INDOLE-3-ACETIC ACID IN ROOT NODULES OF

ALNUS GLUTINOSA (L.) GAERTN.

A thesis submitted to the University of Glasgow
for the degree of Master of Science

by

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December, 1979.

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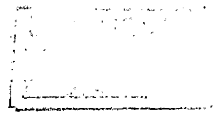
Nodulated seedling of Alnus glutinosa (L.) Gaertn.

To my parents



C O N T E N T S

ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my supervisor, Dr. C. T. Wheeler for much encouragement throughout the course of this work and for invaluable critical comment. I also wish to thank Professor M. B. Wilkins for the use of facilities within the Department of Botany.

For a great deal of technical advice, help and encouragement in developing parts of this project, I am extremely grateful to Mr. D. Reeve and Dr. A. Crozier.

Thanks are also due to Mrs. L. Williamson for her excellent typing, Mr. T. Norman Tait for his exceptional photography and to all former and present fellow staff and post-graduate students in the Department of Botany, who have helped in innumerable ways through advice, assistance and friendship, to make this thesis possible.

Finally, I would like to acknowledge forbearance of friends and relatives during the course of this work, especially my parents, whom I would like to thank for their continued support and encouragement.

SUMMARY

Two aspects of the occurrence of IAA in nodulated plants of *Alnus glutinosa* have been investigated. Endogenous levels of IAA in root nodule extracts were compared to those from *Myrica gale* and *Vicia faba*. The mobility of exogenously-applied radioactive IAA (1-^{14}C-IAA and 5-^{3}H-IAA), mainly to the host plant after application to root nodules, but also within alder seedlings after application to the shoot apex, has also been examined.

Levels of endogenous IAA in root nodules were estimated initially by bioassay and GC-MS(S.I.M.). The purity of the extracts proved insufficient for satisfactory analysis, so further purification techniques were developed involving the use of gel permeation chromatography and preparative and analytical high performance liquid chromatography. Estimates of IAA levels in root nodule and root extracts of *Alnus glutinosa* and *Vicia faba*, were made by U.V. absorbance of analytical HPLC eluates after satisfactory purification had been achieved.

The mobility of radioactive IAA was followed after introduction of the label into the apical bud and nodule lobe by micropipette injection. A second method of label application to the root nodules, by absorption via an exposed vascular strand of a root nodule, was also investigated. Differences in distribution of radioactivity, as a result of the two methods of application of IAA to the nodule, suggested that application by micropipette injection avoided direct penetration of the conducting tissue.

Much of the methanol soluble radioactivity recovered in extracts of the plant parts in all experiments, remained in the aqueous fraction after solvent partitioning. However, movement of IAA-like radioactivity as determined by monitoring of preparative HPLC eluates of acidic ether extracts of the separated plant parts, was shown from the root nodules of alder seedlings to all parts of the host plant. Movement also from the apical bud to the rest of the plant within a 24 hour absorption period, following analysis of acidic ether extracts of plant parts by TLC, was shown. IAA-like radioactivity (as determined by preparative HPLC) was found in both bark and wood of the stem after application of label to the nodule either by micropipette injection or by absorption via an exposed vascular strand.

Since purification of extracts for endogenous IAA by preparative HPLC, showed that contaminants were still present, further evidence to confirm the mobility of radioactive IAA both out of and into the root nodules of *Alnus glutinosa* seedlings is required.

ABBREVIATIONS

BSTFA	N,O-Bis-(trimethylsilyl)-trifluoro-acetamide
DMSO	dimethylsulphoxide
GC	gas chromatography
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IAA-Me	methyl indole-3-acetate
ICA	indole-3-carboxylic acid
m/e	mass-to-charge ratio
M.I.M.	multiple-ion-monitoring (mass framentography)
POPOP (dimethyl)	1,4-Di-[2-(4-methyl-5-phenyloxazolyl)] benzene
PPO	2,5-diphenyloxazole
p.s.i.	pounds per square inch
PVP	polyvinylpyrrolidone
S.I.M.	single-ion-monitoring
THF	tetrahydrofuran
TLC	thin-layer chromatography
U.V.	ultra-violet

INTRODUCTION

I. Endogenous plant hormones in the root nodule

(a) Levels of hormones

Root nodules of both leguminous and non-leguminous plants have been found generally to contain higher levels of plant hormones than the parent roots (auxin, in particular indole-3-acetic acid (IAA), gibberellin and cytokinin Table 1). Exceptions to this general observation are the root nodules of Myrica cerifera and Casuarina cunninghamiana where auxin was not detected (Silver et al., 1966) although gibberellins (Henson in Wheeler et al., 1979) and cytokinins are present in Myrica gale (Bermudez de Castro et al., 1977; Henson and Wheeler, 1977e).

