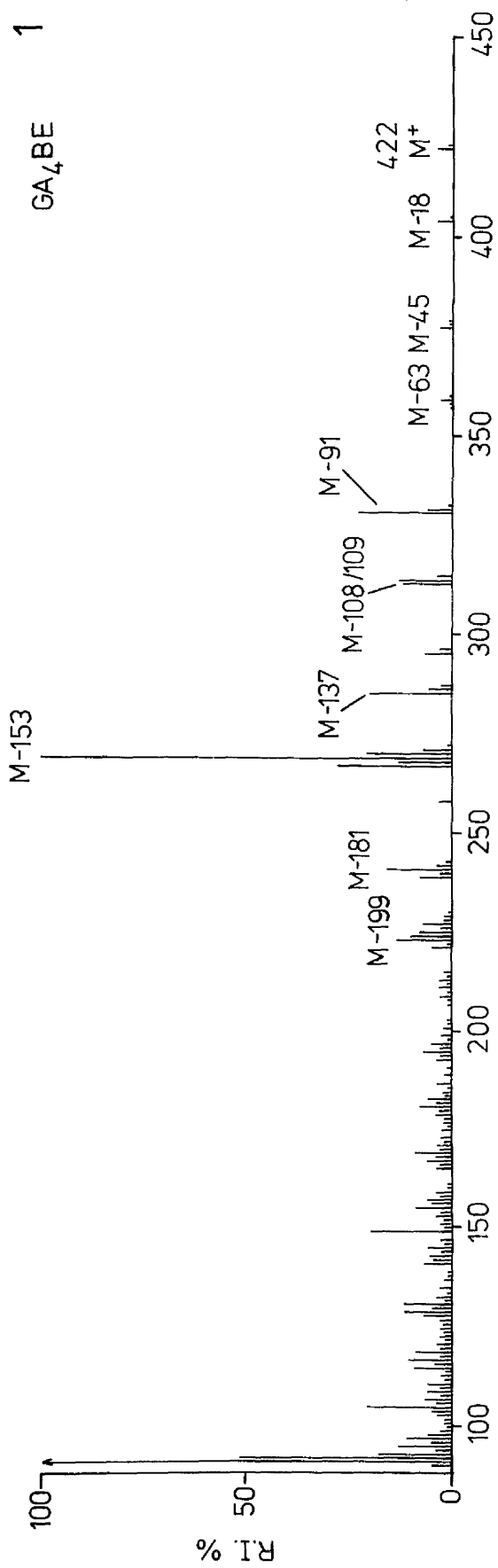


FIGURE 22

Mass spectra of (1) authentic GA₄ benzyl ester, and
(2) purified zone A from LMW acidic ethyl acetate-
soluble fraction.



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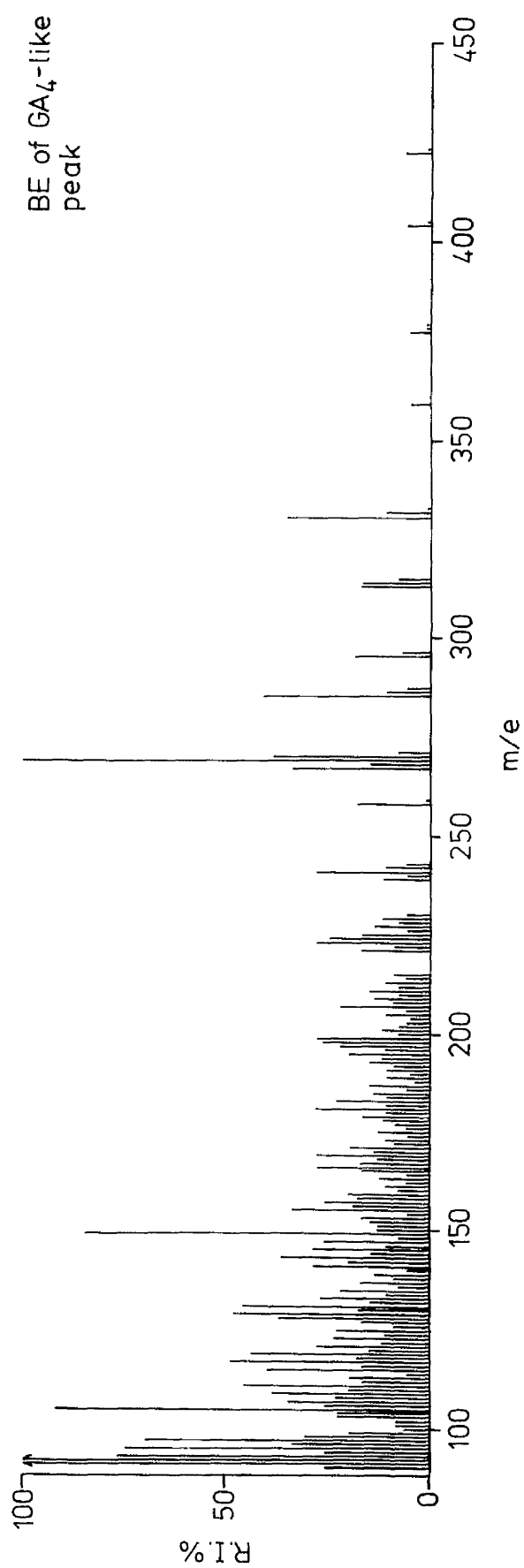


FIGURE 23

[³H]GA₅ feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/50 aliquots), 0.5, 2, 8 and 24h after treatment (5 seedlings, 2.5 µCi per treatment).

Column: 10 x 450 mm Partisil 20

Stationary phase: 40% 0.5M formate

Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)

Flow rate: 5.0 ml. min⁻¹

Detector: radioactivity monitor, FSD = 600 cpm

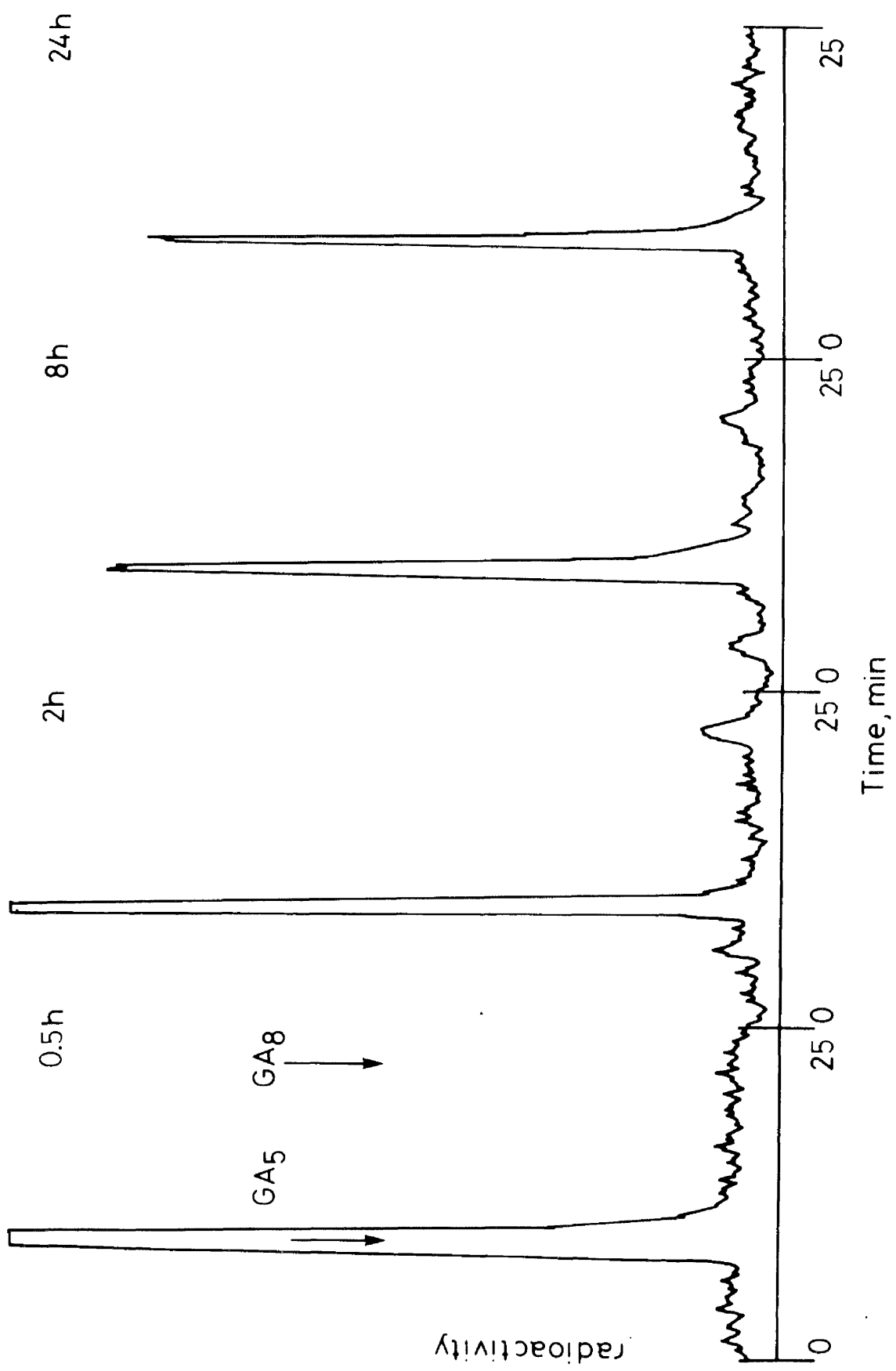


TABLE 23

$[^3\text{H}]\text{GA}_5$ feeds. Variation with applied mass of radioactivity ($\text{dpm} \times 10^{-6}$) in various solvent fractions.

Treatment	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
1	69.1	4.62	0.01	-
2	48.3	2.22	0.01	-
3	54.6	3.62	0.02	-

5.4 $[^3\text{H}]\text{GA}_8$ feeds

5.4.1 Effect of time

Twenty seedlings were treated with $3.6 \mu\text{Ci } [^3\text{H}]\text{GA}_8$ (0.40×10^6 dpm per seedling, specific activity $0.43 \text{ Ci.mmol}^{-1}$) and sets of five seedlings were harvested after 0.5, 2, 8 or 24h. The percentage recovery of radioactivity was lower than for any other $[^3\text{H}]\text{GA}$ (Table 24). Investigation of the acidic ethyl acetate-soluble fraction by GPC and by HPLC (Fig. 26) showed that the radioactivity in this fraction was associated entirely with a zone corresponding to GA_8 . Radioactivity in the crude aqueous fraction accounted for a relatively large proportion of the total at 0.5h and 2h, but declined thereafter.

TABLE 24

$[^3\text{H}]\text{GA}_8$ feeds. Variation with time of radioactivity ($\text{dpm} \times 10^{-6}$) in various solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	7.4	0.14	0.05	-
2	7.9	0.15	0.03	-
8	1.7	0.03	0.01	-
24	1.1	0.02	-	-

FIGURE 24

[³H]GA₅ feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/25 aliquots), 18h after treatment (5 seedlings, 3.0 μCi per treatment). Specific activities: 1, 39.4 μCi.μmol⁻¹; 2, 192.3μCi. μmol⁻¹; 3, 5.3 mCi. μmol⁻¹ (See Table 22).

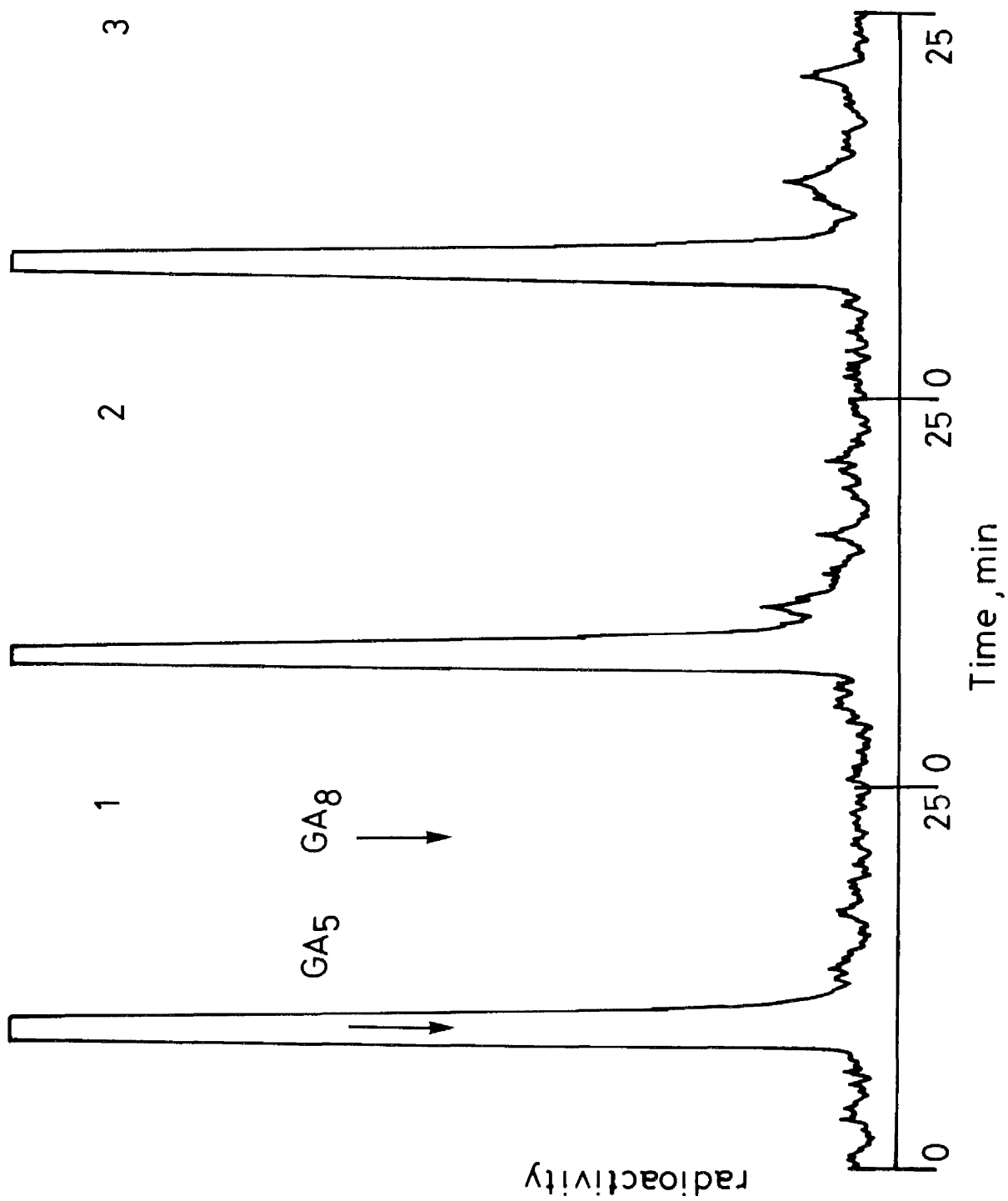
Column: 10 x 450 Partisil 20

Stationary phase: 40% 0.5M formate

Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)

Flow rate: 5.0 ml. min⁻¹

Detector: radioactivity monitor, FSD = 600 cpm



The crude aqueous fractions were pooled and the acidic butanol-soluble fraction obtained (85.11×10^3 dpm). This was hydrolysed to yield an acidic ethyl acetate-soluble fraction (84.57×10^3 dpm) which was shown by HPLC to contain one major radioactive component which co-chromatographed with GA_8 (Fig. 27). However, the amount of [3H] GA_8 available, and the low yield of the single metabolite, precluded a large-scale experiment and, further attempts to confirm the identity of the metabolite with a GA_8 glucoside were not made.

5.5 [3H] GA_9 feeds

The experiments described in this section were carried out using [$17,17'\text{-}^3H$] GA_9 . In addition, the small-scale experiments were repeated using [$2,3\text{-}^3H$] GA_9 adjusted to an equivalent specific activity, and closely similar results (data not presented) were obtained.

5.5.1 Effect of time

Twenty seedlings were treated with $9.0 \mu Ci$ [3H] GA_9 (1.0×10^6 dpm per seedling, specific activity $17.5 \text{ mCi.mmol}^{-1}$), and sets of five seedlings were harvested after 0.5, 2, 8 and 24h. The relatively high levels of radioactivity in the light petroleum fraction (Table 25) were assumed to be a consequence of the partition characteristics of GA_9 (see Durley and Pharis, 1972), and this fraction was not further investigated.

No detectable radioactivity was associated with the HMW fraction of acidic ethyl acetate-soluble extracts. The overall decline in the levels of radioactivity associated with the LMW fraction was reflected by the GA_9 -like zone (A) resolved by HPLC. A more polar component (B) whose retention time corresponded to that of GA_{20} , increased with time, and at 8h, two minor components (C,D) could also be detected (Fig. 28, Table 26).

FIGURE 25

[³H]GA₅ feeds. HPLC (preparative system) of hydrolysed acidic butanol-soluble fraction, 18h after treatment; $\frac{1}{2}$ aliquot, from combined treatments 1 - 3 (see Table 22).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm

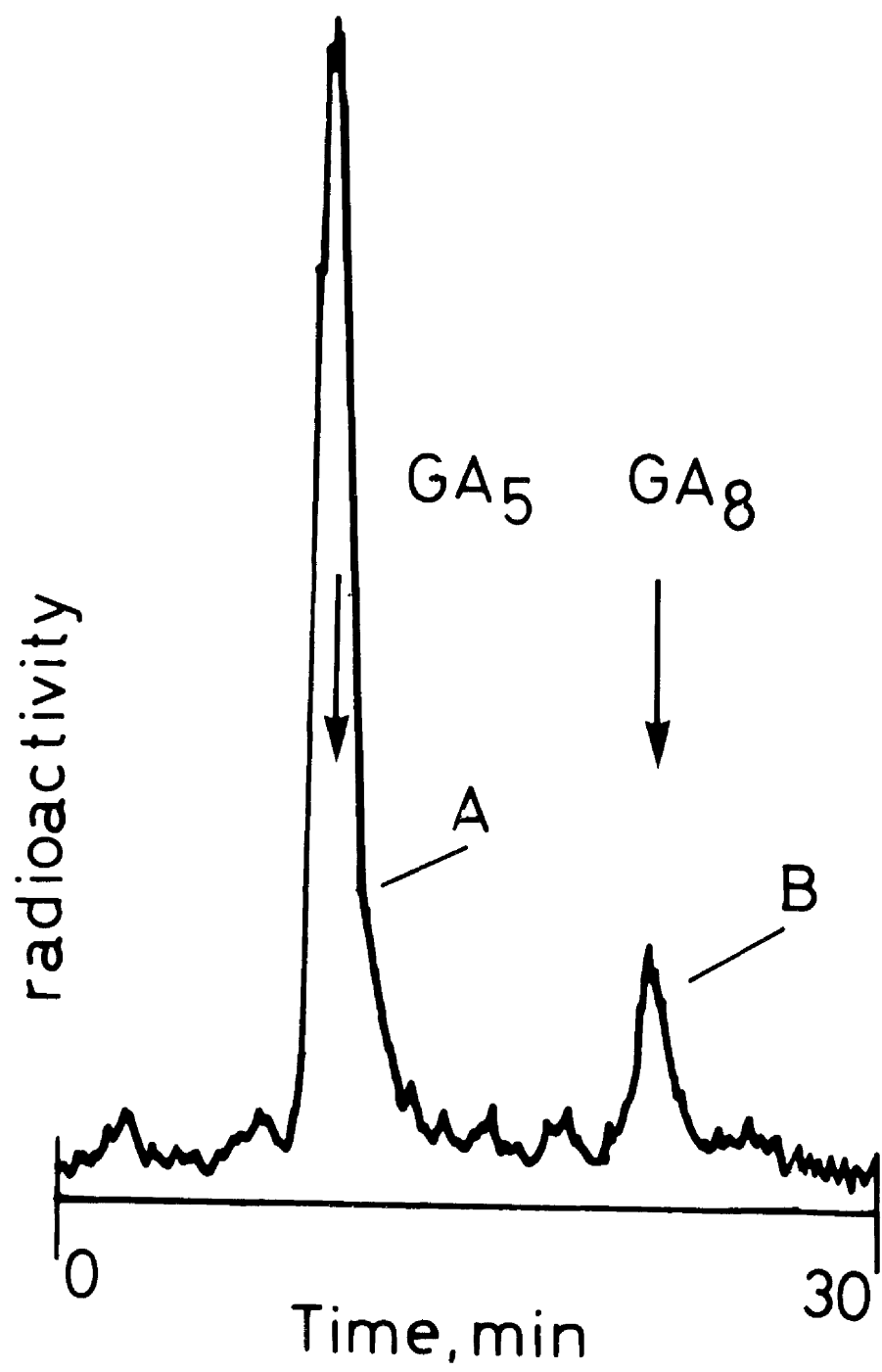


FIGURE 26

[³H]GA₈ feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions ($\frac{1}{2}$ aliquots), 0.5, 2, 8 and 24h after treatment (5 seedlings, 0.9 μ Ci per treatment).