Levels of plant hormones in root nodules have been estimated by bioassay, although in later work on auxins, Dullaart (1967, 1970) measured IAA spectrofluorimetrically. Published estimates of the IAA, gibberellin and cytokinin content of root nodules and roots of nitrogen-fixing plants are summarised in Table 2. Also included in the table are values for indole-3-carboxylic acid (ICA) in the root nodules and roots of Alnus glutinosa and Lupinus luteus. In both these species, larger amounts of ICA were found in the parent roots than in the root nodules. Several factors could be responsible for differences in the auxin complements of roots and nodules. For example, auxins other than IAA may be present in the roots, and these would raise the overall auxin level much closer to that of the nodules. Supporting this possibility, is the work of Burnett et al., (1965), who found four groups of naturally occurring water-soluble auxins in a partially purified extract of Vicia faba roots. In addition, the hormonal complement of many species is mostly conjugated, so that differences in levels of detectable 'free' hormone could be a reflection of differences in the degree of conjugation between plant parts. For example, in dicotyledonous plants (including legumes) IAA is conjugated to peptides, whereas in monocotyledonous plants it is mainly esterified, although in Avena, different conjugates have

Table 1

Plant hormones in root nodules of leguminous and non-leguminous plants.

Auxins		Gibberellins	
Pisum	Thimann (1936)	*Phaseolus vulgaris*, *Pisum sativum*	Radley (1961)
Trifolium	Chen (1938)	*Lupinus luteus*	Dullaart and Duba (1970)
Phaseolus vulgaris, *Soja max*, *Pisum sativum*	Link and Eggers (1940)	*Alnus glutinosa*	Henson and Wheeler (1977a)
Pisum arvense, *Ilex europaea*	Pate (1958)	*Myrica gale*, *Hippophäe rhamnoides*	Henson in Wheeler at al (1979)
Lupinus luteus	Dullaart (1967)		
Alnus serrulata	Silver et al.(1966)		
Alnus glutinosa	Dullaart (1970)		

Cytokinins

Phaseolus vulgaris	Puppo et al.(1974)
Vicia faba	Henson and Wheeler (1976)
Pisum sativum	Syono et al.(1976); Syono and Torrey (1976)
Alnus glutinosa	Henson and Wheeler (1977b)
Alnus rubra (*A. glutinosa* endophyte), *Colletia paradoxa*, *Hippophäe rhamnoides*, *Myrica gale*, *Purshia tridentata*	Henson and Wheeler (1977c)
Alnus glutinosa, *Myrica gale*	Bermudez de Castro et al.(1977)

Table 2

Hormone levels in nodulated roots

(a) Indole-3-acetic acid-like activity

Species	µg IAA equivalents/kg. fr. wt. tissue		Assay	Reference
	Nodules	Roots		
Alnus glutinosa	385-1120 (summer)	90-205	Spectrofluorimetry Corrected for loss	Dullaart (1970)
	630-1430 (winter)	120-185		
Alnus serrulata	20,000	N.D.		
Myrica cerifera	N.D.	N.D. (field, nodulated)	Bioassay - Avena coleoptile	Silver et al. (1966)
		10,000 (young, non-nodulated)		
Casuarina cunninghamiana	N.D.	N.D.		
Lupinus luteus	250-400	70-160 (parental) 30-60 (14 day, non-nodulated)	Spectrofluorimetry Corrected for loss.	Dullaart (1967)
Cycas circinalis	20-50	20-40	Spectrofluorimetry Corrected for loss	Dullaart (1966)

Indole-3-carboxylic acid-like activity

Alnus glutinosa	0	50-90 (Summer)	Spectrofluorimetry Corrected for loss	Dullaart (1970)
		130-150 (Winter)		
Lupinus luteus	50-150	200-400 (parental)	Spectrofluorimetry Corrected for loss	Dullaart (1967)
		25-35 (14 day, non-nodulated)		

(i) N.D. - not detected

Hormone levels in nodulated roots

(b) Gibberellin-like activity

Species	µg GA_3 equivalents/kg. fr. wt. tissue		Assay	Reference
	Nodules	Roots		
Alnus glutinosa	3-5	< 0.5-2	Bioassay-lettuce hypocotyl	Henson & Wheeler (1977a
Myrica gale	11-12	1-2	Bioassay-lettuce hypocotyl	Henson in Wheeler *et al.* (1979)
Hippophäe rhamnoides	2-5	1-2		
Lupinus luteus	420-4280	24-64	Bioassay-lettuce hypocotyl	Dullaart & Duba (1970)
	2	N.D.	-dwarf pea	
Phaseolus vulgaris var. Masterpiece	10 (young, nodulated)	N.D.	Bioassay-dwarf pea	Radley (1961)
	26 (old)	N.D.		
Pisum sativum				
var. Meteor	240	N.D. -0.2		
var. Improved Pilot	35	1		

(i) N.D. - not detected

Hormone levels in nodulated roots

(c) Cytokinin-like activity

Species	µg cytokinin equivalents/kg. fr. wt. tissue		Assay	Reference
	Nodules	Roots		
Alnus glutinosa (mature trees)				
Dormant	85	-	Bioassay-soybean callus (kinetin equivalents)	Henson & Wheeler (1977b)
Budbreak	630	-		
Leaf expansion	43	-		
Alnus glutinosa (young plants)				
(a) Dormant	110	16-23	"	
Budbreak	620	38	"	
Leaf expansion	97	17	"	
(b) Dormant	1110	-		
Budbreak	1980	-		
Leaf expansion	587	-		
Alnus rubra (*A.glutinosa* endophyte)	58-61	8-13	"	Henson & Wheeler (1977e)
Myrica gale	62-95	16-24	"	
Hippophäe rhamnoides	49	35	"	
Purshia tridentata	16	10	"	
Colletia paradoxa	28	10	"	
Vicia faba	140-160	11-12	"	Henson & Wheeler (1976)
Pisum sativum				
effective nodules				
10 day	10	-	Bioassay-soybean callus (zeatin equivalents)	Syono *et al.* (1976)
14 "	30	-		
21 "	50	-		
28 "	50	-		
35 "	40	-		
ineffective nodules				
12 day	20	-		Newcomb *et al.* (1977)
18 "	10	-	"	
24 "	2	-		

(i) - not determined

been found in the shoot and the seed (Labarca et al., 1965; Bandurski and Schulze, 1974, 1977). Other plant hormones also form conjugates; gibberellin glucoside conjugates (see Sembdner et al, 1976 for references) and cytokinin glucoside and peptidyl conjugates are known to occur naturally. More particularly, zeatin riboside was thought to be the major cytokinin in nodules of Vicia faba (Henson and Wheeler, 1976), Purshia tridentata, Hippophäe rhamnoides and Alnus rubra (Henson and Wheeler, 1977e). In Alnus glutinosa root nodules, most cytokinin was present in the form of glucosides (Henson and Wheeler, 1977c) as it was in Myrica gale and Colletia paradoxa (Henson and Wheeler, 1977e).

Estimations of free IAA in plant tissues should therefore ideally take into account the effects of the extraction procedures upon both free and conjugated IAA. Usually the procedure involves an initial extraction in aqueous methanol for up to 24 hours at 0-4°C, conditions which may release some 'bound' auxins (Bentley, 1962), so giving erroneous levels of 'free' IAA. This has been confirmed by Bandurski and Schulze (1974) who found that even using extraction conditions designed to minimise hydrolysis of IAA complexes, 5% hydrolysis of IAA inositol ester standards occurred. This did not apply to peptidyl conjugates which required strongly alkaline extraction conditions (7N alkali for 3 hours at 100°C) for conversion to free IAA. However, although most extraction procedures can be criticised for not taking account of the existence of conjugates, devising the ideal procedure to eliminate conjugate hydrolysis during extraction poses many difficulties. Identification of all IAA complexes in the particular tissue would be necessary, which in turn would require analysis of all the fractions obtained during extraction of the tissue. These problems are likely to be exacerbated by the scarcity of information concerning the structure of naturally occurring IAA conjugates. For example, in vegetative tissue only one has been described, indole-3-acetyl-aspartic acid (Tillberg, 1974) although the conjugates from Avena and Zea seeds are well documented (Ueda and Bandurski, 1974; Percival and Bandurski, 1976).

(b) Origin of hormones

The elevation or lowering of hormone levels, is a common feature of plant tissues following their invasion by parasitic micro-organisms (Sequeira, 1973), suggesting that the hormone levels observed in root nodules may be ascribed in some way to invasion of the roots by the microsymbiont. The highly organised nodule structure produced following infection by nitrogen-fixing bacteria, contrasts markedly with the mass of undifferentiated, sometimes tumourous tissue induced by some parasitic organisms (Posthumus, 1967). It is possible, that in the symbiotic association the synthesis of hormones by the endophyte, or by the host tissue following stimulation by the endophyte, may be subject to some sort of control mechanism , normally absent from the parasitic association, which allows the nodule to develop its characteristic structure.

Whether the host plant or the microsymbiont, or both, produce the hormones is still a matter for conjecture. Rhizobium species, which symbiose with legumes, have long been known to synthesis/ plant hormones in culture, for example, auxins, mainly IAA and ICA (Link, 1937; Chen, 1938; Thimann, 1939; Georgi and Beguin, 1939; Rigaud and Bulard, 1965; Hartmann and Glombitza, 1966; Dullaart, 1970). Similarly cytokinins (Giannattasio and Coppola, 1969; Phillips and Torrey, 1970, 1972) and gibberellin-like substances, which are active in bioassay, have been detected in small amounts (Katznelson and Cole, 1965). Growth substances have been shown also to be released by Rhizobium at the root surfaces of inoculated plants (Dart, 1974), in particular IAA (Allen and Allen, 1968; Kefford et al., 1960) and cytokinins (Puppo and Rigaud, 1978). The origin of the nodule hormones has not been demonstrated directly as yet, although it was concluded by Dullaart (1970a), that a substantial part of the IAA present in the root nodules of Lupinus luteus was produced via plant enzymes as a result of metabolic modification, induced by rhizobial infection. Similarly, two groups of cytokinins (zeatin plus derivatives and small amounts of isopentenyladenine plus derivatives) were found in the root nodules of Pisum sativum,

suggesting that the bacterium and host cells might each contribute to the total cytokinin complement of the nodules (Syono and Torrey, 1976). In non-legumes, the endophyte is not rhizobial, except in a tropical species Parasponia (Akkermans et al., 1978), but is an actinomycete which has only recently been isolated from some host plants (Callaham et al., 1978 ; Berry and Torrey, 1979). Since isolation is so recent, the origin of the hormones in the non-legume association is even more speculative than in the rhizobial association, but the way is now open for physiological investigation of the endophyte.