Column: 10 x 450 mm Partisil20

Stationary phase: 40% 0.5M formate

Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)

Flow rate: 5.0 ml.min⁻¹

Detector: radioactivity monitor, FSD = 600 cpm

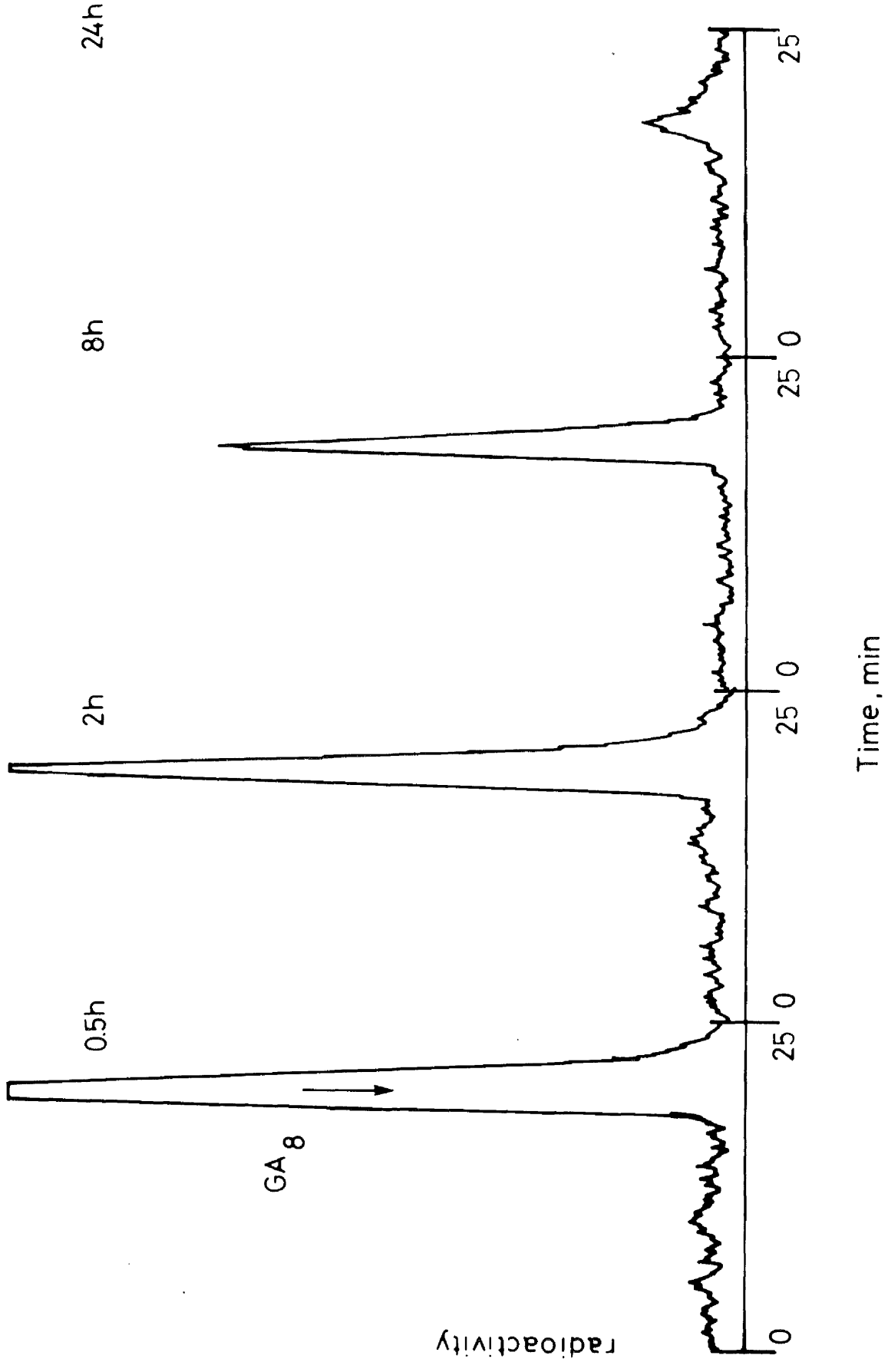


FIGURE 27

[³H]GA feeds. HPLC (preparative system) of
hydrolysed acidic butanol-soluble fraction from
combined 0.5, 2, 8 and 24h feeds.
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig. 5)
Flow Rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm

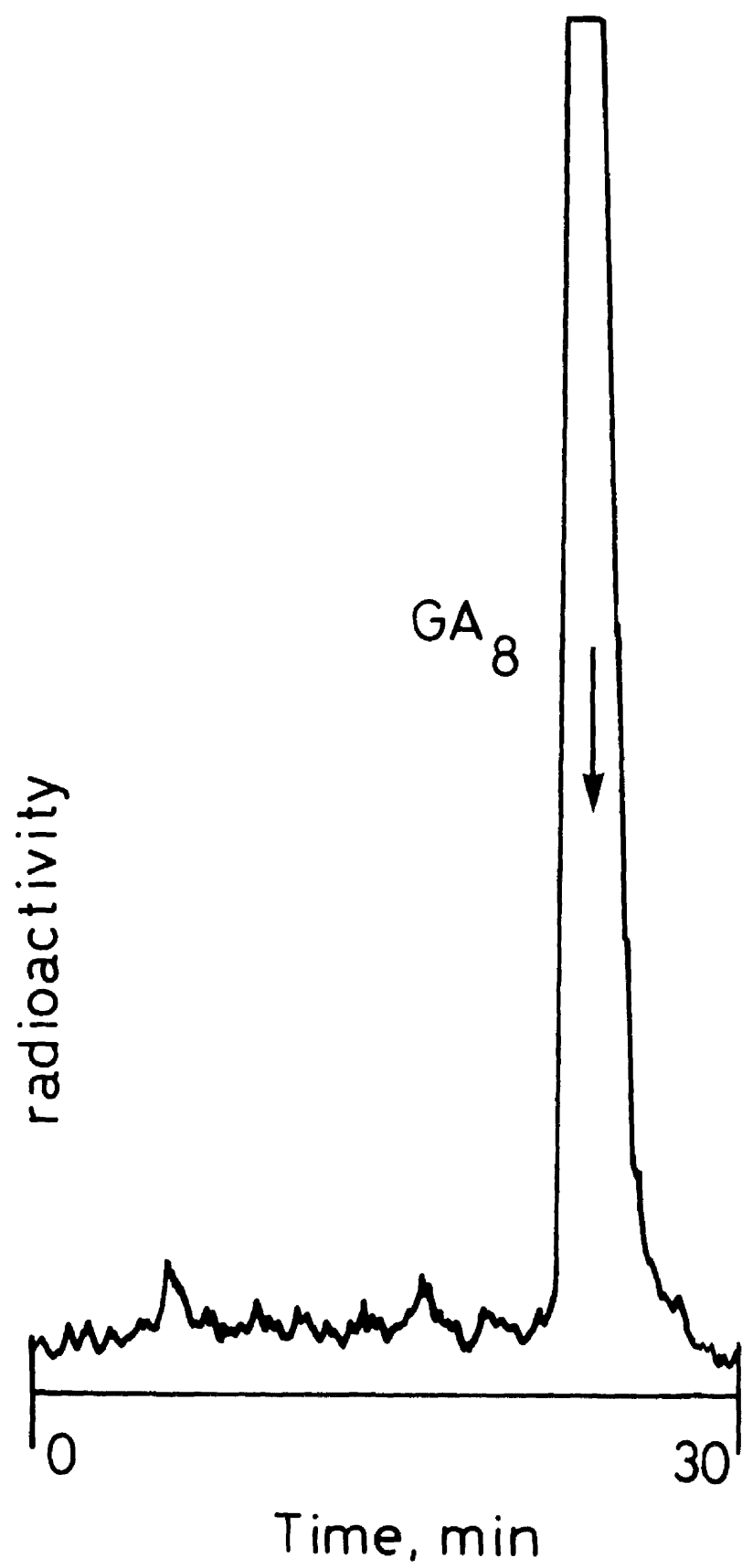


TABLE 25

[^3H]GA₉ feeds. Variation with time of radioactivity (dpm $\times 10^{-6}$) in various solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	30.4	1.50	-	0.01
2	17.4	0.83	-	0.04
8	11.2	0.55	-	0.02
24	9.1	0.44	-	0.01

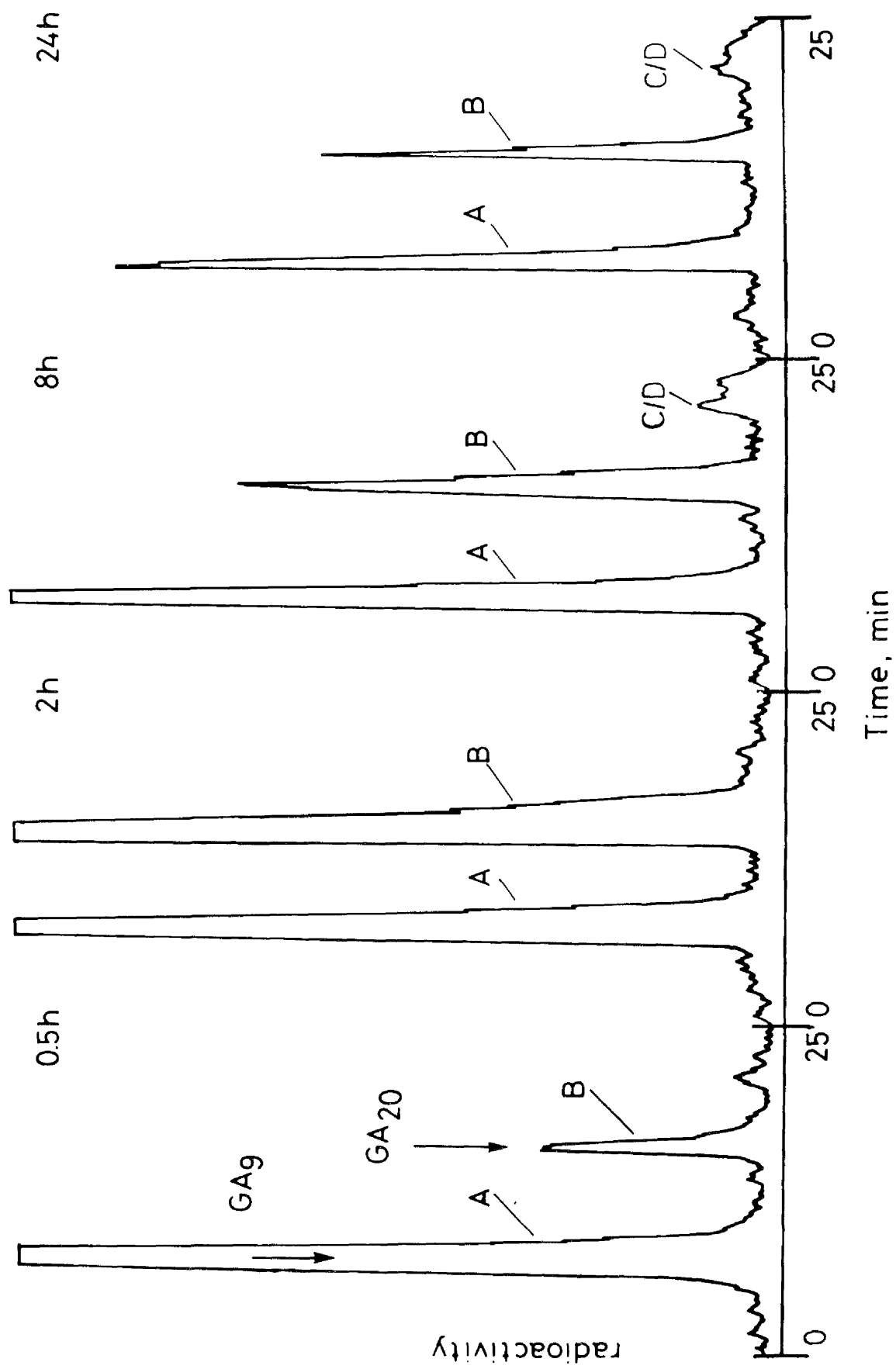
TABLE 26

[^3H]GA₉ feeds. Variation with time of acidic ethyl acetate-soluble metabolites (radioactivity, dpm $\times 10^{-6}$, estimated from HPLC analysis, Fig. 28). Zone A, presumptive GA₉; zone B, presumptive GA₂₀; zones C,D unknown.

Time (h)	Radioactivity zone			
	A	B	C	D
0.5	1.22	0.02	-	-
2	0.57	0.19	-	-
8	0.23	0.25	0.01	0.01
24	0.12	0.30	-	-

FIGURE 28

[³H]GA₉ feeds. HPLC (preparative system) of acidic ethyl-soluble fractions (3/10 aliquots), 0.5, 2, 8 and 24h after treatment (5 seedlings, 2.2 µCi per treatment).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 60 - 80% ethyl acetate in hexane (Fig. 5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm



5.5.2 Effect of dose size

Seedlings were treated with [^3H]GA₉ of three different specific activities (Table 27), and harvested after 8h. The distribution of radioactivity between solvent fractions is summarised in Table 28. The radioactivity associated with the acidic ethyl acetate-soluble fractions could be resolved by preparative HPLC to provide three components (A,B and C) with retention times corresponding to those of GA₉, GA₂₀ and GA₁ respectively (Fig. 29). Quantitative estimates of the radioactivity associated with these three components are given in Table 28. The radioactivity associated with A,B and C did not directly reflect the increasing applied dose, and C could not be detected at the highest dose level (treatment 1). However, on the basis of the specific activity of [^3H]GA₉ in each treatment, it was assumed that the yield of B increased with the dose of applied GA₉.

TABLE 27

[^3H]GA₉ feeds. Adjustment of specific activity.

Treatment	seedlings	Dose per treatment		μg per seedling
		[^3H]GA ₉ (μCi)	GA ₉ (μg)	
1	5	1.0	60	15.6
2	5	1.0	20	7.6
3	5	1.0	0	3.6

TABLE 28

[^3H]GA₉ feeds. Variation with applied mass of radioactivity (dpm x 10⁻⁶) in various solvent fractions.

Treatment	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
1	57.9	1.28	0.20	0.01
2	78.2	1.73	0.06	0.01
3	48.9	1.08	0.10	0.01

FIGURE 29

[³H]GA₉ feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/20 aliquots), 8h after treatment. Specific activities: 1, 4.0 μCi.μmol⁻¹; 2, 8.3 μCi.μmol⁻¹; 3, 17.5 μCi.μmol⁻¹ (see Table 27). (5 seedlings, 1.0 μCi per treatment).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile Phase: 60 - 80% ethyl acetate in hexane (Fig. 5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm

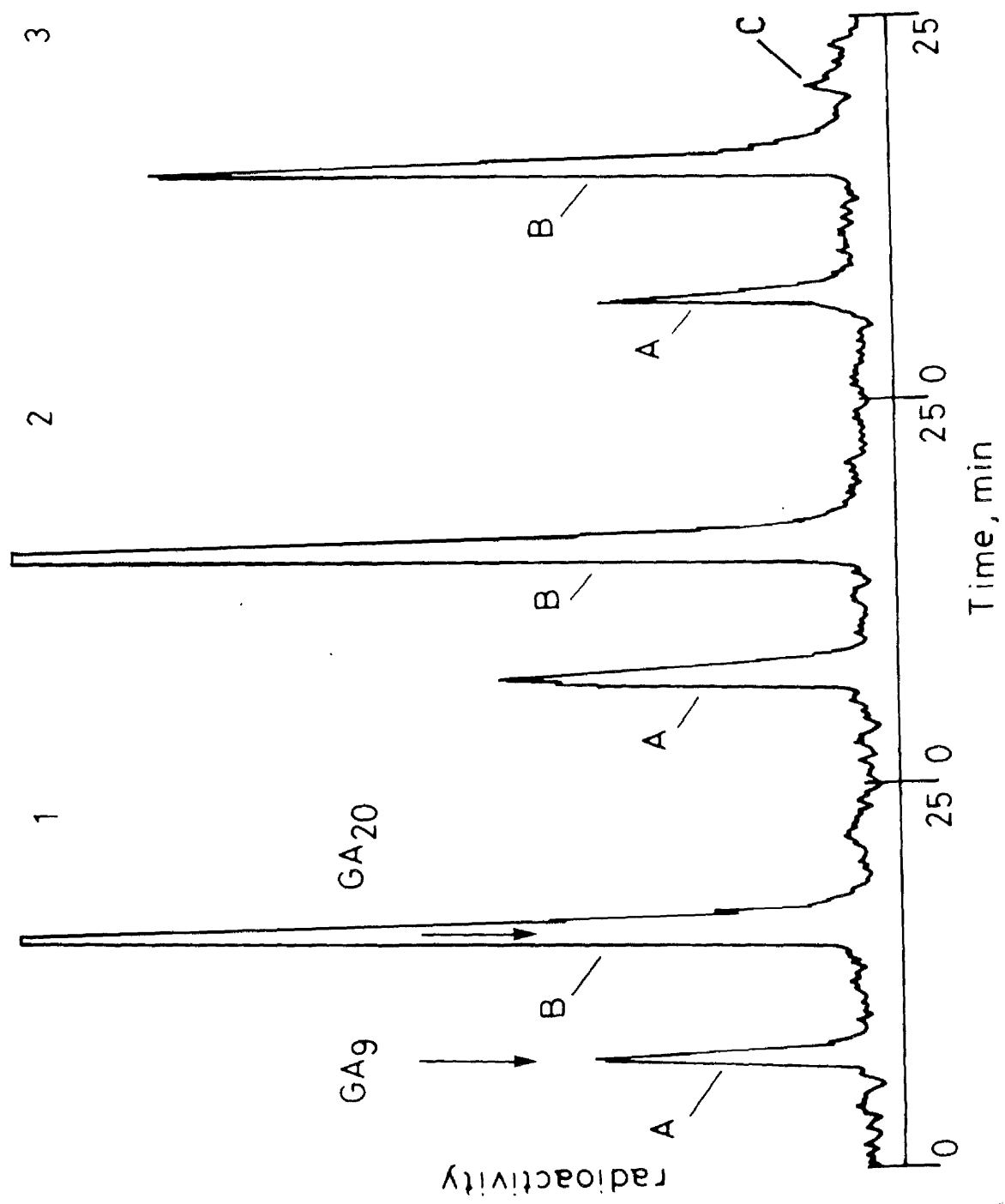


TABLE 29

[³H]GA₉ feeds. Variation according to applied mass of acidic ethyl acetate-soluble metabolites (radioactivity, dpm x 10⁻⁶, estimated from HPLC analysis, Fig. 29). Zone A, presumptive GA₉; zone B, presumptive GA₂₀; zone C, presumptive GA₁.

Treatment	Radioactivity zone		
	A	B	C
1	0.22	0.90	-
2	0.32	1.09	0.03
3	0.16	0.51	0.04

5.5.3 Identity of [³H]GA₉ metabolites

Two hundred seedlings were treated with GA₉ (3.0 mg containing 20 μ Ci [³H]GA₉), and were harvested after 8h. Radioactivity was associated with the light petroleum fraction (70.0 x 10³ dpm, discarded), the acidic ethyl acetate-soluble fraction (22.01 x 10⁶ dpm), the acidic butanol-soluble (0.88 x 10⁶ dpm) and neutral butanol-soluble fractions (0.73 x 10⁶ dpm), representing a recovery of 51% of the applied radioactivity.

Acidic ethyl acetate-soluble fraction

No detectable radioactivity was associated with the HMW fraction (Fig. 30). The LMW fraction (20.9 x 10⁶ dpm) yielded three zones of radioactivity in the preparative HPLC system: A, 5.92 x 10⁶ dpm; B, 5.43 x 10⁶ dpm; C, 0.26 x 10⁶ dpm. The retention times of A, B and C corresponded to those of GA₉, GA₂₀ and GA₁ respectively (Fig. 31). B, when converted to its benzyl ester and purified by preparative HPLC, was found to contain one radioactivity maximum which

co-chromatographed with authentic [^3H]GA₂₀ benzyl ester ($k' = 0.47$ in ethyl acetate - hexane, 40:60). The radioactivity zone in B co-chromatographed with GA benzyl ester in analytical system 1 (Fig. 32). After repeated purification in systems 1 and 2, a one-twentieth fraction of B was sufficient to yield a mass spectrum corresponding in all respects to that of GA₂₀ benzyl ester (Fig. 33).

Acidic and neutral butanol-soluble fractions

After enzymatic hydrolysis, 0.83×10^6 dpm was recovered from the acidic butanol-soluble fraction and 0.63×10^6 dpm from the neutral butanol-soluble fraction. Preparative HPLC showed that the hydrolysed acidic butanol-soluble fraction contained one zone of radioactivity (B, 0.48×10^6 dpm) whose retention times corresponded to that of GA₂₀, and the hydrolysed neutral butanol-soluble fraction contained two components, (A, 0.04×10^6 dpm; B, 0.35×10^6 dpm) corresponding to GA₉ and GA₂₀ respectively (Fig. 31). Component A was not further investigated, but the two GA₂₀-like components were esterified and each was found to contain one zone of radioactivity co-chromatographing with GA₅ benzyl ester and GA₂₀ benzyl ester in the preparative HPLC system ($k' = 1.15$ in ethyl acetate - hexane, 40:60). In both analytical HPLC systems, the recovered radioactivity was associated with a UV-absorbing component co-chromatographing with GA₂₀ benzyl ester (Fig. 32). After repeated purification, each of the two components provided a mass spectrum identical in all respects to that of GA₂₀ benzyl ester (see Fig. 33).

GA₂₀ glucosyl ether would be expected to partition into butan-1-ol, but not ethyl acetate, at pH 2.5, and may therefore be the source of the GA₂₀ obtained by hydrolysis from this fraction. However, no partition data for GA₂₀ glucosyl ester are available, and it cannot therefore be unequivocally assumed that this compound

is the source of the GA₂₀ obtained by hydrolysis of the neutral butanol-soluble fraction.