While the evidence supports the synthesis of hormones by the endophyte and host plant individually, there is no direct evidence concerning the relative contributions of the symbionts to a hormonal control of nodule differentiation. The main piece of indirect evidence supporting involvement of the microsymbiont in the hormonal control of nodule development, is the occurrence of cell division ahead of the developing infection thread (legumes), suggesting the involvement of a diffusible stimulus (Libbenga and Harkes, 1973). The cell proliferation patterns produced in the early stages of root nodule formation,are similar to those which occur in explants of pea root cortex incubated in a medium containing both auxin and cytokinin (Libbenga et al., 1973). The only direct analyses relating hormones to nodule development have been those of Syono et al. (1976) who found that levels of cytokinin activity in pea root nodules were positively associated with nodule growth rate, and Henson and Wheeler (1977b) where in the perennial nodules of alder, high levels of cytokinin activity were associated with the breaking of dormancy. In both cases, cytokinins may have been concerned with regulation of mitotic activity of the nodule meristem. In non-legumes, a tenuous relation of hormonal activity to nodule development was observed in Alnus glutinosa with the induction by cytokinins of undifferentiated 'pseudonodules' on seedling roots (Rodriguez - Barrueco and Bermudez de Castro, 1973); the phenomenon has been observed also on tobacco roots which are never nodulated (Arora et al., 1959).

Although higher levels of auxin have been found in the root

nodules of many species, whether they arise as a result of IAA synthesis in the bacteroid or host plant cells or by a low rate of breakdown in the host cells, it is unlikely that IAA, being such a potent growth substance, would occur as a free compound at any great concentration. Controls which might operate to confine the effect of IAA include compartmentation or inactivation by means of conjugate formation. For example, confinement of the IAA oxidase (peroxidase) of mature bacteroid-containing cells to the cell walls, has been suggested as a possible cause of high IAA levels in pea nodules (Oostrom et al., 1975). However, in alder (non-legume) nodules, which also show elevated IAA levels (Table 2a) peroxidase activity has been demonstrated both in the cell wall and cytoplasmic fractions (Wheeler et al., 1979a). In the non-legumes, Myrica cerifera and Casuarina cunninghamiana, the absence of detectable auxin in the root nodules has been attributed to higher levels of IAA oxidase activity in the root nodules compared to the roots (Silver et al., 1966).

Phenolic compounds which are present in high amounts in non-legume nodules, could affect IAA levels by modulation of the activity of IAA degrading enzymes. For example, monophenols seem to act as co-factors and dihydric and polyphenols act as inhibitors of IAA oxidase activity (Schneider and Wightman, 1974); the latter include high molecular weight 'auxin protectors' (Stonier, 1976; Atsumi and Hayashi, 1978; Syono, 1979). In some tissues, a major proportion of extractable IAA is present in conjugated form, for example, 80% of the acetone extractable IAA from Avena kernels occurs as high molecular weight, polymeric esters (Percival and Bandurski, 1976). It has been suggested that such compounds are a normal storage form of IAA, and perhaps serve to control IAA levels in the particular tissue (Berger and Avery, 1944; Bandurski et al., 1969). Excess IAA might be conjugated so that the hormone remains limiting and therefore regulating (Bonner and Thimann, 1935). Conjugates might also protect IAA from oxidation by peroxidase. Cohen and Bandurski (1978) showed that all IAA conjugates tested (both glucoside and peptidyl) were resistant to peroxidase-catalysed oxidation, whereas IAA was not. The widespread distribution of IAA esters and peptides,

and the fact that they are often present in greater concentration than IAA, would suggest some metabolic function, perhaps in the nodule.

To overcome the problems associated with bioassay, in the estimation of endogenous IAA levels, physico-chemical techniques have been used with varying degrees of success. These include GLC (e.g. Grunwald et al., 1968; Champault, 1975; DeYoe and Zaerr, 1976), radio-immunoassay (Pengelly and Meins, 1977), indole-α-pyrone fluorescence (Eliasson et al., 1976), HPLC (e.g. Durley et al., 1978; Sweetser and Schwartzfager, 1978) and GC-MS. Mass spectrometry has been used in a number of studies to identify the very small quantities of hormones which can be extracted from plant tissues (for example in the case of IAA, Igoshi et al., 1971, and Greenwood et al., 1972). This technique has also been used in the S.I.M. and M.I.M. modes, in this Department and elsewhere, to quantify a particular compound in an extract (for example, IAA, S.I.M. - Hall and Medlow, 1974; M.I.M. - Rivier and Pilet 1974; Caruso et al., 1978; Allen et al., 1979). Considerable effort was expended in this project, to develop techniques which would allow application of physico-chemical procedures to the analysis of IAA in extracts of root nodules from a variety of plant species. The species chosen for study ranged from a legume (Vicia faba), nodule extracts of which were low in phenols, to two other woody, non-legume species, the root nodules in one (Alnus glutinosa) reported to contain high auxin levels (Dullaart, 1970) and in the other (Myrica gale) to contain no detectable auxin (Silver et al., 1966); impure extracts of both woody species are rich in phenols which would interfere with characterisation and quantification.

II . IAA in the nodulated plant

Apart from their probable role in the control of nodule development, plant hormones may be involved in various other aspects of the physiology of the mature nodule. Assuming the absence of a direct effect of IAA upon nitrogenase, for which there is no good evidence, there are two main ways in which IAA present in the nodule could affect nitrogen fixation:-

(1) by direction of assimilates to the nodule either by an effect upon transport or by stimulation of sink activity.

(2) by an effect upon host plant metabolism, e.g. photosynthesis, following translocation from the nodule.

(1) Direction of assimilates to the nodule

Nitrogen fixation is a process with high energy requirements which can only be met in the green plant by the translocation of a steady supply of photoassimilates from the shoot to the nodules, where they are used to generate reducing power and ATP used in nitrogen fixation (Ching et al., 1975) and to provide carbon skeletons for amino acid synthesis from the fixed nitrogen. The demands placed upon the host plant by nitrogen fixation are considerable. For example, Minchin and Pate (1973) calculated that in terms of the carbon taken up by the leaves, 32% was translocated to the nodules of Pisum sativum under normal vegetative conditions (9% in Vigna unguiculata, Herridge and Pate, 1977; 24% declining to 8% in Lupinus albus, Pate and Herridge, 1978). For every mg of nitrogen fixed, pea nodules consume a substantial amount of carbohydrate (3 - 19 mg; in Vigna unguiculata the energy consumption is 6.8 mg carbon/mg nitrogen fixed and in Lupinus albus 4.0 - 6.5 mg carbon/mg nitrogen fixed). This high 'sink' activity of nodules, in addition to attracting substrates for the support of nitrogen fixation, could also serve indirectly to control

the numbers of nodules formed in the root system. Existing nodules may sequester carbohydrate so that the portion of root nearest already formed nodules would contain too low a concentration to support further nodule development (Dart, 1974).

Root nodules, active in nitrogen fixation, therefore form substantial sinks for photoassimilates in the nodulated plant. It is conceivable that the significant quantities of plant hormones in root nodules, in particular IAA, may perform one of the main functions which has been suggested frequently for these substances, in directing the long-distance transport of assimilates to the nodules. Many of the experiments designed to demonstrate hormone-directed transport of metabolites in organs other than nodules, have involved replacement of tissues, normally considered rich in auxin or other plant hormones, with hormone incorporated into a lanolin paste and following the movement of radioactive metabolites applied in the vicinity of the treated region. Until fairly recently, however, these experiments have not distinguished between the formation of a growth centre in the tissue over a period of time as a result of exogenous hormone application, and a direct effect of the applied hormone on metabolite transport processes. As far as the root nodules are concerned, any endogenous hormone, if it affects movement of assimilates at all, could act in either or both of these ways. Firstly, through an enhancement of metabolism resulting either from a direct effect on respiration (Bonner, 1933) or by the creation of a growth centre in the nodule producing a high rate of metabolism. Secondly, by movement of the hormone into the vascular tissues where the movement of assimilates could be directly affected by an influence on the transport processes within the sieve elements (Kursanov, 1963; Phillips, 1975).

A large body of evidence exists to support the suggestion that hormones can influence the direction of transport of metabolites in both herbaceous and woody plants, irrespective of whether this is achieved by a 'sink' effect or an effect on assimilate transport. IAA, has been extensively investigated in this respect (Went, 1936; Mitchell and Martin, 1937; Penot, 1961; Booth *et al.*, 1962; Davies and

Wareing, 1965; Hew et al., 1967; Woolley and Wareing, 1972; Patrick and Woolley, 1973; Altman and Wareing, 1975; da Cruz and Audus, 1978; Patrick and Wareing 1976, 1978; Patrick, 1979), and can attract metabolites to its site of application. More than one hormone is likely to be involved in the direction of assimilates to the application site, for example, a synergistic effect of IAA and kinetin in stimulating the transport of ^{32}P in Phaseolus stems was observed by Seth and Wareing (1967). This stimulation was greater than that of IAA alone and kinetin had no detectable effect on transport in this system.

One explanation for the experimental data described above, could be that an IAA-stimulated assimilate demand, acts at the 'sink' to influence a pressure-flow mechanism of assimilate translocation (Münch, 1930), by attracting available assimilate from the source to the site of hormone application; the two processes would then be complementary. The general applicability of this hypothesis is questionable, however, since Wardlaw and Moncur (1976) using wheat found no correlation either between the auxin content of a sink and its demand for assimilates, or between ^{14}C-auxin movement and assimilate transport. Their observations of ^{14}C-photosynthate movement fitted a mechanism of translocation depending almost entirely on a source-sink concentration gradient, without hormonal involvement, similar to that of the pressure-flow mechanism of Münch (1930).

A number of studies, mainly on the development of fruits and seeds, have attempted to relate directly endogenous hormone levels of plant organs with their accumulation of photosynthates during development. In seeds of Pisum sativum, levels of some gibberellins reached a peak during seed development and declined at seed maturity (Frydman et al., 1974); levels of gibberellins, auxins and abscisic acid in developing pea seeds were found to be closely correlated with changes in the growth rates of pea pod wall and seeds (Ewens and Schwabe, 1975). Similarly, in tomato fruits and Ceratonia seeds, the level of growth promoting substances decreased with maturation of the seed (Abdel-Rahman et al., 1975; Ilahi and Vardar, 1975).

However, in some fruits no clear relationship has been observed between levels of endogenous growth regulators and fruit development (Nitsch, 1970). It was suggested by Nitsch (1970) that for auxins, diffusates (Nitsch, 1961) may show a closer correlation than extractable auxins (Nitsch et al., 1960).

Inaccurate estimation of endogenous hormone levels may provide a reason why in some studies, no correlation with development or sink strength has been demonstrated. Inaccuracies could stem from two main sources. Firstly, in the majority of studies, methods used for estimation of hormone levels have been based on bioassays, in which inaccurate assessments may result from the presence of inhibitors, masking hormonal activity. Secondly, in any tissue, including nodules, factors such as intracellular compartmentation (Patrick, 1976) and chemical conjugation of hormones may result in differing estimates of hormonal activity, depending on the extraction methods used.