FIGURE 30
[³H]GA₉ feed. Gel permeation chromatogram of
acidic ethyl acetate-soluble fraction (1/200
aliquots), 8h after treatment (200 seedlings,
20.0 μCi).

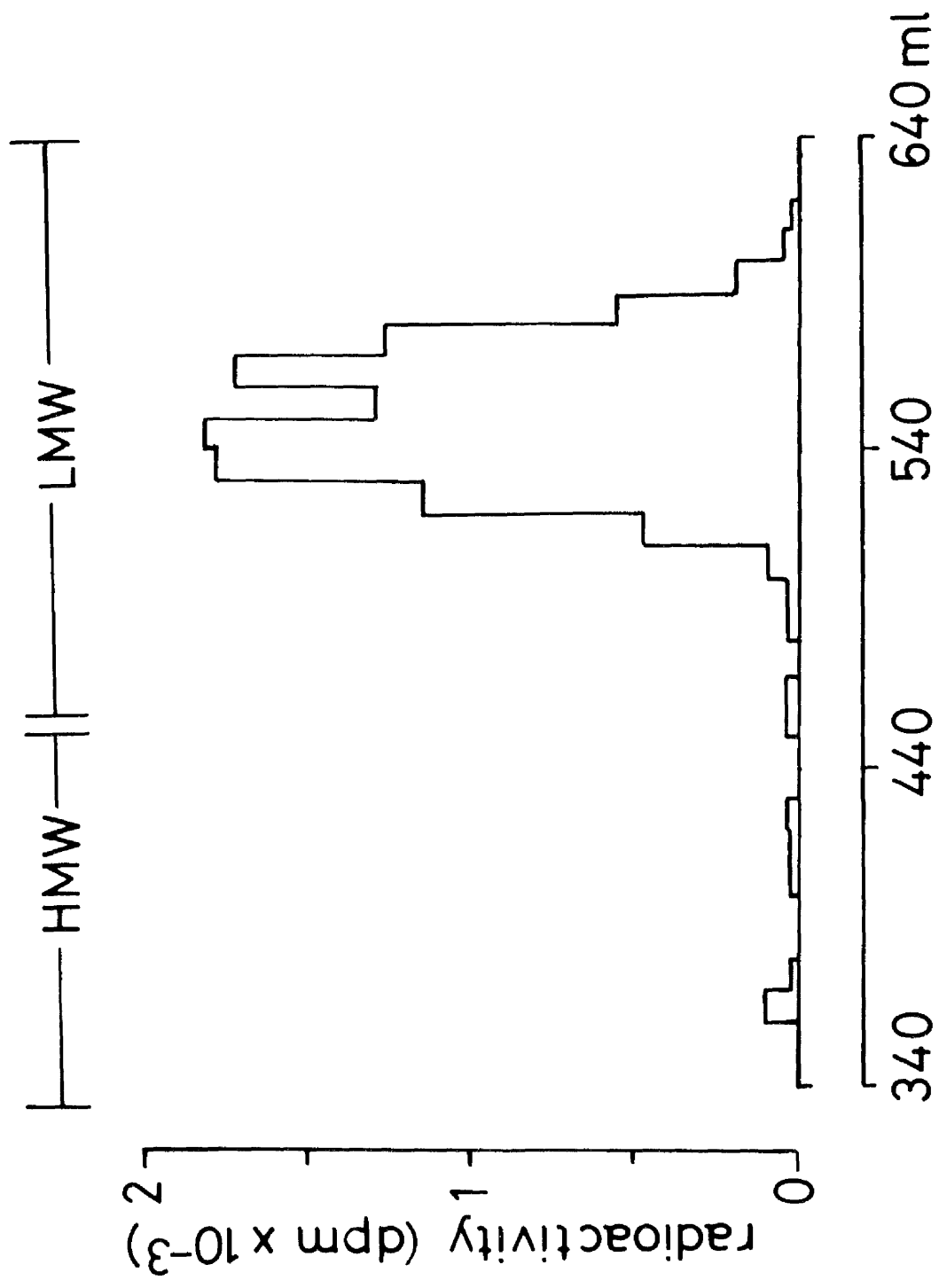


FIGURE 31

[³H]GA₉ feed. HPLC (preparative system) of (1) acidic ethyl acetate-soluble fraction (1/500 aliquot); (2) hydrolysed acidic butanol-soluble fraction (1/20 aliquot); (3) hydrolysed neutral butanol-soluble fraction (1/20 aliquot); 8h after treatment (200 seedlings, 20.0 μCi).
Column: 10 x 450 mm Partisil 10
Stationary phase: 40% 0.5M formate
Mobile phase: 60 - 80% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml. min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm

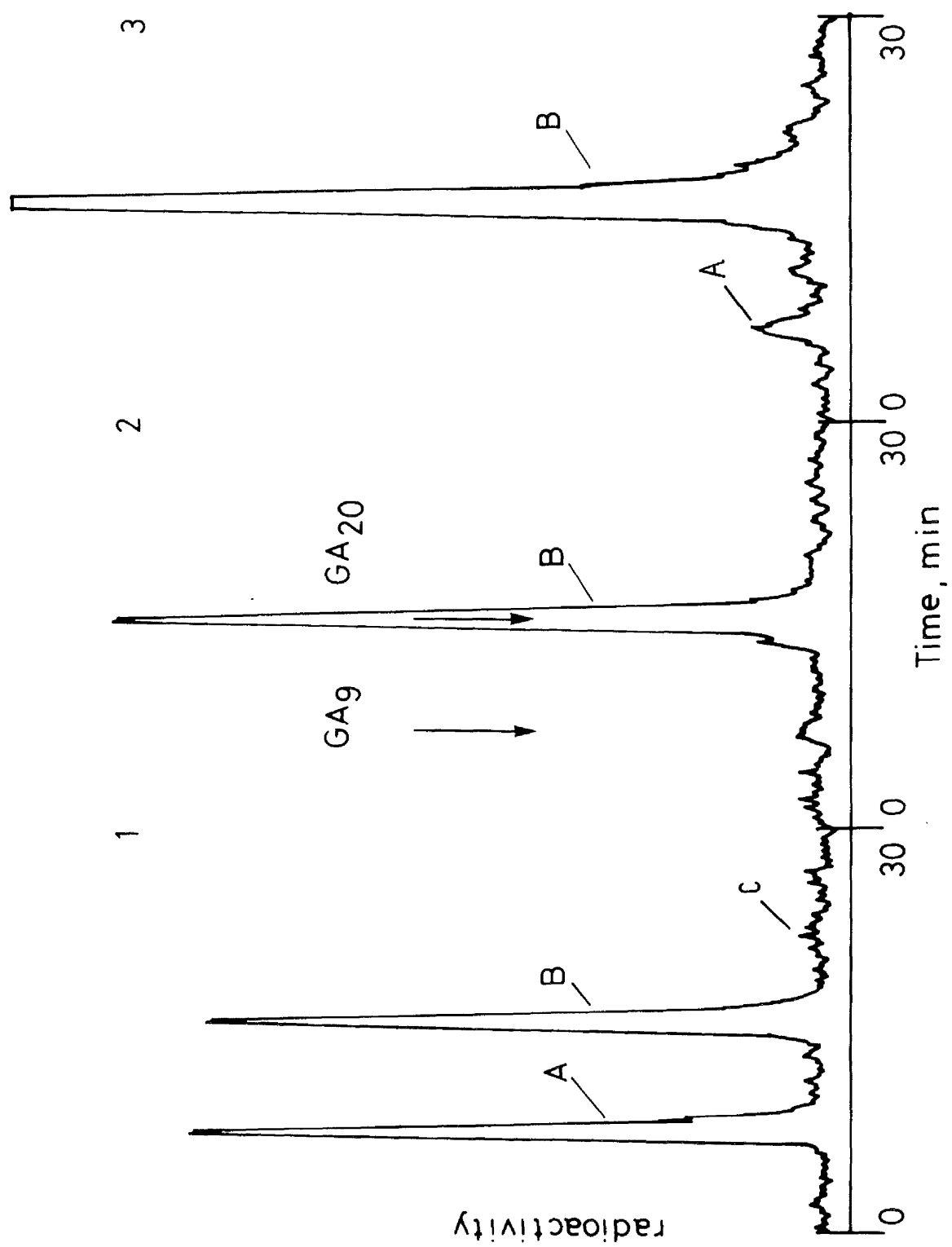


FIGURE 32

[³H]GA₉ feed. Analytical HPLC of (1) zone B from acidic ethyl acetate-soluble fraction; (2) zone A from hydrolysed acidic butanol fraction; (3) zone B from hydrolysed neutral butanol-soluble fraction.

Column: 4.6 x 500 mm. Partisil 10

Mobile phase: 1% dimethylsulphoxide in hexane -
dichloromethane, 1:1

Detector: UV monitor and on-line radioactivity detector.

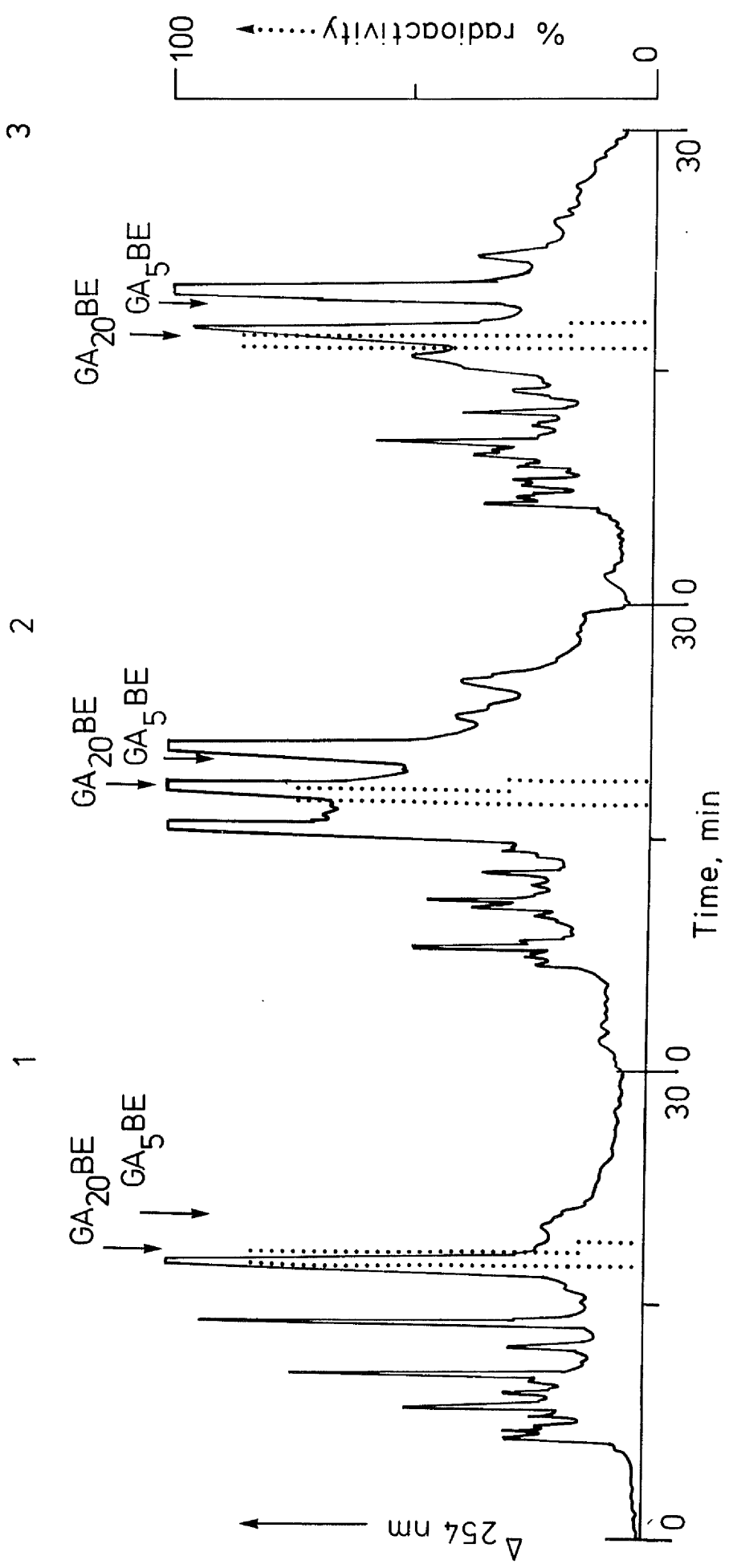
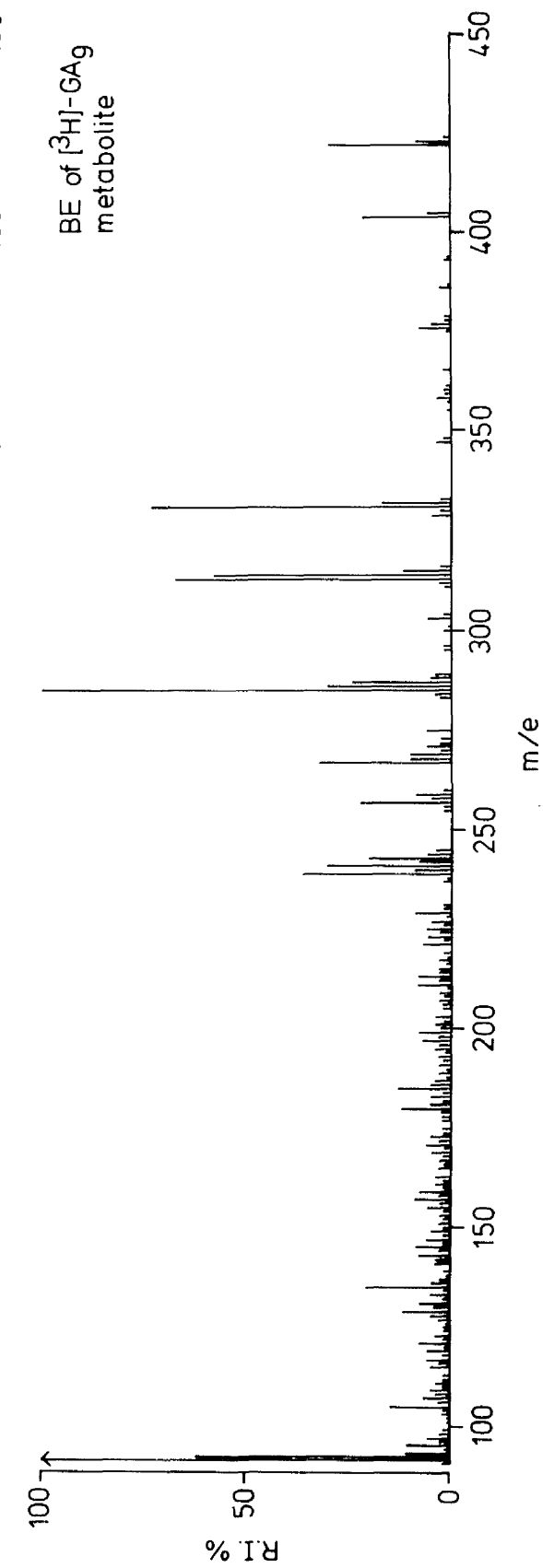
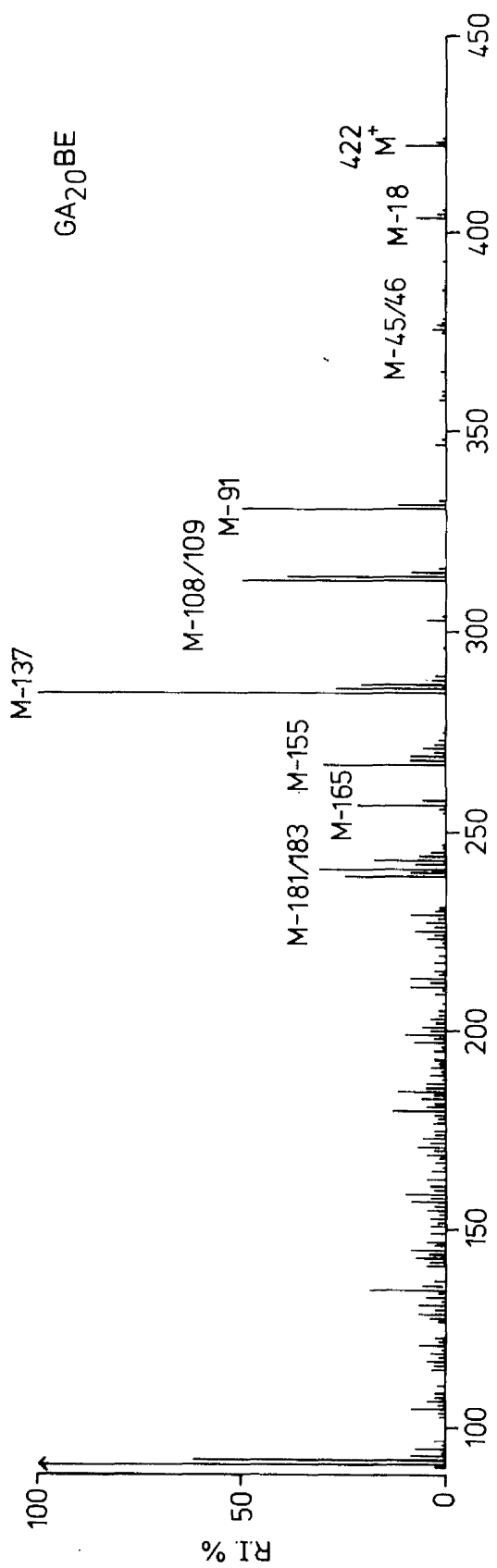


FIGURE 33

$[^3\text{H}]\text{GA}_9$ feeds. Mass spectra of (1) authentic GA_{20} benzyl ester, and (2) purified zone B from acidic ethyl acetate-soluble fraction.



5.6 $[^3\text{H}]\text{GA}_{12}$ aldehyde feeds

5.6.1 Effect of time

Fifty seedlings were treated with 50 μCi $[^3\text{H}]\text{GA}_{12}$ aldehyde (2.22×10^6 dpm per seedling, specific activity $25.0 \text{ mCi.mmol}^{-1}$), and sets of ten seedlings were harvested at 0.5, 2, 4, 8 and 24h. A considerable proportion of the recovered radioactivity was associated with the light petroleum fraction (Table 30). A zero-time extraction provided a value of $K_d = 0.73$ for the partition coefficient of $[^3\text{H}]\text{GA}_{12}$ aldehyde between light petroleum and 0.5M phosphate buffer and chromatographic investigation confirmed that the radioactivity in the light petroleum fraction was associated only with components resembling $[^3\text{H}]\text{GA}_{12}$ aldehyde itself.

TABLE 30

$[^3\text{H}]\text{GA}_{12}$ aldehyde feeds. Variation with time of radioactivity ($\text{dpm} \times 10^{-6}$) in various solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	43.4	8.53	—	1.11
2	38.9	6.16	0.01	2.47
4	18.4	2.71	0.01	1.36
8	14.7	2.02	0.03	1.23
24	10.6	1.76	0.02	0.57

The levels of radioactivity in the HMW fraction of acidic ethyl acetate-soluble extracts remained relatively constant with time, but there was a decline in the radioactivity associated with the LMW fraction (Table 31).

TABLE 31

[³H]GA₁₂ aldehyde feeds. Variation with time of HMW and LMW fractions of acidic ethyl acetate-soluble extracts. Estimates (dpm x 10⁻⁶) based on radioactivity maxima in GPC eluates.

Time (h)	HMW fraction	LMW fraction
0.5	0.44	8.02
2	0.59	5.47
4	0.53	2.12
8	0.71	1.30
24	0.60	1.12

Preparative HPLC of the LMW fraction revealed a considerably larger number of metabolites than were formed from any other [³H]GA tested (Fig. 34). Quantitative estimates of the radioactivity associated with the ten major components (A - J) are given in Table 32.