In nodulated plants, changes in endogenous hormone levels with the demands of host plant processes have been described in lupin nodules, where the level of free IAA in a soluble fraction reached a peak at the onset of flowering (Alekseeva and Shramko, 1977). Dart (1975) suggested that changes in soybean nitrogenase activity associated with flowering (Day, 1972), which occurred before any competition with developing fruits for photosynthate could have developed, might be due to a hormonal influence upon relative sink strengths in the nodulated plant, although direct measurements of hormone levels were not made. By contrast, in non-legumes, in a seasonal study including the dormant period of Alnus glutinosa root nodules, no significant changes were detected in auxin content of the nodules of mature trees (Dullaart, 1970) even though large changes in relative 'sink' strengths must have occurred over this period. The relationship between endogenous hormone levels and the 'sink' activity of plant parts, is obscured further by the failure to detect auxin in nitrogen-fixing root nodules of Myrica and Casuarina (Silver et al., 1966). This suggests either that auxin is not involved in the attraction of photoassimilates to the root nodule, or that auxin is present in other

forms, undetected by the extraction and assay methods employed by Silver et al (1966). It would seem that a pre-requisite for further elucidation of the relationship, if any, between endogenous hormone levels and sink strength, is the complete extraction of the hormone, in all its forms, with subsequent purification to allow accurate and precise estimation of levels, together with identification and estimation of any conjugates present. The sub-cellular compartmentation and localisation of the various hormone forms could then be examined further with the added confidence of sound analytical techniques.

(2) Translocation of nodule IAA

Most information concerning the translocation of hormones in higher plants has been obtained by following the movement of radioactive hormone, applied by various means to the plant tissues. This approach was adopted in the only two published studies of hormone movement in nodulated plants. In Phaseolus vulgaris, substantial movement was observed to all plant parts, including the nodules, of radioactivity derived from ^{14}C-ABA applied to a leaf, and chromatographic analysis suggested that some radioactivity remained as ABA in the receiving organs (Hocking et al., 1972). Radioactivity derived from ^{14}C-zeatin moved to all parts of an Alnus glutinosa seedling, within 24 hours of application to a root nodule; chromatography showed that some of this radioactivity was similar to zeatin (Henson and Wheeler, 1977d). However, although this data supported the occurrence of some translocation of cytokinin from alder nodules, the possibility that nodules constitute a major source of hormone for the plant, was discounted by a comparative analysis of the cytokinin activity of nodulated and non-nodulated plants (Wheeler and Henson, 1978). In this experiment, higher levels of cytokinin activity were found in non-nodulated plants and were related to the greater root development of these plants. Despite the lack of firm evidence concerning the occurrence or importance of hormone translocation in nodulated plants, several authors have speculated on the effects which translocation of hormones from the nodules to other plant parts might have upon the host plant. For example, it was suggested by Bouma (1970) that the higher rates of dark respiration he

measured in the shoots of nodulated compared to non-nodulated plants of Trifolium subterraneum, may be a result of IAA transport from the nodule. The greenness of the leaves of alder, Myrica gale and Hippophäe rhamnoides at abscission, was commented upon by Rodriguez-Barrueco (1968) who suggested that this may be due to the export of cytokinins from the root nodules to the leaves, although in view of the results already described, the nodules in alder at least, seem unlikely to supply large amounts of cytokinins to the shoot.

Although information concerning translocation of hormones within nodulated plants is very limited, and movement of IAA from root nodules to host plant, or from shoot to nodules has not been studied previously, much data has accumulated concerning the movement of hormones in other plants, mostly seedlings, and in excised segments. The direction and route of IAA transport varies according to the experimental system used and can be in an acropetal and a basipetal direction in both the phloem and the xylem. Basipetal polar transport of IAA which occurs in isolated tissues of stem, petiole, leaf and coleoptile has been well reviewed (McCready, 1966; Goldsmith, 1968, 1977). Acropetally orientated polar movement of applied auxin has been demonstrated in root segments of several species (Pilet, 1964; Kirk and Jacobs, 1968; Scott and Wilkins, 1968; see also reviews by Scott, 1972; Batra et al., 1975; Goldsmith, 1977).

One of the first attempts to study IAA movement in intact plants was by Eschrich (1968) who applied 2-^{14}C-IAA to fully expanded leaves of Vicia faba. There was movement of label into the stem, roots and young expanding leaves, although not into mature leaves, showing both upward and downward movement of label from treated leaves. The patterns of auxin distribution were consistent with the transport of auxin together with assimilates in the phloem. Morris et al., (1969) applied ^{14}C-IAA to the apices of intact, light-grown dwarf pea seedlings in an attempt to investigate auxin transport from its site of synthesis in the stem apex and also to avoid the possibility of transport along with photo-assimilates in the phloem which leaf-applied auxins might be likely to accompany, and which could mask the normal endogenous auxin