TABLE 32

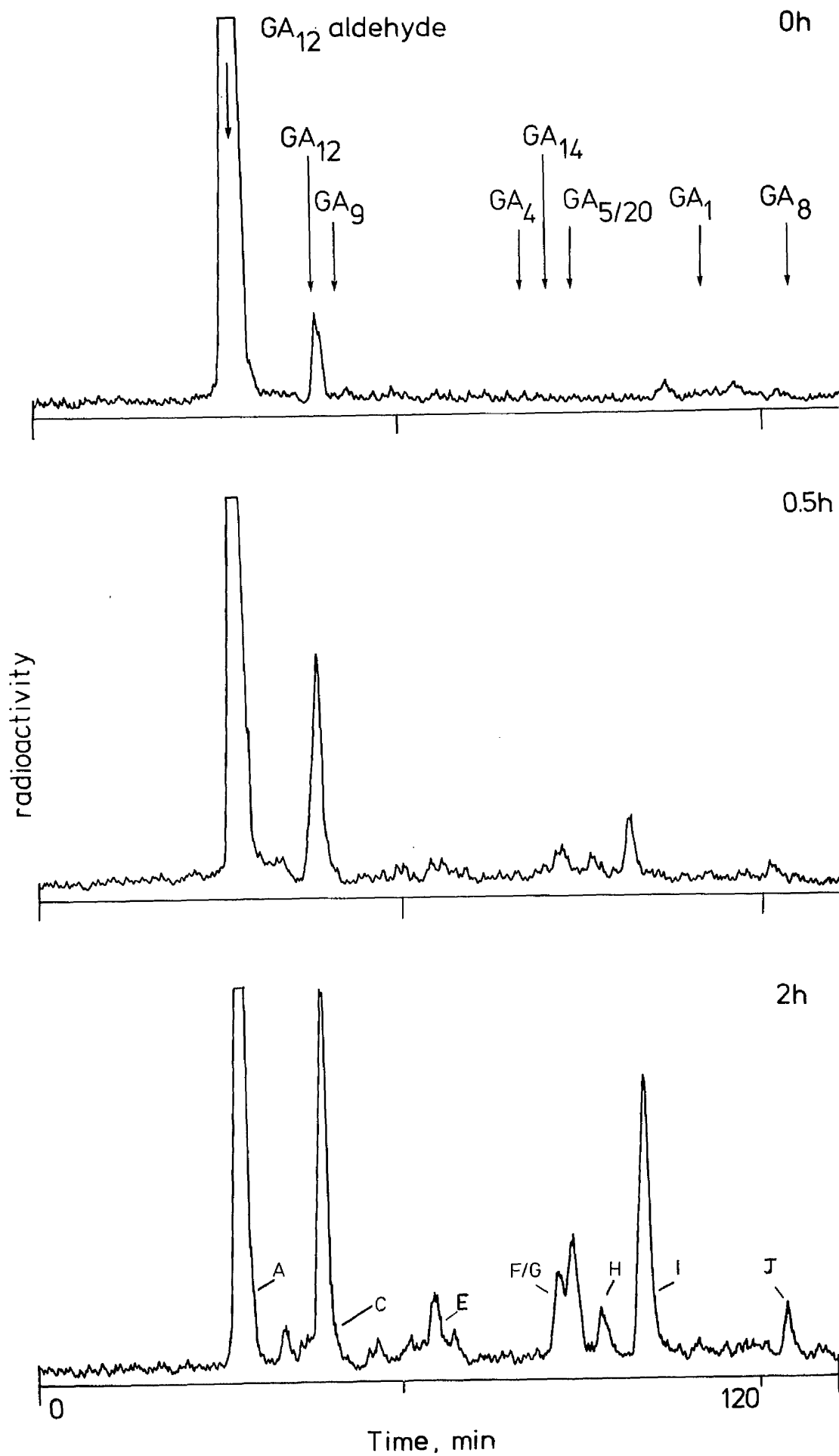
[³H]GA₁₂ aldehyde feeds. Variation with time of acidic ethyl acetate-soluble metabolites (radioactivity, dpm x 10⁻⁶, estimated from HPLC analysis, Fig. 34).
Zone A, presumptive GA₁₂ aldehyde.

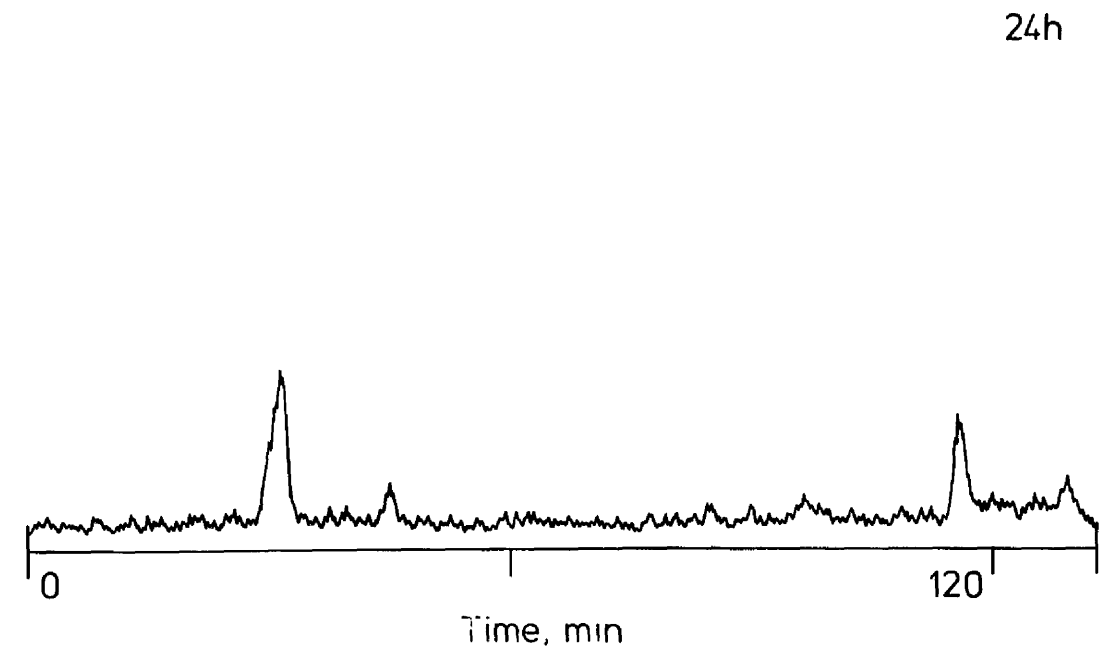
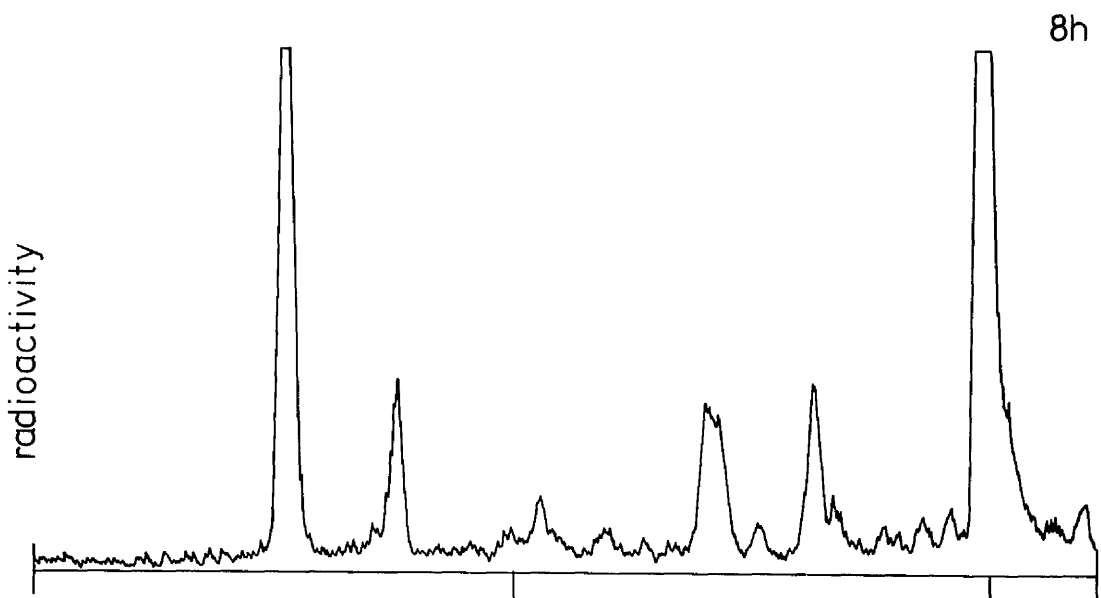
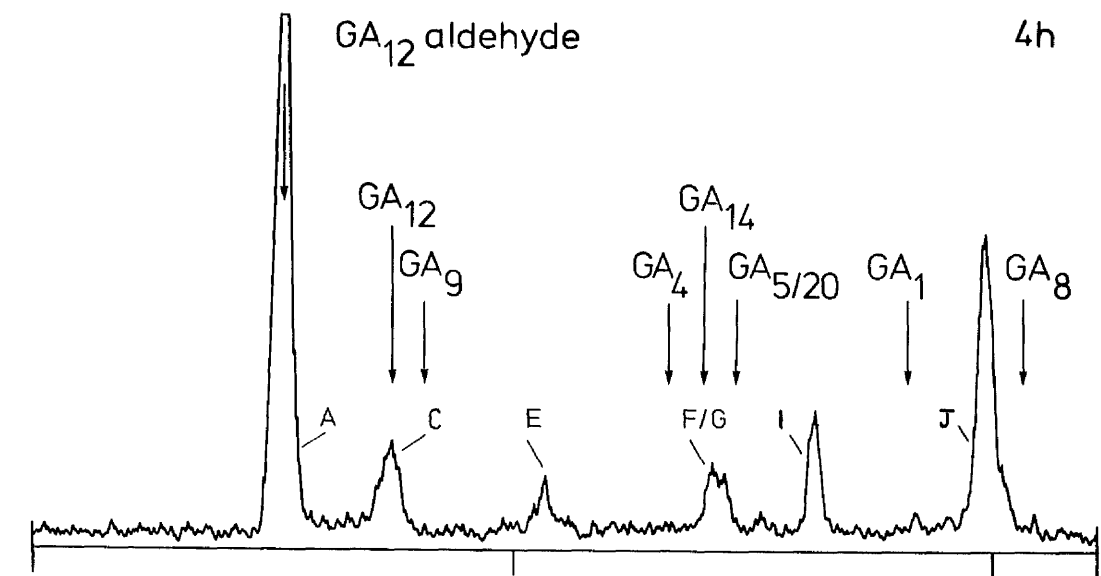
Time (h)	Radioactivity zone								
	A	B	C	D	E	F/G ^a	H	I	J
0.5	5.95	0.16	1.52	0.10	0.12	0.32	0.11	0.38	0.09
2	2.42	-	1.03	0.26	0.07	0.68	0.22	0.99	0.19
4	1.22	-	0.25	0.08	-	0.23	-	0.21	0.06
8	0.47	-	0.10	0.02	-	0.15	0.01	0.10	0.71
24	0.57	-	0.12	-	-	0.03	0.01	0.05	0.31

^a Incompletely resolved in this system.

FIGURE 34

[³H]GA₁₂ aldehyde feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/25 aliquots), 0.5, 2, 4, 8 and 24h after treatment (10 seedlings, 10 µCi per treatment).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile Phase: 0 - 100% ethyl acetate in hexane (Fig. 5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm





5.6.2 Effect of dose size

The specific activity of [^3H]GA₁₂ aldehyde (25.0 mCi.mmol⁻¹) was adjusted (Table 33) to three different levels and treated seedlings were harvested at 8h. In this experiment, the amount of radioactivity associated with the light petroleum fraction (Table 34), represented a smaller proportion of the total recovered than in the time-course experiments, probably as a result of the lower radioactive dose. The percentage recovery increased with dose size, while the effect of increasing applied mass on the distribution of radioactivity between the HMW and LMW fractions of the ethyl acetate-soluble extracts appeared to be to increase the levels of radioactivity in the LMW fraction (Table 35).

TABLE 33

[^3H]GA₁₂ aldehyde feeds. Adjustment of specific activity.

Treatment	Seedlings	Dose per treatment		µg per seedling
		[^3H]GA ₁₂ ald.	GA ₁₂ ald.	
		(µCi)	(µg)	
1	5	1.0	60	14.6
2	5	1.0	20	6.6
3	5	1.0	0	2.6

On the basis of the specific activity of [^3H]GA₁₂ aldehyde in treatments 1 - 3, it was assumed that the increased radioactivity in the HMW fractions represented a considerable increase in HMW metabolite mass. No similar trend was visible, however, in the radioactivity associated with any of the LMW metabolites (Table 36), except in the case of B. A radioactivity zone with the same

retention time as B occurred in some zero-time extracts, representing approximately 0.5% of the total radioactivity, and this zone was assumed to contain a breakdown product, possibly $[^3\text{H}]\text{GA}_{12}$. GA_{12} is readily formed from GA_{12} aldehyde in aqueous solution (see Cross et al., 1968). However, the amount of B produced in feeding experiments was relatively large and B may therefore be a metabolite rather than an artefact.

TABLE 34

$[^3\text{H}]\text{GA}_{12}$ aldehyde feeds. Variation with increasing applied mass of radioactivity ($\text{dpm} \times 10^{-6}$) in various solvent fractions.

Treatment	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
1	36.6	0.71	0.05	0.05
2	29.9	0.62	0.02	0.02
3	28.2	0.54	0.02	0.06

TABLE 35

$[^3\text{H}]\text{GA}_{12}$ aldehyde feeds. Variation with applied mass of HMW and LMW fractions of acidic ethyl acetate-soluble extracts. Estimates ($\text{dpm} \times 10^{-6}$) based on radioactivity maxima in GPC eluates.

Treatment	HMW fraction	LMW fraction
1	0.44	0.27
2	0.41	0.19
3	0.19	0.35

FIGURE 35

[³H]GA₁₂ aldehyde feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions, 8h after treatment (5 plants, 1.0 µCi per treatment). Specific activities: 1, 4.3 µCi.µmol⁻¹; 2, 9.6 µCi.µmol⁻¹; 3, 25.0 µCi.µmol⁻¹ (see Table 33).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 0 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm

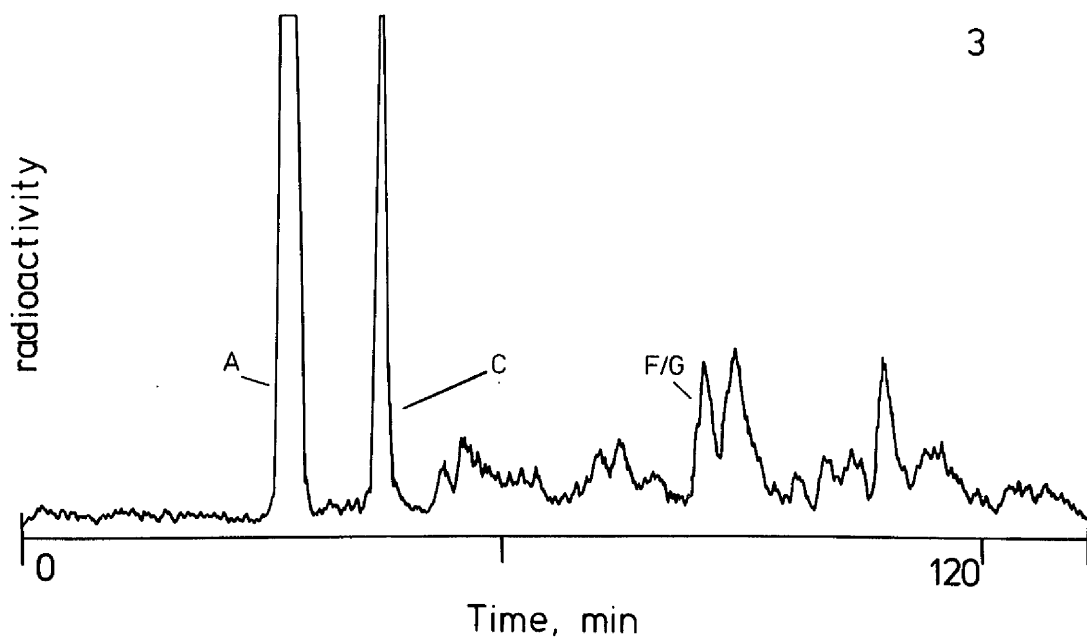
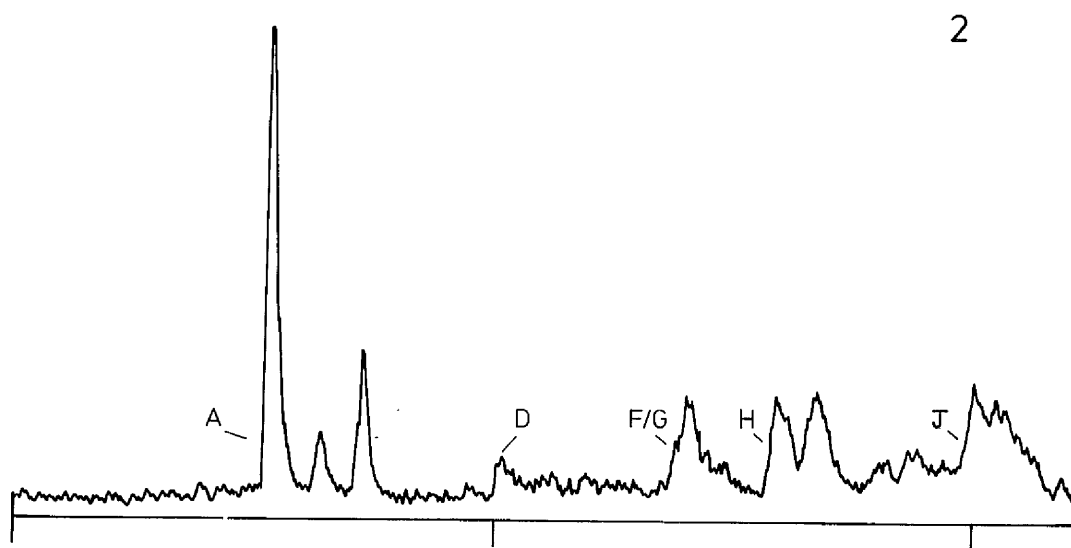
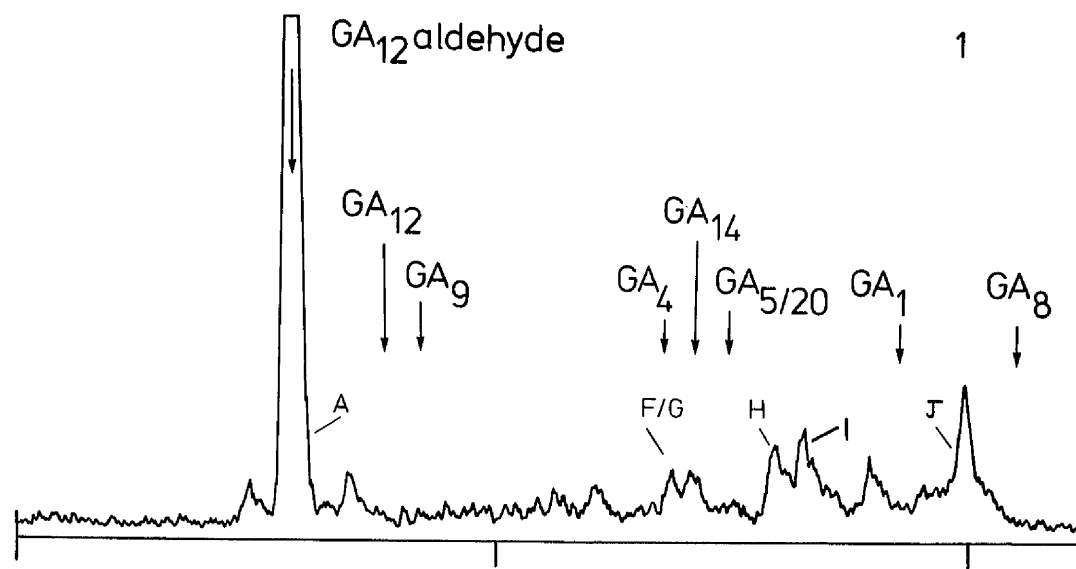


TABLE 36

[³H]GA₁₂ aldehyde feeds. Variation with applied mass of acidic ethyl acetate-soluble metabolites. Estimates (dpm x 10⁻³) based on HPLC analysis (Fig. 35).

Treatment	Radioactivity zones						
	A	B	C	F/G	H	I	J
1	64.42	5.33	12.03	7.01	10.27	11.99	14.56
2	35.46	3.99	10.61	5.16	10.59	13.13	5.40
3	90.29	-	66.47	11.48	13.39	22.40	15.33

5.6.3 Identity of [³H]GA₁₂ aldehyde metabolites

Four hundred seedlings were treated with 9.5 mg GA₁₂ aldehyde containing 350 µCi [³H]GA₁₂ aldehyde, and harvested at 8h. The light petroleum fraction (31.90 x 10⁶ dpm) was investigated by TLC and HPLC and found to contain one zone of radioactivity corresponding to [³H]GA₁₂ aldehyde. There was no indication of the presence of [³H]GA₉. The remaining recovered radioactivity was distributed between the acidic ethyl acetate-soluble fraction (308.0 x 10⁶ dpm), and the acidic butanol-soluble (12.05 x 10⁶ dpm) and neutral butanol-soluble fractions (0.43 x 10⁶ dpm, discarded).

Acidic ethyl acetate-soluble fraction

Gel permeation chromatography (Fig. 36) showed that 47.4 x 10⁶ dpm was associated with the HMW fraction and 258.0 x 10⁶ dpm with the LMW fraction. After enzymatic hydrolysis of the HMW fraction, five zones of radioactivity were isolated by preparative HPLC (Fig. 37). Of the two major components, F/G (5.0 x 10⁶ dpm) appeared to consist of two unresolved compounds, both having

similar retention times to GA_4 , while B (3.5×10^6 dpm) corresponded to GA_{12} . The LMW fraction contained at least seven major radioactivity zones (Fig. 38): A (175.0×10^6 dpm); B (6.32×10^6 dpm); C (19.75×10^6 dpm); D (7.74×10^6 dpm); F/G (unresolved, 12.75×10^6 dpm); H (5.06×10^6 dpm); I (1.12×10^6 dpm).

Acidic butanol-soluble fraction

After enzymatic hydrolysis of this fraction, a similar spectrum of metabolites to those occurring in the LMW acidic ethyl acetate-soluble fraction could be isolated by preparative HPLC (Fig. 39). These were: A (0.34×10^6 dpm); B (0.22×10^6 dpm); C (0.13×10^6 dpm); D (0.17×10^6 dpm); F/G (2.32×10^6 dpm); H (0.67×10^6 dpm); I (0.16×10^6 dpm).

When the 19 metabolites were individually examined in the preparative HPLC system, it was found that each could be further resolved into two or more distinct radioactive zones having closely similar retention times, and complex mixtures of closely similar radioactive products resulted from benzyl esterification of the radioactive components. The time allotted to this project was however insufficient to allow the characterisation of the large number of metabolites.

5.7 $[^3H]GA_{14}$ feeds

5.7.1 Effect of time

Twenty seedlings were treated with $9.0 \mu Ci [^3H]GA_{14}$ (1.0×10^6 dpm per seedling, specific activity 61 mCi.mmol^{-1}), and sets of five seedlings were harvested after 0.5., 2, 8 and 24h. The greatest proportion of the recovered radioactivity was associated with the acidic ethyl acetate-soluble fraction (Table 37), and this could be resolved by GPC into HMW and LMW fractions (Table 38).

FIGURE 36

[³H]GA₁₂ aldehyde feed. Gel permeation chromatogram of acidic ethyl acetate-soluble fraction (1/100 aliquots), 8h after treatment (400 seedlings, 350 μCi).

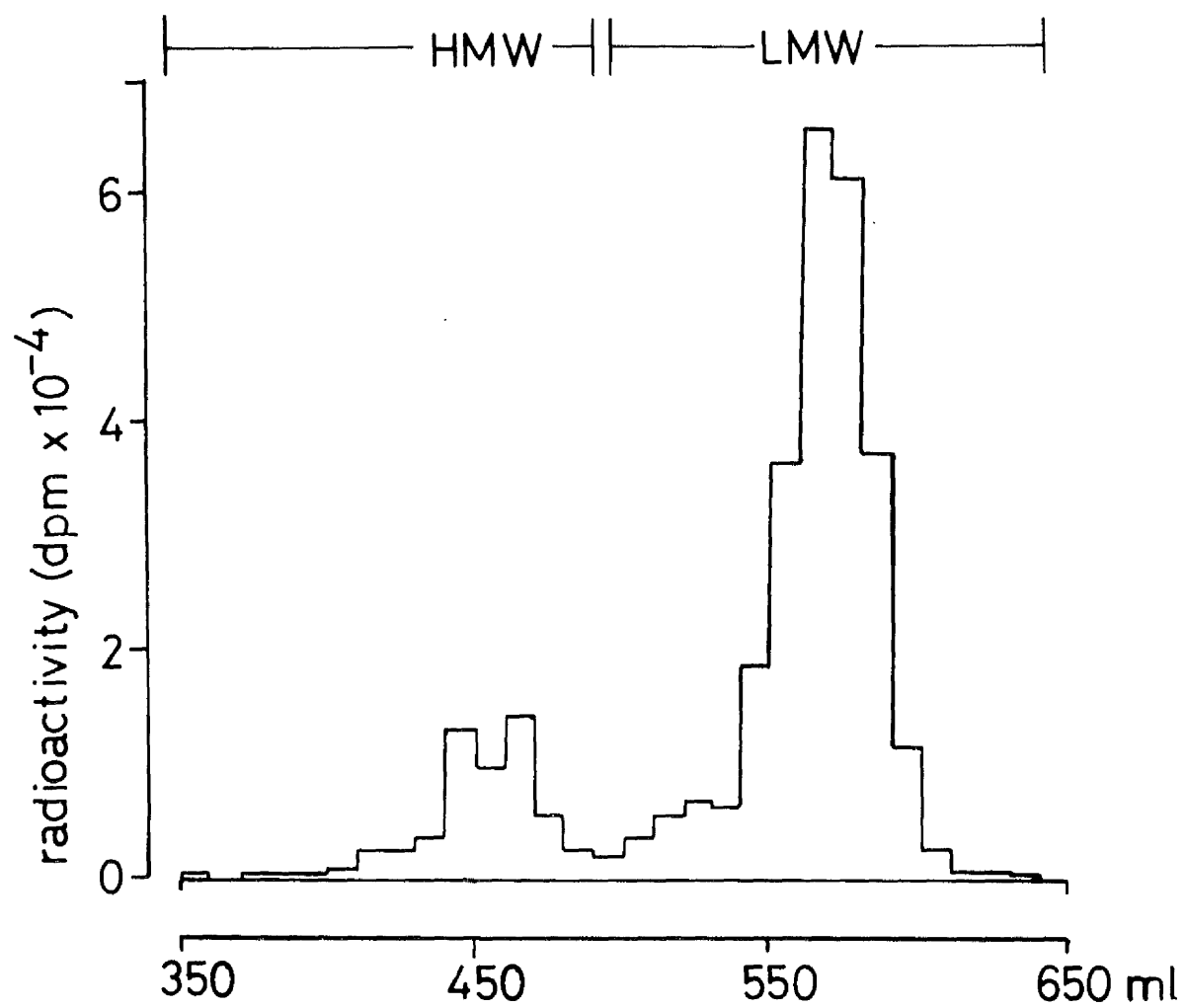


FIGURE 37

[³H]GA₁₂ aldehyde feed. HPLC (preparative system)
of hydrolysed acidic ethyl acetate-soluble HMW
fraction (1/50 aliquot) 8h after treatment (400
seedlings, 350 µCi).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 0 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.

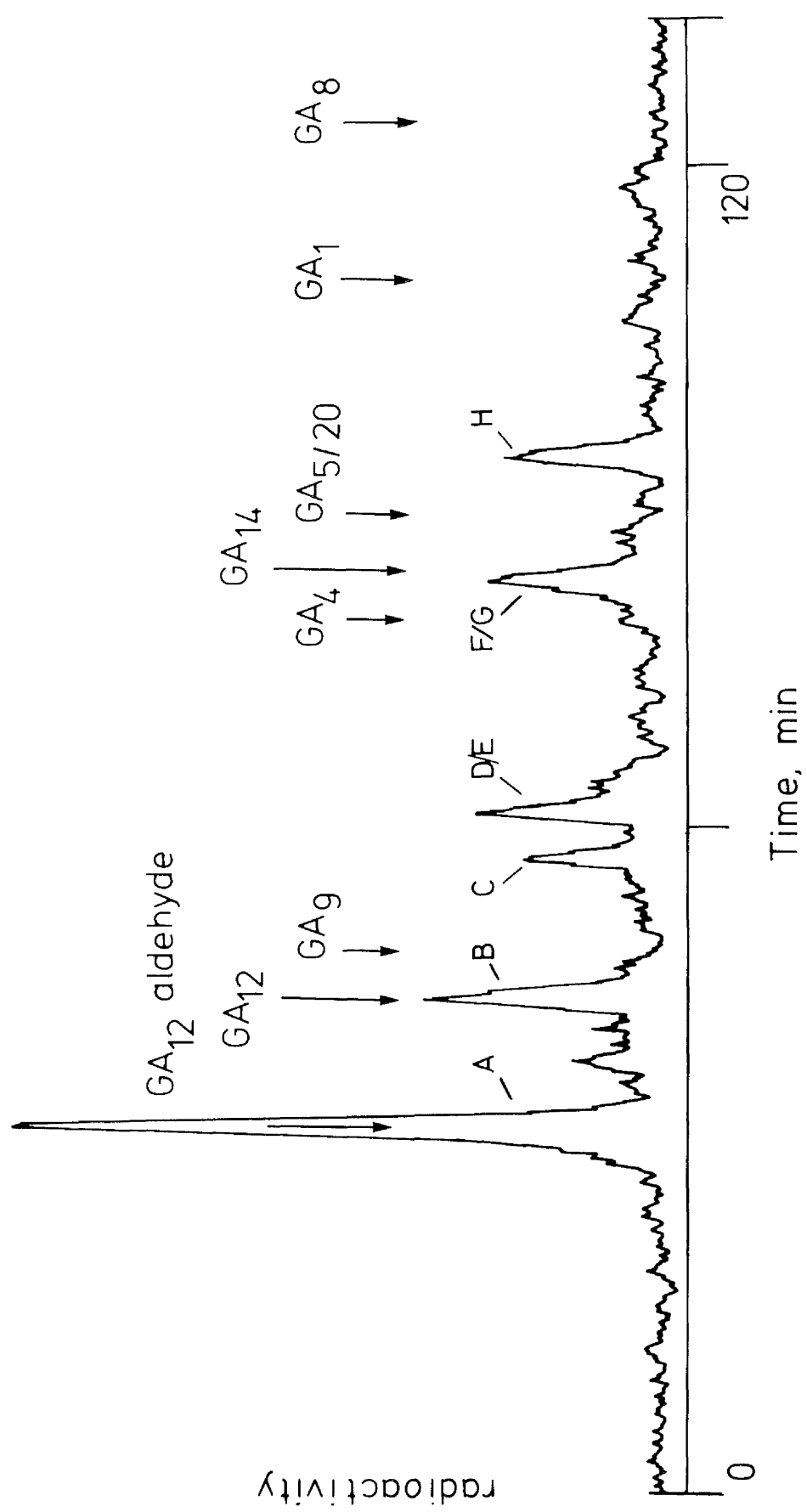


FIGURE 38

[³H]GA₁₂ aldehyde feed. HPLC (preparative system)
of acidic ethyl acetate-soluble LMW fraction
(1/500 aliquot) 8h after treatment (400 seedlings,
350 µCi).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 0 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.

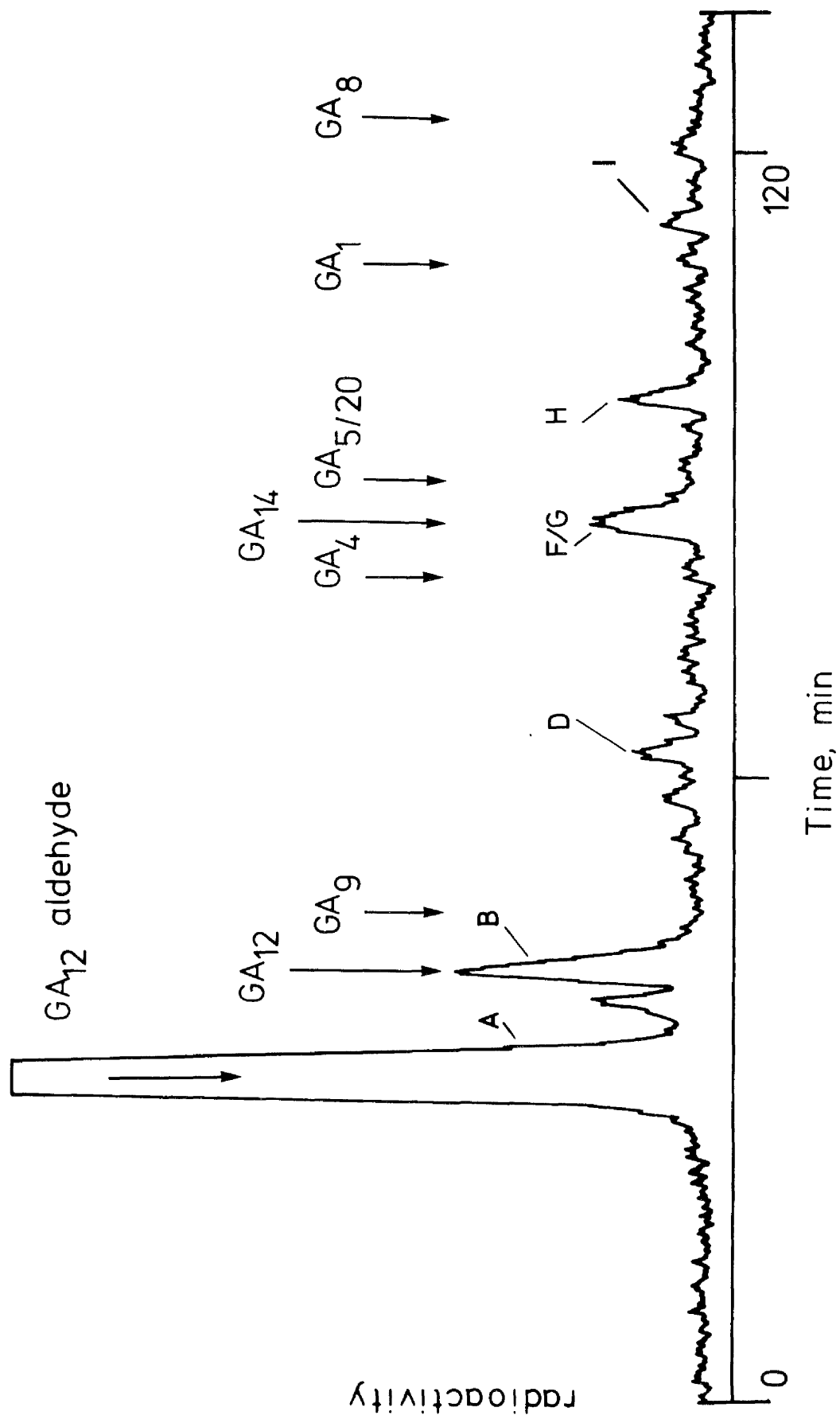
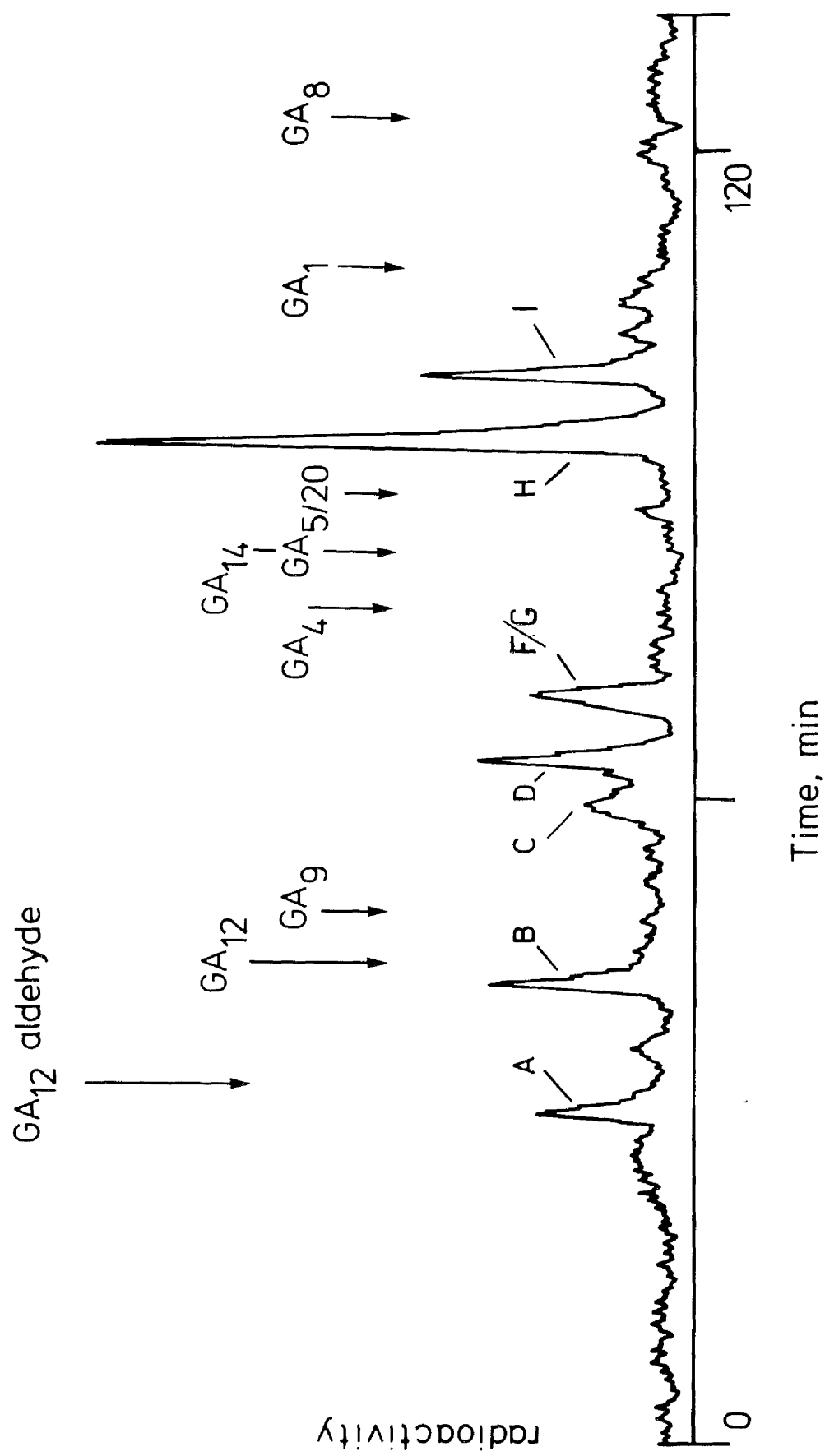


FIGURE 39

[³H]GA₁₂ aldehyde feed. HPLC (preparative system)
of hydrolysed acidic butanol-soluble fraction (1/50
aliquot) 8h after treatment (400 seedlings, 350 µCi).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 0 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.



Radioactivity in the HMW fraction increased with time, but that in the LMW fraction declined rapidly, accounting for the overall fall in radioactivity associated with the acidic ethyl acetate-soluble fraction as a whole.

The major radioactive component in the LMW fraction (A) was assumed from its retention time in the preparative HPLC system to be [^3H]GA₁₄ itself (Fig. 40). While A declined with time, a minor component, B, with similar retention time to GA₁, reached maximal concentration at 2h (Table 39). However, the levels of radioactivity in the HMW fraction at 2h were relatively low, and subsequent experiments were therefore carried out at 8h.

TABLE 37

[^3H]GA₁₄ feeds. Variation with time of radioactivity (dpm $\times 10^{-6}$) in various solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	43.2	2.12	0.03	0.01
2	33.9	1.68	0.06	0.01
8	34.2	1.69	0.02	0.01
24	24.8	1.19	0.03	0.02

FIGURE 40

[³H]GA₁₄ feeds.. HPLC (preparative system) of
acidic ethyl acetate-soluble fractions (1/10
aliquots), 0.5, 2, 8 and 24h after treatment
(5 seedlings, 2.2 µCi per treatment).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.

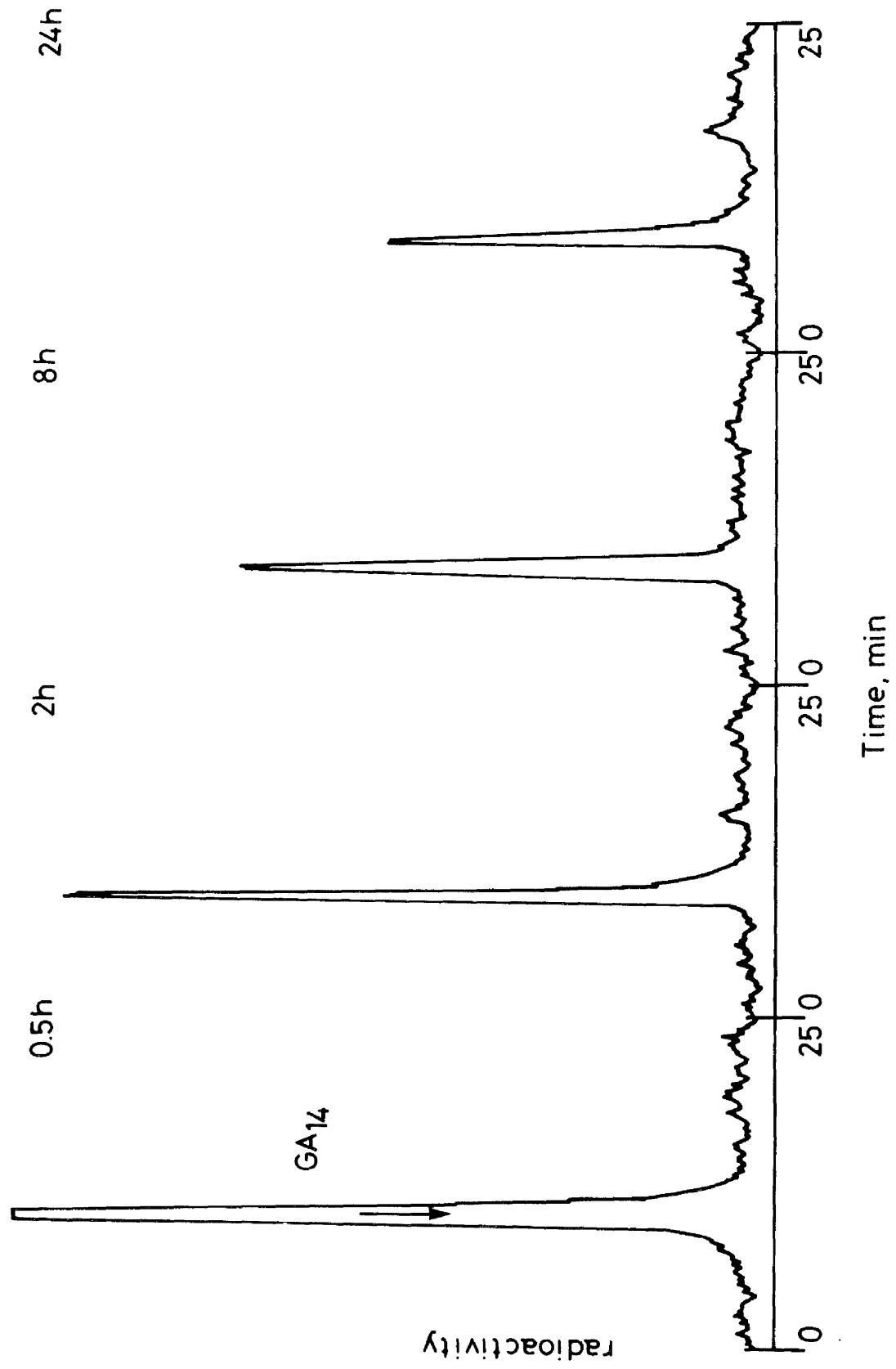


TABLE 38

[^3H]GA₁₄ feeds. Variation with time of HMW and LMW fractions of acidic ethyl acetate-soluble extracts. Estimates (dpm $\times 10^{-6}$) based on radioactivity maxima in GPC eluates.