transport route. The velocity and characteristics of auxin transport corresponded with that previously reported for excised coleoptile, stem and petiole segments (McCready and Jacobs, 1963; and also review by Goldsmith, 1977) suggesting a similar pathway. Part of the applied IAA was transported unchanged to the root system, although a large amount was converted by the apical bud, stem and root tissues to indole-3-acetylaspartic acid (IAAsp) which was not thought to be transported. In a later paper (Morris and Kadir, 1972) IAAsp was shown to be mobile, again using intact pea seedlings, and the existence of two physically separate pathways for long-distance auxin transport was proposed. ^{14}C-labelled IAA, fed to leaves, was presumed to be exported in the phloem, and when fed to the apical bud was thought to be exported not in the phloem but by some other pathway. Autoradiography has now provided evidence that this is the vascular cambium and differentiating vascular elements (Bonnemain, 1971; Morris and Thomas, 1978). A hypothesis involving two separate pathways for long distance auxin transport had been put forward previously by Smith and Jacobs (1969) who suggested a relatively rapid transport of auxin in the phloem and slower, polar auxin transport in a non-phloem pathway. Additional evidence for this hypothesis was provided by aphid feeding experiments which showed the absence of detectable auxin from the sieve tubes of apical bud fed plants (Morris and Kadir, 1972), whereas when exporting leaves were fed labelled IAA, the presence of auxin in aphid stylets suggested transport in the phloem (Eschrich, 1968; Morris and Kadir, 1972). Microautoradiographic evidence for the phloem pathway was given by Bourbouloux and Bonnemain (1973) and Goldsmith *et al.* (1974). In the phloem pathway, velocities of IAA transport of 10-24 cm.h^{-1} have been recorded (Little and Blackman, 1963; Bonnemain, 1971; Goldsmith *et al.*, 1974) in contrast to the non-phloem pathway in which velocities of 1 cm.h^{-1} are more usual (Morris *et al.*, 1969; Bourbouloux and Bonnemain, 1973; Rowntree and Morris, 1979). Such velocities are also found in woody shoots (Gregory and Hancock, 1955). The rate of transport of IAA in woody shoots provides additional evidence for the involvement of the cambium since it is closely correlated with

seasonal changes in cambial activity, being more rapid in active shoots (Hollis and Tepper, 1971; Zamski and Wareing, 1974). It has been suggested that if auxin transport is to be controlled as part of its role in developmental processes, then the main endogenous route for auxin transport would be unlikely to be the phloem (Morris and Kadir, 1972). Little evidence was found for a lateral interchange of auxin between the two pathways, at least in an herbaceous plant such as pea.

In nodulated plants, the most likely route of hormone transport from the nodules would be in the xylem transpiration stream along with assimilated nitrogen (Oghoghorie and Pate, 1972; Lewis and Pate, 1973; Pate and Flinn, 1973). This is supported by evidence from the only study on movement of a hormone, in this case zeatin, from the root nodules, where it was shown that stem ringing did not affect transport, suggesting the xylem as the probable route by which applied nodule hormones would move into the host plant (Henson in Wheeler and Henson, 1978). Processes in the host plant which might be affected by translocation of IAA from the root nodule include photosynthesis. IAA has been shown to enhance the activity of photosynthetic enzymes, for example, Turner and Bidwell (1965) and Bidwell and Turner (1966) found that IAA increased the rate of photosynthesis in bean leaves 30-100% within 30 minutes of its application to leaves. Bidwell *et al.* (1968) showed that IAA can move sufficiently rapidly, from its point of application to the site of increased photosynthesis, to be responsible for the increased rate of photosynthesis. They also found that IAA stimulated the translocation of recent products of photosynthesis from photosynthetically active leaves.

The large body of evidence concerning the occurrence of hormone transport in higher plants and the relative paucity of information relating to the movement of hormones in the nodulated plant, were the main factors which initiated the studies of movement of IAA in nodulated alder seedlings reported here.

GENERAL MATERIALS AND METHODS

Plant culture

(a) *Alnus glutinosa*

Plants were glasshouse grown in 'Peralite' from locally collected seed (> 2 mm). The roots of young seedlings (3 weeks old) were inoculated with crushed *Alnus glutinosa* root nodules and the growing plants fed once weekly with half-strength nitrogen-free Crone's solution, supplemented with minor elements (Hoagland's A-Z solution with added molybdenum, Templeman, 1941). In later experiments, plants were grown in water culture using the same nutrient solution, in a controlled environment room (photoperiod 16h, 19°C; dark period 8h, 15°C) illuminated by a mixture of warm white, white and daylight fluorescent lights (75/85 W), supplemented with tungsten bulbs (150 W) 60-90 cm. above the growing surface. The natural illumination of the glasshouse was supplemented with mercury vapour lamps (400 W) during the winter months. Plants were used for experiments when they were 4-5 months old and 15-40 cm in shoot height, although within each experiment, the plant heights were matched to within 5 cm. Prior to the radiotracer experiments, glasshouse grown plants were acclimatised in the controlled environment room for 2-4 weeks.

(b) *Vicia faba*

Plants were grown in a controlled environment room, as specified for *Alnus glutinosa*. Clean seed (var. 'Midget', supplied by Dobies, Llangollen) of uniform size was inoculated at sowing with *Rhizobium leguminosarum* strain WA-174 (supplied by the Landbouwhogeschool, Wageningen, Netherlands), after the seed had been surface sterilised with 2% sodium hypochlorite. The plants were grown for 15-20 weeks in 'Peralite' and fed once weekly with the nutrient solution described for *Alnus glutinosa*. The growing period of the beans was extended, and inhibition of nodule development prevented, by carefully removing the flowers at the bud stage. Other varieties of broad bean were field grown as detailed in the relevant results section.