Time (h)	HMW fraction	LMW fraction
0.5	0.29	1.90
2	0.40	1.28
8	0.84	0.71
24	0.87	0.45

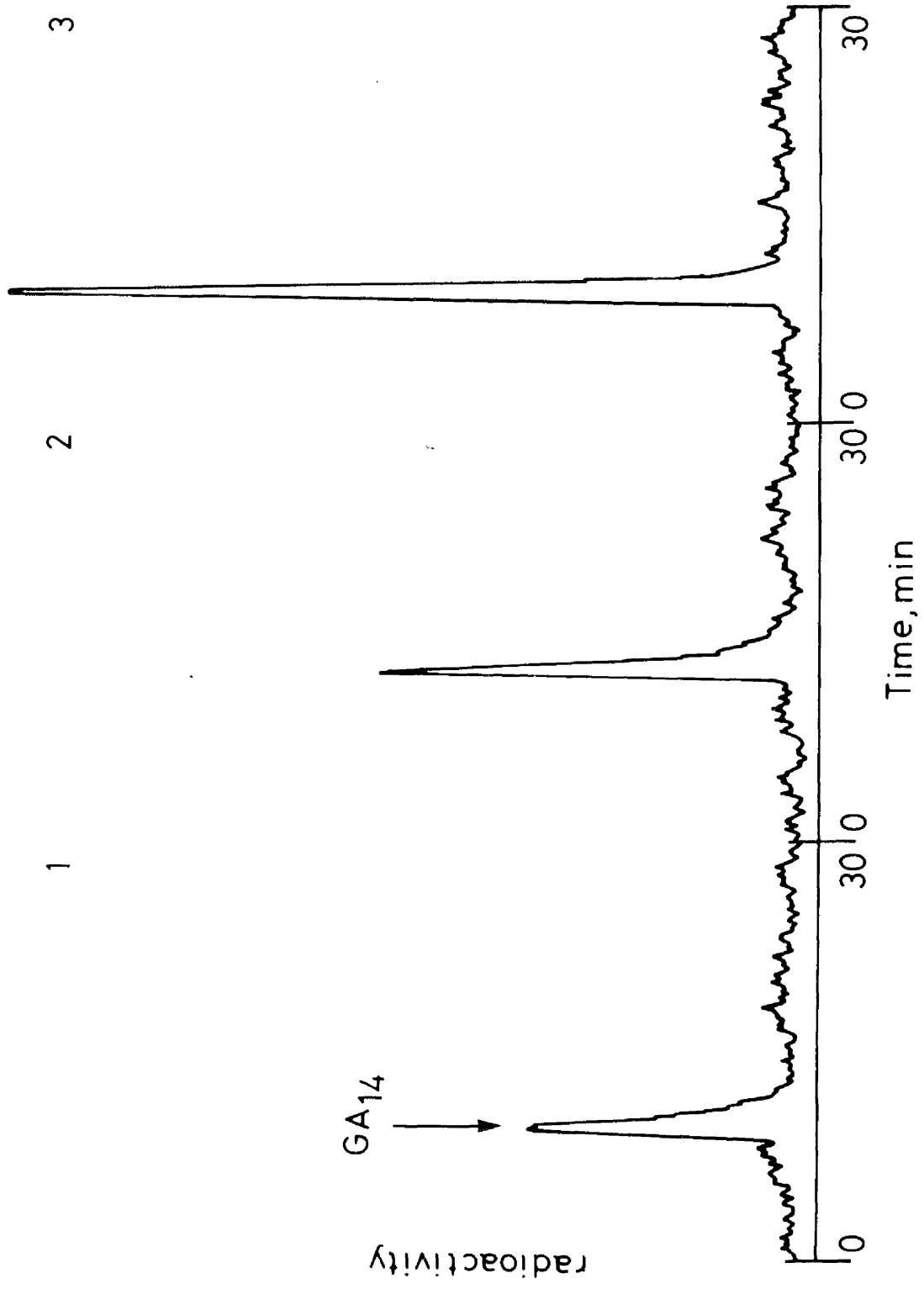
TABLE 39

[^3H]GA₁₄ feeds. Variation with time of acidic ethyl acetate-soluble metabolites (radioactivity, dpm $\times 10^{-6}$, estimated from HPLC analysis, Fig. 40).
Zone B, presumptive GA₁₄, Zone B, presumptive GA₁.

Time (h)	Radioactivity zone	
	A	B
0.5	1.65	0.05
2	0.92	0.09
8	0.72	0.02
24	0.34	0.02

FIGURE 41

[³H]GA₁₄ feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/10 aliquots) 8h after treatment (5 seedlings, 1.0 µCi per treatment). Specific activities: 1, 5.3 µCi.µmol⁻¹; 2, 15.3 µCi.µmol⁻¹; 3, 61.0 µCi.µmol⁻¹ (see Table 40).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD =600 cpm



5.7.2 Effect of dose size

Seedlings were treated with [^3H]GA₁₄ of varying specific activity as shown in Table 40, and harvested after 8h.

TABLE 40

[^3H]GA₁₄ feeds. Adjustment of specific activity

Treatment	Seedlings	Dose per treatment		µg per seedling
		[^3H]GA ₁₄ (µCi)	GA ₁₄ (µg)	
1	5	1.0	60	17.7
2	5	1.0	20	9.7
3	5	1.0	0	5.7

The distribution of radioactivity between solvent fractions (Table 41), and that of ethyl acetate-soluble radioactivity between HMW and LMW fractions (Table 42) was similar in all three treatments despite the disparity in specific activity of [^3H]GA₁₄. Investigations of the LMW fraction by preparative HPLC (Fig. 41) failed to reveal metabolite B found in the time-course experiments, the single zone of radioactivity detected corresponding to GA₁₄.

TABLE 41

[^3H]GA₁₄ feeds. Variation with applied mass of radioactivity (dpm x 10⁻⁶) in various solvent fractions.

Treatment	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
1	29.3	0.43	0.07	0.01
2	25.2	0.47	0.07	0.01
3	23.2	0.43	0.07	0.01

FIGURE 42

[³H] GA₁₄ feed. . Gel permeation chromatogram of acidic ethyl acetate-soluble fraction (1/1000 aliquots) 8h after treatment (200 seedlings, 15.0 µCi).

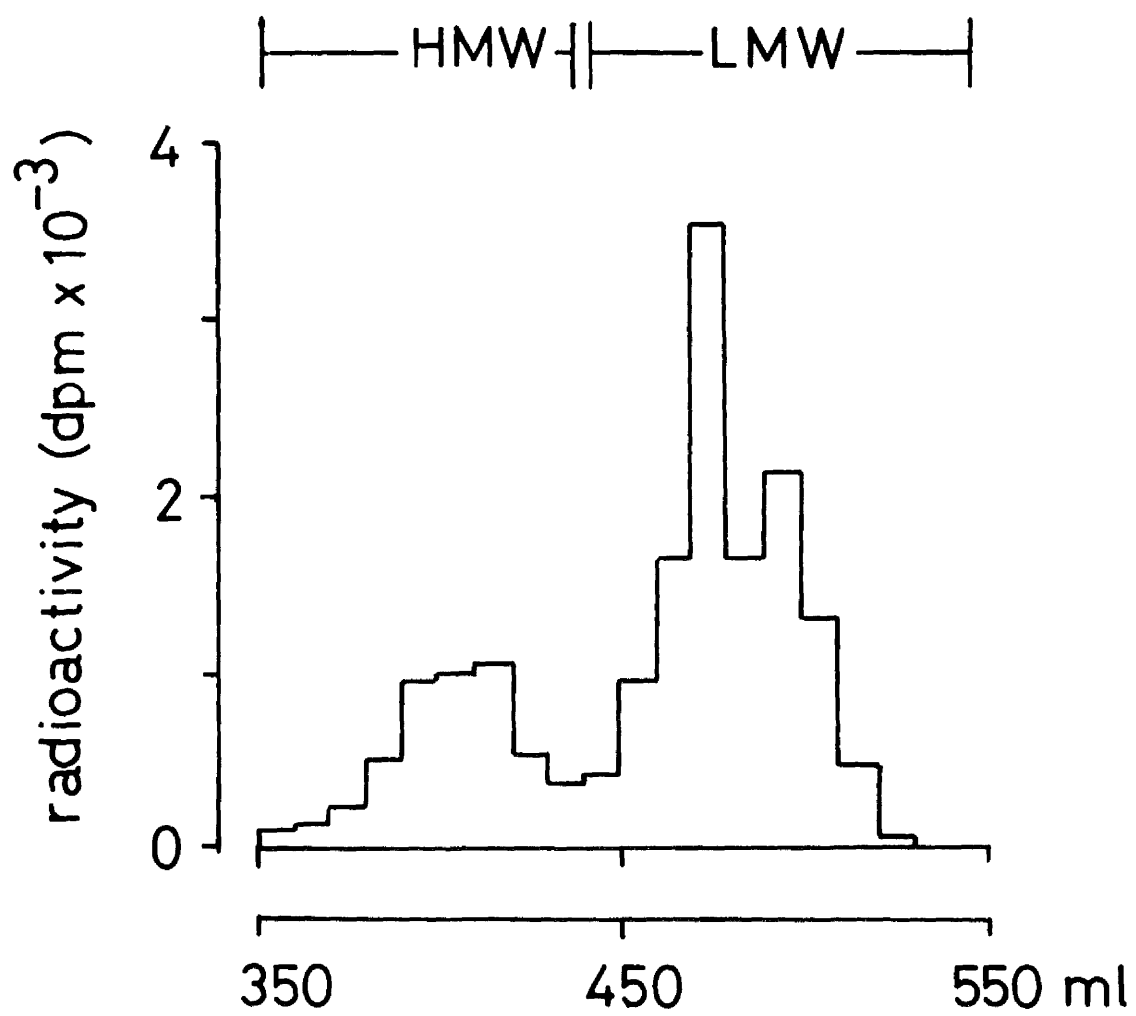


TABLE 42

[³H]GA₁₄ feeds. Variation with applied mass of HMW and LMW fractions of acidic ethyl acetate-soluble extracts. Estimates (dpm x 10⁻⁶) based on radioactivity maxima in GPC eluates.

Treatment	HMW fraction	LMW fraction
1	0.19	0.24
2	0.14	0.33
3	0.19	0.23

5.7.3 Identity of [³H]GA₁₄ metabolites

Two hundred seedlings were treated with 3.0 mg GA₁₄ containing 15.0 µCi [³H]GA₁₄, and harvested at 8h. Radioactivity was distributed between solvent fractions as follows: acidic ethyl acetate, 16.76 x 10⁶ dpm; light petroleum, 0.03 x 10⁶ dpm (discarded); acidic butanol, 0.50 x 10⁶ dpm; neutral butanol, 0.11 x 10⁶ dpm, representing a recovery of 52.7% of the applied radioactivity.

Acidic ethyl acetate-soluble fraction

Gel permeation chromatography (Fig. 42) showed that 5.09 x 10⁶ dpm was associated with the HMW fraction and 11.67 x 10⁶ dpm with the LMW fraction. The enzyme-hydrolysed HMW fraction contained only one radioactive component (A) with the same retention time as GA₁₄ (Fig. 43). This was converted to its benzyl ester, which co-chromatographed with authentic [³H]GA₁₄ BE in the preparative HPLC system (k' = 0.84 in ethyl acetate - hexane, 15:85) and also in analytical HPLC system 1 (Fig. 44). It is therefore assumed that A is the aglycone of either GA₁₄ glucosyl ester or the glucosyl ester of a closely similar GA.

FIGURE 43

[³H]GA₁₄ feed. . HPLC (preparative system) of (1) acidic ethyl acetate-soluble LMW fraction, 1/100 aliquot; (2) hydrolysed HMW fraction, 1/100 aliquot; (3) hydrolysed acidic butanol-soluble fraction, 1/10 aliquot, 8h after treatment (200 seedlings, 15.0 µCi).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.

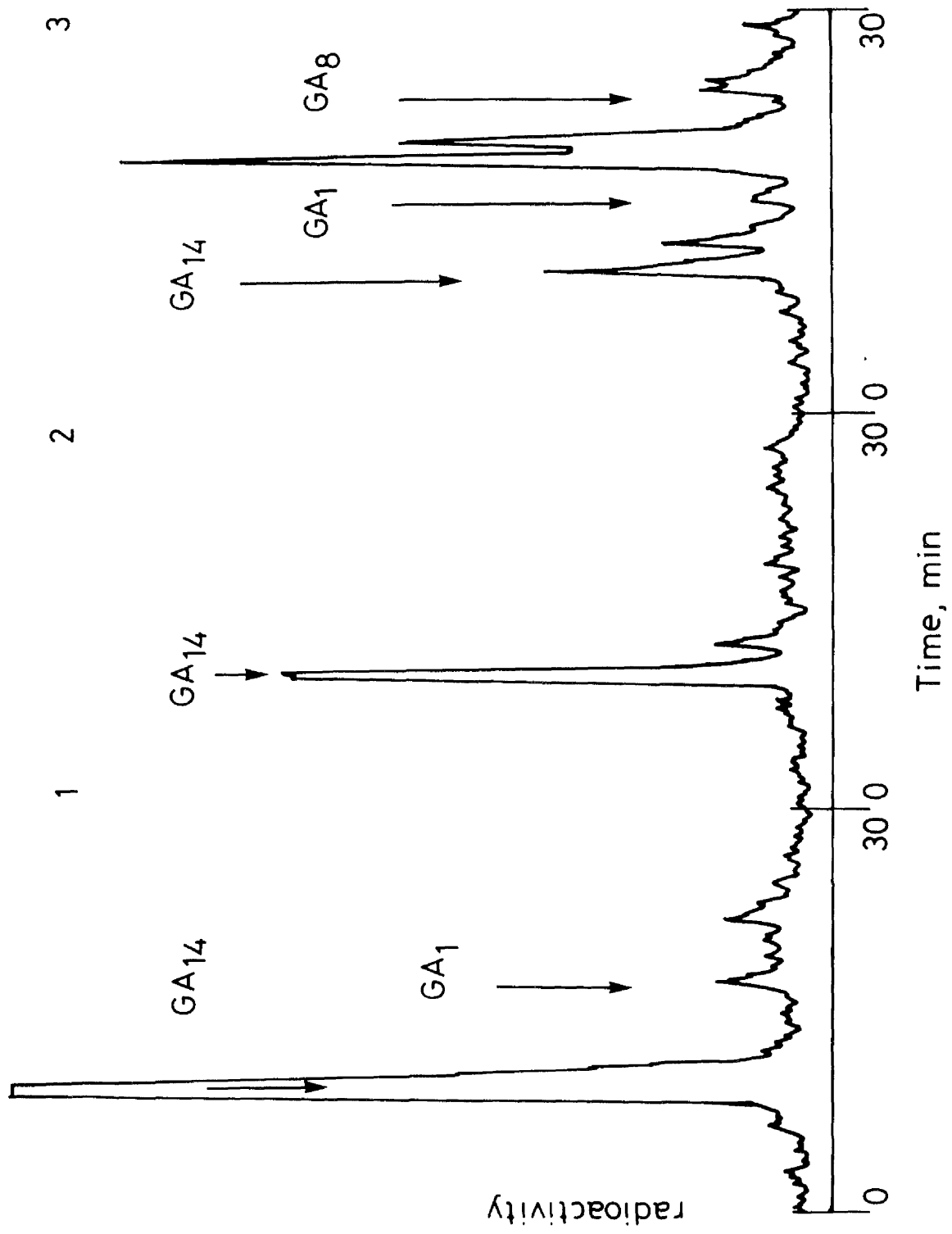


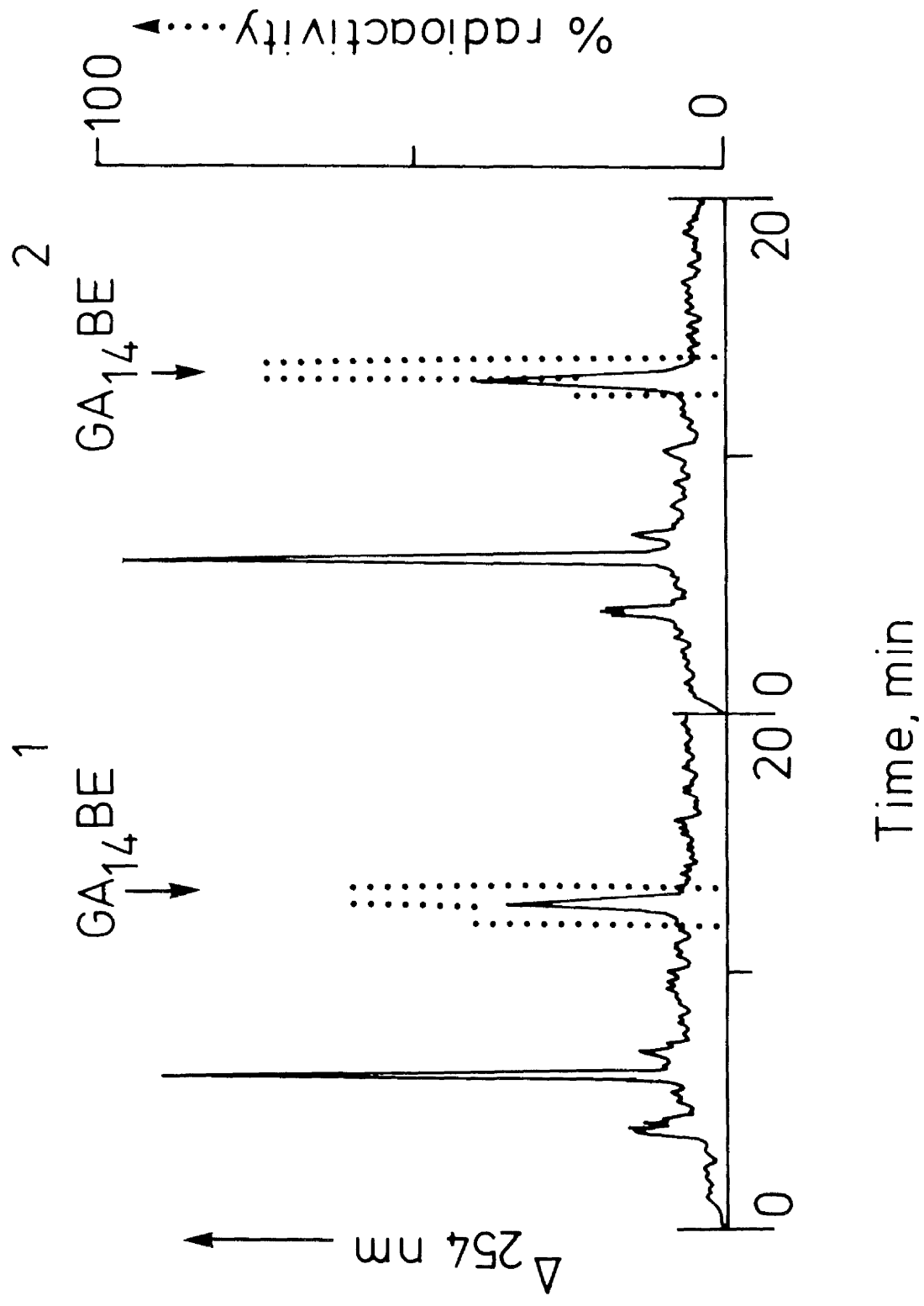
FIGURE 44

[³H]GA₁₄ feed. Analytical HPLC of (1) zone A from hydrolysed HMW acidic ethyl acetate-soluble fraction, and (2) zone A from LMW acidic ethyl acetate-soluble fraction.

Column: 4.6 x 500 mm Partisil 10.

Mobile phase: 70% dichloromethane in hexane

Detector: UV monitor



The major radioactive component in the LMW fraction (A, 5.29×10^6 dpm) also had the same retention time as GA₁₄, and its benzyl ester co-chromatographed with GA₁₄ benzyl ester in the preparative HPLC system, and in analytical HPLC system 1 (Fig. 44). In addition to A, two minor radioactivity zones (B,C) at retention times corresponding to GA₁ and GA₈, were detected by preparative HPLC, but their small size precluded further analysis (Fig. 43).

Acidic and neutral butanol-soluble fractions

After hydrolysis, the acidic butanol-soluble fraction (4.80×10^6 dpm) was shown by HPLC to contain two major and several minor, incompletely resolved radioactive components (Fig. 43). The major components, A (0.16×10^6 dpm) and B (0.34×10^6 dpm), had retention times corresponding to those of GA₁₄ and GA₁ respectively. The benzyl ester of A co-chromatographed with [³H]GA₁₄ benzyl ester ($k' = 0.84$ in ethyl acetate - hexane, 15:85), but the benzyl ester of B had a shorter retention time than that of [³H]GA₁ benzyl ester in the preparative HPLC system (B, $k' = 0.42$; GA₁, BE, $k' = 1.42$ in ethyl acetate - hexane, 55:45), and did not correspond to any available standard. The source of A may be a glucoside of GA₁₄ or a closely similar GA, but the parent glucoside of B could not be identified.

The hydrolysed neutral butanol-soluble fraction (0.11×10^6 dpm) contained one major radioactive component (A, 0.08×10^6 dpm), which had a retention time corresponding to that of GA₁₄, and a minor GA₁-like component (B, 0.02×10^6 dpm). However, the low levels of radioactivity associated with these suggested that their presence in the neutral butanol-soluble fraction resulted from incomplete partitioning, and they probably represent residual A and B from the acidic butanol-soluble fraction rather than independent metabolites.

FIGURE 45

[³H]GA₂₀ feeds. HPLC (preparative system) of acidic ethyl acetate-soluble fractions (1/30 aliquots), 18h after treatment. (5 seedlings, 2.9 μCi per treatment). Specific activities: 1, 38.2 $\mu\text{Ci} \cdot \mu\text{mol}^{-1}$; 2, 181.2 $\mu\text{Ci} \cdot \mu\text{mol}^{-1}$; 3, 2.3 $\text{mCi} \cdot \mu\text{mol}^{-1}$ (see Table 44).
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 $\text{ml} \cdot \text{min}^{-1}$
Detector: radioactivity monitor, FSD = 600 cpm

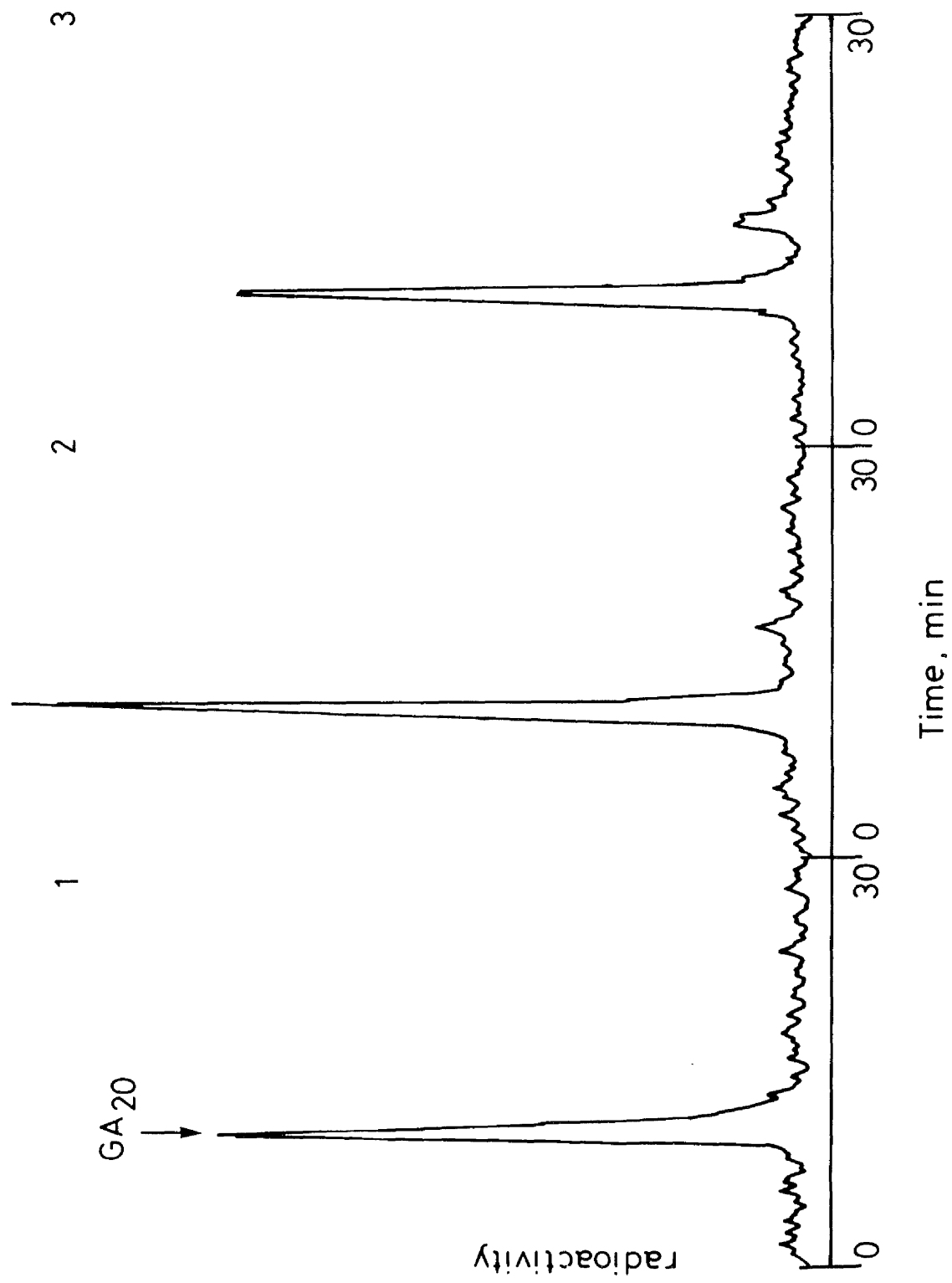
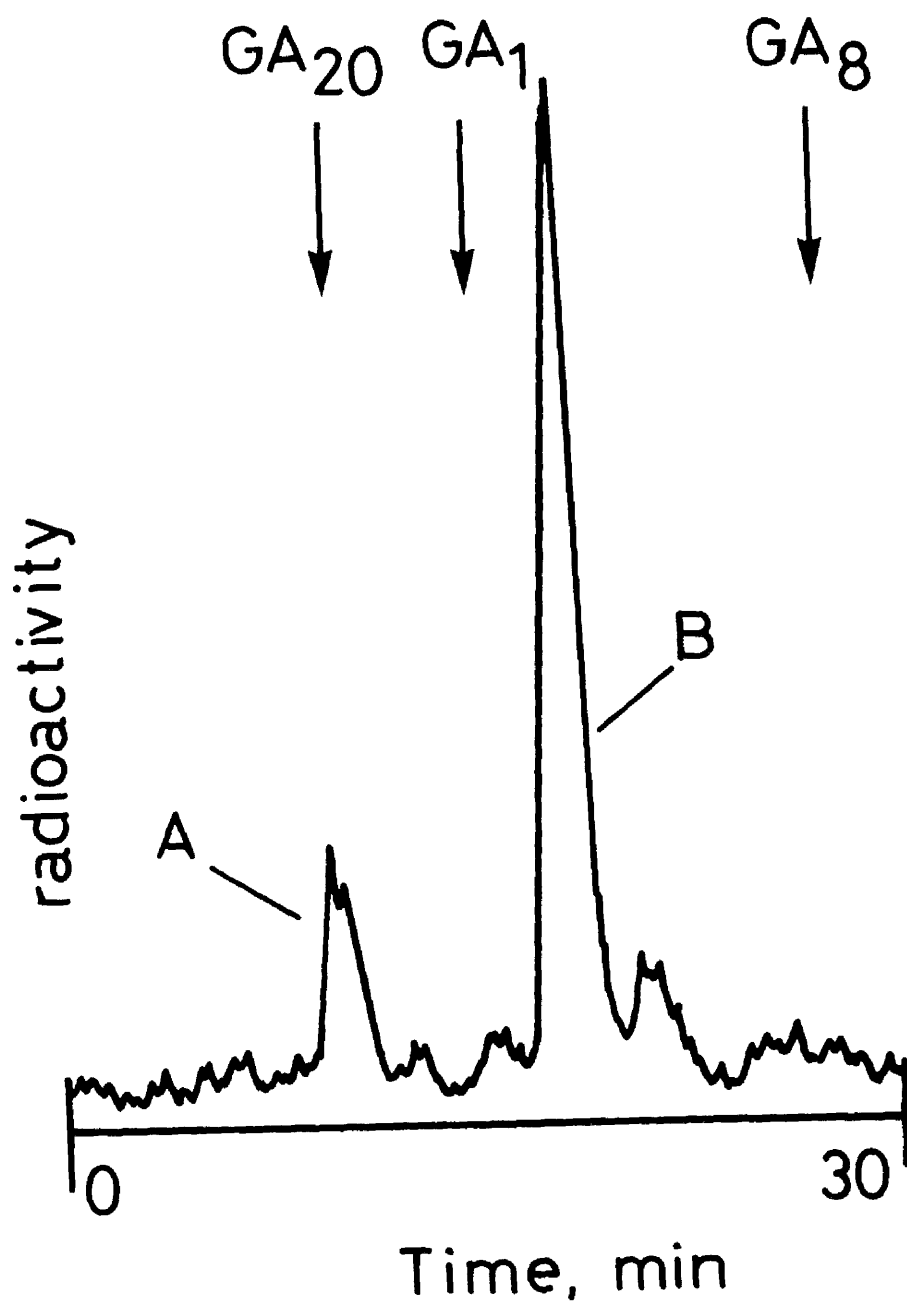


FIGURE 46

[³H]GA₂₀ feeds. HPLC (preparative system) of hydro-
lysed acidic butanol-soluble fraction, 18h after
treatment, from combined treatments 1 - 3.
Column: 10 x 450 mm Partisil 20
Stationary phase: 40% 0.5M formate
Mobile phase: 80 - 100% ethyl acetate in hexane (Fig.5)
Flow rate: 5.0 ml.min⁻¹
Detector: radioactivity monitor, FSD = 600 cpm.



5.8 [³H]GA₂₀ feeds

In preliminary time-course experiments on [³H]GA₂₀, (Table 43) it was noted that the radioactivity in extracts declined with time at approximately the same rate as in [³H]GA₄ feeds, but that there was no evidence of accumulation of radioactivity in solvent fractions other than the acidic ethyl acetate-soluble, nor of the conversion of [³H]GA₂₀ to chromatographically distinct radioactive product. The effect of increasing the applied dose was therefore investigated. Seedlings treated with [³H]GA₂₀ at three different specific activities (Table 44) were harvested at 18h. Recovery of applied radioactivity increased with applied mass in the acidic ethyl acetate-soluble fractions, but no radioactivity exceeding the background level could be detected in other solvent fractions (Table 45).

TABLE 43

[³H]GA₂₀ feeds. Variation with time of radio-
activity (dpm x 10⁻⁶) in various solvent fractions.

Time (h)	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
0.5	51.3	2.24	-	-
2	50.1	2.20	-	-
8	31.9	1.40	-	-
24	24.2	1.04	-	-

TABLE 44

[³H]GA₂₀ feeds. Adjustment of specific activity.

Treatment	Seedlings	μg per seedling		
		[³ H]GA ₂₀ (μCi)	GA ₂₀ (μg)	
1	5	2.92	25	5
2	5	2.92	5	1
3	5	2.92	0	-

TABLE 45

[³H]GA₂₀ feeds. Variation with applied mass of
radioactivity (dpm x 10⁻⁶) in various solvent fractions.

Treatment	% recovery	acidic ethyl acetate	crude aqueous	light petroleum
1	47.8	3.10	-	-
2	29.4	1.91	-	-
3	17.4	1.13	-	-

Investigation of the acidic, ethyl acetate-soluble extracts by GPC showed that the recovered radioactivity was confined to the LMW fractions. Preparative HPLC of these fractions resolved one major zone of radioactivity (A) in the case of treatment 1, but in treatments 2 and 3 an additional, minor zone (B) occurred which had the same retention time as GA₁ (Fig. 45). Recovery of A increased with applied mass, but the radioactivity associated with B decreased (Table 46).

TABLE 46

[³H]GA₂₀ feeds. Variation of acidic ethyl acetate-soluble metabolites with applied mass. Estimates (dpm × 10⁻⁶) based on HPLC analysis, Fig. 45. Zone A, presumptive GA₂₀; zone B, presumptive GA₁.

Treatment	Radioactivity zone	
	A	B
1	2.88	-
2	1.02	0.03
3	1.00	0.12

The crude aqueous fractions were pooled (34.00 × 10³ dpm), hydrolysed and investigated by preparative HPLC. Three radioactivity zones were resolved (Fig. 46): A, 4.02 × 10³ dpm; B, 20.56 × 10³ dpm; D, 1.40 × 10³ dpm. The retention times of A and D corresponded to those of GA₂₀ and GA₈ respectively, but B could not be related to any available GA standard and the low yield of these metabolites precluded further attempts at identification.

TABLE 47
[³H]GA metabolites

[³ H]GA fed	Metabolites	Basis of Identification ^a
A ₁	A ₈	1
	A ₁ glucosyl ether	1, 2, 3, 4 (aglycone)
	A ₈ glucosyl ether	1, 2 (aglycone)
A ₄	A ₁	1, 2, 3, 4
	A ₈	1, 2
	A ₄ glucosyl ester	1, 2, 3 (aglycone)
	three unidentified glucosyl esters	1, 2 (aglycones)
	A ₁ glucoside	1, 2 (aglycone)
	two unidentified glucosyl ethers.	1, 2
A ₅	A ₅ glucosyl ether	1 (aglycone)
A ₈	A ₈ glucosyl ether	1 (aglycone)
A ₉	A ₂₀	1, 2, 3, 4
	A ₂₀ glucosyl ether	1, 2, 3, 4 (aglycone)
A ₁₂ aldehyde	seven unidentified GA-like	1, 2
	five unidentified glucosyl esters.	1, 2 (aglycones)
	seven unidentified glucosyl ethers	1, 2 (aglycones)
A ₁₄	A ₁₄ glucosyl ester	1, 2, 3 (aglycone)
	A ₁	1
	A ₈	1
	A ₁₄ glucosyl ether	1, 2 (aglycone)
	unidentified glucosyl ether	1, 2 (aglycone)
A ₂₀	A ₂₀ glucosyl ether	1 (aglycone)
	two unidentified glucosyl ethers	1 (aglycone)

^a 1, prep. HPLC of free GA; 2, prep. HPLC of GABE; 3, analytical HPLC of GABE; 4, MS of GABE.

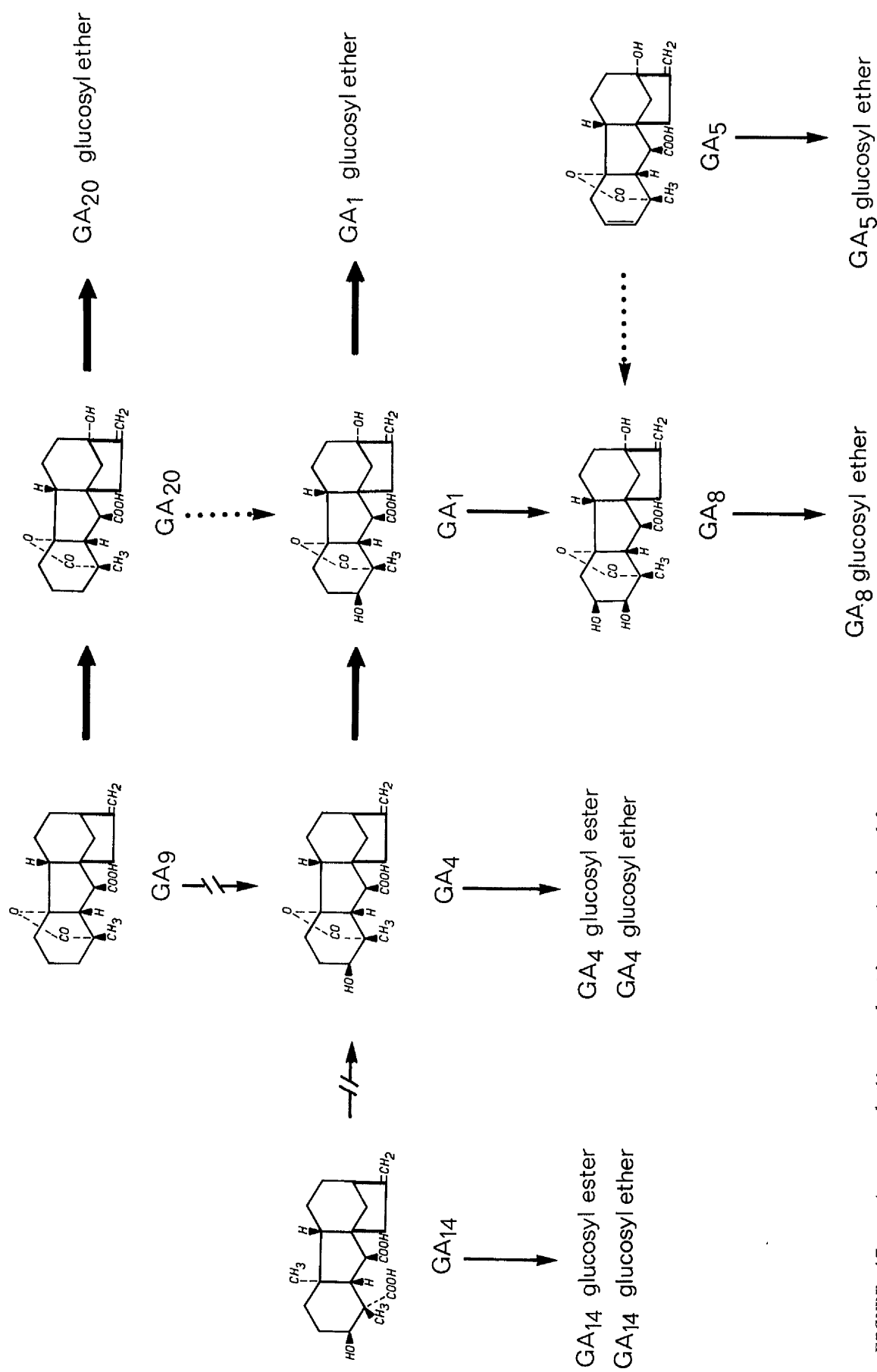


FIGURE 47. Proposed GA metabolic relationships in *P. coccineus* seedlings.

DISCUSSION

The redistribution of radioactivity from [^3H]GAs in intact P. coccineus seedlings is consistent with the view that GAs originating in the shoot apex have a relatively limited distribution, whereas root-synthesised GAs are readily translocated to all parts of the plant. Consequently, the influence of apically synthesised GAs may be restricted to tissues in the vicinity of the apical bud, which would include the subapical meristem responsible for shoot elongation (Sachs, 1965). While there was no evidence of a preferential accumulation of radioactivity at the elongating zone after treatment of apical buds with [^3H]GAs, detectable amounts of radioactivity were found in this region within 24h, by which time the promotive effects of apically applied GAs on the subapical elongation of shoots could also be observed. The low percentage of the applied radioactivity associated with the elongating zone may imply that the subapical meristem is sensitive to a correspondingly small proportion of the applied dose, but an alternative possibility is that little radioactivity accumulates because of rapid turnover of GAs by actively enlarging cells.

A regulatory role for root-synthesised GAs, on the other hand, would require facile upward translocation. Available evidence for the occurrence of GA-like activity in bleeding sap (e.g. Carr et al., 1964) suggests that this occurs in the xylem. The pattern of distribution of [^3H]GAs following basal application to P. coccineus seedlings implies that they readily gained access to the xylem conduits, a possibility which is supported by the appearance of radioactivity in bleeding sap 4h after basal application. Furthermore, the upward movement of radioactivity was not inhibited by heat-girdling treatments (data not presented).

Although the rate of upward translocation of [^3H]GAs in intact plants appeared to be considerably greater than that of basipetal export from apical buds, approximately similar amounts of radioactivity were found to be associated with the elongating region of shoot explants 72h after application of [^3H]GAs to either the apical bud or the base of the explants. This is perhaps consistent with the finding that elongation of explants was promoted to the same extent by apically - and basally-supplied GAs, and would suggest that, although the rate of upward GA translocation estimated from the acropetal export of basally-supplied [^3H]GAs is high, only a relatively small proportion of the upward-moving GAs is involved in internode elongation. This in turn may be a result either of the more rapid metabolism of basally-supplied GAs or of their diversion to the primary leaves. The lack of accumulation of radioactivity in the subapical region, in contrast to the preferential accumulation of [^3H]GA₁ and [^3H]GA₅ reported by Musgrave et al. (1969) for etiolated pea shoots, may perhaps reflect differences in the rate of utilisation of applied GAs by etiolated and light-grown tissue, such as those shown in light-grown and etiolated P. coccineus seedlings treated with [^3H]GA₄ (Bown et al., 1975).

It has been proposed that the physiological effectiveness of certain GAs may depend on the efficiency of their conversion to a more active structure (Reeve and Crozier, 1974). GA₁ is more active than GA₄, GA₅ or GA₂₀ in a number of bioassay systems (Reeve and Crozier, 1975) and it could perhaps be argued that the equivalent growth responses evoked in P. coccineus shoot explants by GA₁ and GA₄ are a consequence of the conversion of GA₄ to GA₁ which was established in later experiments. However, GA₅ and GA₂₀, which also induced the same degree of response as GA₁ in this system, gave rise

mainly to glucosyl ether-type metabolites rather than detectable amounts of GA_1 . Glucosyl ethers are relatively ineffective in promoting growth (Yokota et al., 1971) and consequently the observed response to GA_1 , GA_4 , GA_5 and GA_{20} may well be due to these compounds themselves. Alternatively, metabolites which do not accumulate in quantities sufficient to permit detection may represent the physiologically active species. However, the relationship between metabolic transformations of GAs and their biological activity is at present poorly understood and there are no clear indications as to possible requirements for either pool sizes or turnover rates.

It is possible that GAs are imported from the xylem by apical buds and that they or their metabolites are subsequently re-exported in a basipetal direction by a symplastic pathway at the same rate as GAs synthesised de novo. Some evidence for such a process is provided by a comparison of chromatograms of apical-bud and stem extracts from basally-treated seedlings. In the case of [3H] GA_1 , [3H] GA_5 and [3H] GA_{20} treatments, the two profiles correspond closely, suggesting an accumulation in apical buds of acropetally-moving metabolites. After [3H] GA_4 treatments, however, a polar metabolite which was the major radioactive component of stem extracts was found in relatively small quantities in the apical bud, whereas a less polar compound, probably GA_4 itself, had accumulated.

After applications of [3H]GAs to the hypocotyl, radioactivity was found in roots, cotyledons and aerial parts, but mostly did not correspond chromatographically to the original [3H]GA, except in the case of [3H] GA_5 treatments. The upward movement of GAs and their metabolites from the hypocotyl region can readily be explained in terms of xylem movement, but a downward translocation, presumably in the assimilate stream, is implied by the occurrence of small

amounts of radioactive compounds corresponding to each of the applied [^3H]GAs in root extracts.

Apical buds of both intact seedlings and explants exported very little applied [^3H]GA in a basipetal direction. In the case of shoot explants it could furthermore be shown that the amount of radioactivity reaching basal agar receivers from the treated apical buds depended on the length of the intervening internode, suggesting a diffusion-type movement. The low levels of exported radioactivity are in agreement with the low levels of endogenous diffusible GA-like activity obtained from excised P. coccineus shoots (Crozier and Reid, 1972). Extracts of P. coccineus seedlings which had been partially purified by silicic acid partition chromatography could be shown by bioassay to contain at least six distinct zones of GA-like activity, including one whose retention characteristics corresponded to those of GA₁. Diffusates, in contrast, contained only the GA₁-like component, and after 24 hours' diffusion, the GA₁-like activity in agar receivers represented only 5% of that extractable from the tissue. Discrepancies between the extractable and diffusible GAs of several species have previously been reported (e.g. Jones, 1968; Jones and Lang, 1968; Cleland and Zeevaart, 1970). However, estimates of these discrepancies based on TLC and bioassay of crude extracts are probably conservative. Since bioassay responses are markedly affected by impurities, the levels of GA-like activity in crude extracts may be considerably underestimated relative to those in diffusates, which generally contain few impurities. Consequently, the diffusible GA-like activity may in reality represent a still smaller proportion of the total GA-like activity of a tissue than appears from the bioassay data.

Estimates of GA levels based on diffusion and bioassay can therefore not be regarded as providing an accurate reflection of the GA content of a tissue, and the identity of diffusible GAs with GAs undergoing normal translocation processes is also open to question, since it seems likely that these processes must themselves be affected by the excision of explants. Since a number of conclusions, such as the identification of apical buds and root tips as sites of GA biosynthesis, rest principally on the evidence of diffusion experiments (Jones and Phillips, 1966), a critical re-evaluation of the relationship of diffusible GAs to the endogenous GA pools of intact plants would seem to be essential.

The cotyledons of developing seedlings have been implicated as an additional source of endogenous GAs, since there is some evidence that the GA glucosyl derivatives which accumulate in seeds may be hydrolysed on germination to release free GAs (see Lang, 1970; Sembdner et al., 1972, 1974). Hartung and Phillips (1974) observed that [^3H]GA₁ injected into the cotyledons of three-day-old P. coccineus seedlings moved into the roots in an acropetal direction, and this was taken as evidence for the corresponding translocation of endogenous GAs. In the present study, in contrast, when [^3H]GAs were injected into cotyledons at various stages of seedling development, the amount of radioactivity recoverable from the remainder of the plant at any one time was so small as to suggest that the cotyledons do not supply a significant proportion of the seedling's GA requirements. However, isolated, as well as attached, cotyledons accumulated relatively large amounts of a [^3H]GA₄ conversion product, probably GA₄ glucosyl ester. This finding is perhaps consistent with the high levels of glucosyl transferase activity reported to occur in P. coccineus cotyledons,

although incubation of this enzyme system with GA₃ produced GA₃ glucosyl ether rather than its glucosyl ester (Müller *et al.*, 1974).

Attempts to estimate the rates of conversion of [³H]GA₄ by isolated apical buds and roots were inconclusive, as a result of a rapid loss of radioactivity to the incubation medium. However, when the apical buds, hypocotyls and roots of intact plants were treated with [³H]GAs, considerably greater utilisation of the GAs, as estimated from the chromatographic distribution of radioactivity in extracts, took place after basal than after apical treatments. The ability to convert, as well as to export, applied GAs therefore appears to differ in different parts of seedlings. It is probably that the extensive redistribution of [³H]GAs following basal application facilitated their conversion, perhaps by bringing them into contact with metabolically active sites, but the appearance of radioactive conversion products in bleeding sap within 4h of application to the hypocotyl indicated that at least some conversion of [³H]GAs took place near the point of application.

Analysis of extracts of whole seedlings at various times following basal [³H]GA feeds generally showed a progressive increase in the radioactivity associated with most of the metabolites listed in Table 47, with a concomitant decrease in radioactivity associated with the precursor GA. However, in some instances, notably after [³H]GA₁₂ aldehyde feeds, the variation in amounts of recovered radioactive metabolites with time was more complex, and maximal radioactive concentration was reached at different times by different metabolites (Fig. 34). Glycosylated metabolites generally accumulated with increasing time but the free GA-like metabolites corresponding to the aglycones of certain of the conjugates could often not be detected, even at short time intervals after feeding the precursor GA. Examples of glucosyl derivatives whose aglycones did not accumulate in quantities sufficient for detection

were found after feeding GA_4 (Figs. 19, 20), GA_{14} (Fig. 43) and GA_{20} (Figs. 45, 46). Thus the analysis of extracts at various times after feeding [3H]GAs, while of practical value in locating maximal concentrations of metabolites, also provided evidence for the existence of reaction sequences involving short-lived intermediates.

The amount of radioactivity recoverable after [3H]GA feeds decreased markedly with time, and from those metabolic transformations which could be established, it was assumed that this decrease resulted at least in part from the hydroxylation of tritiated positions and the subsequent elimination of tritium from the system. The conversion of [3H]GA₁ to [3H]GA₈ catalysed in vitro by an enzyme system from P. vulgaris cotyledons has been shown to yield tritiated water as a quantitative by-product (Patterson et al., 1975), although analogous reactions occurring in vivo would result in the loss of tritiated water by transpiration, and its production can therefore not be used as an assay in open systems.

The synthesis of [3H]GAs by selective hydrogenation results in labelling principally at the C-1, C-2 and C-3 positions. Consequently, hydroxylations involving these three loci would be expected to result in a loss of radioactivity the magnitude of which depends mainly on the configuration of the labelled atoms and the stereospecificity of the hydroxylation mechanism. In practical terms, a conversion such as that of [$1,2-^3H$]GA₄ to [$1,2-^3H$]GA₁, representing 13-hydroxylation, would not be expected to result in a significant difference between the respective specific activities of precursor and product. At the opposite extreme, a reaction sequence such as the conversion of [$2,3-^3H$]GA₂₀ to GA₈ via GA₁ would involve hydroxylations at both C-2 and C-3. All of the radioactive label originally associated with [3H]GA₂₀ could theoretically be lost as a result,

although in practice non-specific labelling and stereo chemical considerations would probably ensure that a significant proportion of label remained associated with GA₈.

The radioactive GAs produced by a Wittig-type synthesis are labelled principally at C-17, and consequently the specific activity of conversion products formed by hydroxylations at positions on the A or C rings should be the same as that of the precursor, always provided that there is no significant dilution with endogenously produced material. However, it has been shown that GA₇ can be converted to GA₇ noraketone by non-specific hydroxylating enzyme systems (Jones et al., 1971). Analogous reactions occurring in vivo have not to date been demonstrated, but represent at least a potential means whereby the radioactivity associated with [17,17'-³H]GAs could be eliminated from the gibbane skeleton. A comparison of the rates of disappearance of radioactivity from different [³H]GAs applied to P. coccineus seedlings suggests that in this system the loss of radioactivity, as well as the accumulation of radioactive metabolites is determined primarily by specific metabolic transformations. This contention is supported by the observed similarities between the rates of utilisation of [2,3 -³H]GA₉ and [17,17' -³H]GA₉, and the similar yields of radioactive conversion products found in each case.

The rate of disappearance of labelled precursor provided a relatively accurate means of estimating the effects of dose size on the utilisation of individual [³H]GAs, and also permitted comparisons between GAs. The effect of dose size on the rate of conversion within any GA treatment was less accurately determinable from the accumulation of products, as result firstly of the loss of label from the system, and secondly of the further metabolism of

products, the rate of synthesis not necessarily corresponding to the observed accumulation. However, there was no evidence of a qualitative variation in the metabolites detectable at a given time as a result of increasing the dose size. This is in contrast to the apparent artefacts produced by maturing *P. vulgaris* seeds in response to high doses of [^3H]GA₈ (Yamane *et al.*, 1975). Quantitative estimates of the effect of dose size on precursor utilisation suggested that seedlings were able to accommodate amounts of applied GAs within the range tested (up to 25 µg per seedling) with no apparent evidence of the distinct increase in the ratio of dose to metabolite yield which would result from saturation of the conversion mechanism. The increasing applied dose led to an increase in metabolite pool sizes, and this was of practical value since the increased mass associated with metabolites facilitated their characterisation.

Possible metabolic relationships between the applied GAs and the conversion products listed in Table 47 are outlined in Fig. 47. The most marked accumulation of free GA-like metabolites resulted from the conversions of GA₄ to GA₁ and of GA₉ to GA₂₀. Accordingly, possible explanations for the accumulation of these two metabolites were sought in the rates of further conversion of GA₁ and GA₂₀ themselves. Applied [^3H]GA₁ gave rise to GA₁ glucosyl ether and smaller amounts of presumptive GA₈ and GA₈ glucosyl ether. Feeding of [^3H]GA₈, however, resulted in the rapid elimination of radioactivity and a small accumulation of a conjugate resembling GA₈ glucosyl ether. This rapid loss of radioactivity from [^3H]GA₈ probably accounts for its failure to accumulate as a metabolite of [^3H]GA₁. Although none of the metabolites of [^3H]GA₁ accumulated in large quantities relative to the applied dose, the disappearance within 18h of up to 90% of the applied radioactivity indicated that the GA₁ was readily converted.

Clearly the accumulation of GA_1 after $[^3H]GA_4$ treatment is not a consequence of the plant's being unable to metabolise GA_1 . It can, however, be ascribed to a rate of synthesis of GA_1 from GA_4 which is more rapid than the rate of conversion of GA_1 to other products, including GA_8 . Similarly the high rate at which seedlings metabolised $[^3H]GA_8$ is in keeping with the transient appearance of small quantities of GA_8 after $[^3H]GA_1$ and $[^3H]GA_4$ feeds.

The accumulation of GA_{20} as the major metabolite of GA_9 can likewise not be explained in terms of a low rate of further conversion of GA_{20} , since between 50% and 85% of the applied radioactivity had been eliminated within 18h of feeding $[^3H]GA_{20}$. Most of the remaining radioactivity was associated with GA_{20} itself, but small amounts of glucosyl ether-type conjugates could be detected, and in some experiments a compound with retention properties resembling those of GA_1 was also present. Thus the possibility that both GA_4 and GA_{20} are precursors of GA_1 in P. coccineus cannot be ruled out. The low recovery of $[^3H]GA_{20}$ indicates that it is efficiently utilised, but as there was no appreciable accumulation of metabolites it appears that their rate of metabolism was at least equal to their rate of synthesis. The metabolites of GA_4 , on the other hand, including GA_1 , accumulated in considerable quantities. If it is assumed that the rate of utilisation of GA_1 formed from GA_4 is the same as that of GA_1 formed from GA_{20} , the discrepancy in yield can most conveniently be explained in terms of a more rapid conversion of GA_4 to GA_1 than of GA_{20} to GA_1 . Some evidence that the former, 13 α -hydroxylation, mechanism is favoured in P. coccineus is seen in the conversion of GA_9 to its 13 α -hydroxy-derivative, GA_{20} , whereas the 3 β -hydroxylation product, GA_4 , could not be shown to be formed. An alternative possibility is that the GA_1 formed from GA_{20} is more readily metabolised than is the GA_1 formed from GA_4 . However, this hypothesis is difficult to evaluate in view of the lack of information

regarding the intracellular localisation of GA pools and associated enzyme systems.

A trihydroxy-GA such as GA₈ could be regarded as the end product of a metabolic pathway proceeding in the direction of increasing hydroxylation. Small quantities of presumptive GA₈ and its glucosyl ether were detected after feeding all the [³H]GAs tested, with the exception of GA₉ and GA₂₀, although the apparent rapid turnover of metabolites of GA₂₀ might well preclude the detection of GA₈ if, as may be possible, it were formed via GA₁. The failure of GA₈ to accumulate, however implies the existence of efficient catabolic mechanisms. Clearly these must involve the loss of the radioactive label at C-1, and consequently [¹⁴C]-ring labelled precursors would be required in order to investigate their nature. However, the rapid disappearance of radioactivity associated with [³H]GA₈, together with indications that GA₈ represents the last detectable free GA intermediate in a number of reaction sequences, strongly suggests that much of the loss of radioactivity observed to result from the metabolism of [³H]GAs may take place as a result of the further conversion of GA₈ or its conjugate.

The behaviour of applied [³H]GA₅ was anomalous in that the loss of radioactivity and the accumulation of the single, glucosyl ether-like metabolite were both low relative to the other GAs tested. The small amount of [³H]GA₅ converted during 18h indicates the opposite extreme to GA₈ in the wide range of variation in the rates of utilisation of applied [³H]GAs, but is also of interest because it implies that seedlings can tolerate the presence of a relatively large pool of unmetabolised GA₅. The pool size of the GA₅ conjugate at 18h implies that only small amounts of GA₅ were glycosylated in comparison with the considerable accumulation of glucosides after GA₄

feeds, for instance, and this raises the question of whether glycosylation does indeed represent the most significant mechanism whereby the reactivity of GAs is reduced.

The presence of GA₁, GA₄, GA₅, and GA₂₀ in P. coccineus seedlings has been demonstrated by GC-MS (Bowen et al., 1973). GA₈ has been shown to occur in the seeds (Durley et al., 1971), but neither GA₈ nor GA₉ has been conclusively established as a seedling component, although biologically active compounds with similar retention characteristics to GA₈ and GA₉ are found in seedling extracts (Crozier and Reid, 1971; Crozier et al., 1973). On the basis of observed transformations, GA₄ is clearly a precursor of endogenous GA₁, while endogenous GA₂₀ could be derived at least in part from GA₉. The origins of GA₄, GA₅, and GA₉ themselves could not however be established. GA₄ appears not to be formed from GA₉, as in Cucurbita maxima, nor from GA₁₄, as in Gibberella fujikuroi (see Fig. 2). The relative amounts of endogenous GA₁, GA₄, GA₅ and GA₂₀, and also of presumptive GA₈ and GA₉ in seedlings are in good agreement with the rates at which seedlings convert GAs of exogenous origin. Thus, GA₉ is converted in high yield to GA₂₀, while the endogenous GA₂₀ pool is large relative to endogenous GA₉-like compounds; similarly, the large endogenous GA₅ pool corresponds to the low rate of utilisation of [³H]GA₅. The rapid conversion of [³H]GA₄ to [³H]GA₁ is reflected in the relative pool sizes of endogenous GA₄ and GA₁; [³H]GA₈, which is apparently readily formed from [³H]GA₁, does not accumulate as a product of this reaction and rapidly disappears when fed directly to the seedling, while appreciable quantities of endogenous GA₈ cannot be shown to occur. The large number of free GA-like and glycosylated metabolites which were detected after feeding [³H]GA₁₂ aldehyde suggests that GA₁₂ aldehyde is readily utilised as a precursor in P. coccineus

seedlings. Members of the reaction sequences leading to the four characterised endogenous GAs may well be amongst this array of metabolites. However, until GA₁₂ aldehyde has been established to be an endogenous component of P. coccineus seedlings and conclusive identifications of its metabolites have been obtained, it will not be possible to determine the role of this compound with any degree of certainty. All of the GAs applied underwent conversion to more polar compounds from which free GAs could again be released by enzymatic hydrolysis. On the basis of their partition and chromatographic characteristics and their susceptibility to β -glucosidase, these conversion products were taken to represent both GA glucosyl ethers and esters (Sembdner et al., 1972, 1974; Takahashi, 1974). The relative proportions of the two types of glucoside varied considerably depending on the GA fed. Each GA, with the exception of GA₉, and each free GA-like metabolite gave rise to a corresponding glucosyl ether-like conjugate. In addition, glucosyl ethers whose aglycones were not detected amongst the free GA-like metabolites were formed after feeding [³H]GA₄ and [³H]GA₂₀. In the case of GA₉, the absence of hydroxyl functions would preclude the formation of a glucosyl ether, and, in keeping with this prediction, no GA₉-like components were to be found amongst the hydrolysis products of the glucosyl ether-containing fraction after [³H]GA₉ feeds.

Glucosyl ester-like conjugates, unlike glucosyl ethers, were apparently not formed when [³H]GA₁, [³H]GA₅, [³H]GA₈ or [³H]GA₂₀ were fed, but a considerable proportion of the radioactivity recovered after feeding [³H]GA₄, [³H]GA₁₂ aldehyde or [³H]GA₁₄ represented glucosyl ester-type conjugates. The free GAs released by enzymatic hydrolysis suggested that at least four glucosyl esters were formed from [³H]GA₄ feeds. One of these appeared to be GA₄ glucosyl ester,

but the remainder gave rise to unknown aglycones which did not correspond to observable free GA-like metabolites. In contrast, the five aglycones obtained by enzymatic hydrolysis of the glucosyl ester-like products of [^3H]GA₁₂ aldehyde could all be related to free GA-like metabolites, while GA₁₄ gave rise to a single glucosyl ester-like metabolite, apparently GA₁₄ glucosyl ester.

Both glucosyl ethers and esters of GAs have been shown to occur in vegetative tissues (Harada and Yokota, 1970; Lorenzi et al., 1976), the type of conjugate formed perhaps being determined by local conditions as well as by the structure of the GA undergoing glycosylation. The glycosylation of applied GAs may consequently reflect an endogenously-occurring sequestration process, rather than an induced, artificial response to supra-optimal GA levels (see Parke, 1968), but the possible influence of conjugation of GAs on endogenous GA pools remains to be finally established.

Some of the transformations of [^3H]GAs carried out by P. coccineus seedlings, such as the conversion of GA₄ to GA₁ and of GA₁ to GA₈, also occur in other vegetative tissues (e.g. Durley and Pharis, 1973; Davies and Rappaport, 1975b). However, the utilisation of some GAs differs considerably from that reported for other systems. For instance, although there were indications that [^3H]GA₁₄ was readily metabolised by P. coccineus seedlings, there was no appreciable accumulation of free GA-like metabolites. In contrast, in etiolated dwarf Pisum sativum seedlings, there was a considerable accumulation of GA₁₈, GA₃₈, GA₂₃, GA₁ and GA₈ after supplying [^3H]GA₁₄ (Durley et al., 1974). It is possible that distinctly different metabolic pathways operate in the two systems. However, the observed qualitative differences in detectable intermediates could equally well be a consequence of quantitative disparities in

relative turnover rates of metabolites, which would allow the accumulation of the metabolites in one instance but not in the other. These disparities could in turn reflect inherent differences between the two species, or, at least in the case in question, could also be related to the fact that the Pisum seedlings were grown in darkness and the Phaseolus coccineus seedlings in the light.

A more critical comparison of GA metabolism in different species can be made in the case of GA₂₀, which, unlike GA₁₄, is known to occur endogenously in P. coccineus (Bowen et al., 1973) and also in Bryophyllum daigremontianum (Gaskin et al., 1973). Light-grown Bryophyllum plants readily converted [³H]GA₂₀ to GA₂₉, which accumulated in appreciable quantities (Durley et al., 1975), whereas there was no comparable accumulation in P. coccineus. Species differences such as these are sufficient to cast some doubt on the suggestion that the observed metabolites of applied GAs represent the products of non-specific transformations (see MacMillan, 1974b).

Differences in the general metabolism of seeds and seedlings may well be reflected in the GA metabolism of vegetative and reproductive tissues. There were a number of qualitative similarities between the products of applied [³H]GAs in maturing P. vulgaris seeds (Yamane et al., 1975) and in P. coccineus seedlings, but as far as could be estimated from the available data, the quantitative differences in metabolite yields were considerable. The high yields of radioactive metabolites often obtained in seed systems reflect the high levels of endogenous GAs typically accumulated by developing seeds (e.g. Durley et al., 1971). Conversely, the low endogenous GA levels of P. coccineus seedlings may be related to a substantially higher rate of turnover evidenced by the high rate of utilisation of applied [³H]GAs. Much of the radioactivity

associated with [^3H]GAs, as has been seen, appears to be eliminated during the further conversion of [^3H]GA₈, which consequently appears to be the last free GA in the metabolic sequence. An explanation for the accumulation of metabolites by seeds but not by seedlings may therefore be sought in the relative rates of removal of GA₈ in the two types of system. In P. vulgaris seeds [^3H]GA₈ is indeed found to accumulate (Yamane et al., 1975), whereas in P. coccineus seedlings [^3H]GA₈ is rapidly eliminated, and it is clearly relevant that endogenous GA₈ levels in P. vulgaris and P. coccineus seeds are relatively high (Durley et al., 1971; Hiraga et al., 1974a), whereas endogenous GA₈ could not be detected in identifiable quantities in P. coccineus seedlings (Bowen et al., 1973).

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