(c) *Myrica gale*

Plants were glasshouse grown in water culture, from locally

collected seed, after the seedling roots had been inoculated with crushed Myrica gale root nodules. The culture medium was the same as that described for Alnus glutinosa. The plants were harvested, while they still showed acetylene-reducing activity, at the end of the second growing season.

Preparation of solvents for HPLC

(a) Hexane

This solvent was obtained commercially in an aromatic-free HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Innerleithen, Peebleshire).

(b) Ethyl acetate

Ethyl acetate was obtained commercially in a technical grade (A. and J. Beveridge, Edinburgh), but was further treated to remove acidic and alcoholic impurities before final redistillation. This was achieved by first washing the ethyl acetate with 5% aqueous sodium carbonate solution for 30 min., followed by three separate washes with 2% saturated calcium chloride solution, each of 30 min., and finally, by the addition of 1% anhydrous sodium carbonate and allowing the mixture to stand overnight. The ethyl acetate was then redistilled, and the distillate collected at 74-77°C.

Scintillation fluid for HPLC

The scintillation fluid consisted of 150g. naphthalene, 12g PPO and 100 ml Triton-X made up to 1 ℓ. with redistilled toluene.

Solvents and Chemicals

All solvents used for extraction of plant material and in scintillation fluid, except phenylethylamine, were obtained in a technical grade and were redistilled before use. The sources of supply were:-
Diethyl ether, tetrahydrofuran and dichloromethane - May and Baker, Dagenham; toluene and methanol - A. and J. Beveridge, Edinburgh; dioxane-BDH Chemicals Ltd., Poole, Dorset; phenylethylamine - Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Other solvents and chemicals used were at least analytical reagent grade.

Crone's salts

	g
KCℓ	375
$CaSO_4.2H_2O$	250
$MgSO_4.7H_2O$	250
$Ca_3(PO_4)_2$	125
$Fe_3(PO_4)_2.8H_2O$	125

The salts were ground individually before mixing thoroughly.
For a solution of half-strength, Crone's salts were used at the rate of 10.5g. $10\ell^{-1}$ distilled water.

Hoagland's A-Z solution

	$g.\ell^{-1}$ distilled water
H_3BO_3	0.62
$Na_2\ Si\ O_3$	0.43
$MnCl_2.4H_2O$	0.40
K_2MnO_4	0.40
$Cu\ SO_4.5H_2O$	0.055
$Zn\ SO_4.7H_2O$	0.055
$Al_2(SO_4)_3.18H_2O$	0.055
$Ni\ SO_4.H_2O$	0.055
$Co\ Cl_2.6H_2O$	0.055
TiO_2	0.055
$Li_2\ SO_4.H_2O$	0.035
$Sn\ Cl_2.2H_2O$	0.035
KI	0.035
KBr	0.035
$Na_2Mo\ O_4.2H_2O$	0.030

The minor elements solution was used at the rate of 1 ml. ℓ^{-1} half-strength Crone's solution.

PART I ENDOGENOUS IAA IN ROOT NODULES OF ALNUS GLUTINOSA, MYRICA GALE AND VICIA FABA.

1. Preliminary assays

Initially, extracts of root nodules and roots of Alnus glutinosa, Myrica gale and Vicia faba, were partially purified using classical procedures of solvent partitioning and paper chromatography, with the inclusion of a PVP chromatographic step, thought to be especially useful in the purification of phenol-rich extracts such as those of Alnus glutinosa (Glenn et al., 1972).

1.1 Methods

Extraction and partial purification of endogenous IAA

Field or glasshouse grown plant material was harvested, washed, and the nodules and roots separated and placed in 80% aqueous methanol. In addition, in glasshouse-grown Myrica gale the nodule roots were also separated from the nodules (in field Myrica gale the nodule roots are not well developed and could not be collected separately). Harvest of sufficient material for analysis frequently required two or more days. During the course of a day, plant material was collected in batches and these were weighed and placed in aqueous methanol in the cold room. At the end of each day, the batches were bulked, homogenised and extracted in aqueous methanol overnight as described below. At a later stage in the purification procedure, several days' harvests from the same batch of plants were combined for analysis.

The bulked plant material in cold 80% aqueous methanol was homogenised at high speed for 30 seconds, and the homogenate extracted, with stirring, for 15-24 hours at $4^{\circ}C$ in the dark (Extraction volume (ml) = 6 times fresh weight (g) root tissue and 10 times fresh wt(g) nodule and nodule root tissue). After filtration, the residue was washed twice with aqueous methanol, and the combined extracts were reduced to the aqueous phase and partitioned three times against equal volumes of diethyl ether at pH3. Foaming of the aqueous methanolic extracts of Myrica gale roots during rotary evaporation, was controlled by the addition of a few drops of n-octanol to the extract. Ethereal extracts were reduced to dryness, resuspended in

a small volume of buffer (0.1M K_2HPO_4 at pH8), and then loaded on to a piece of filter paper on the top of a column (30 ml) of insoluble PVP (2g fresh weight of tissue per ml column volume), the PVP having been previously washed with several changes of distilled water followed by buffer, each for 15 minutes, to remove 'fines'. The column was washed with 120 mls 0.1M K_2HPO_4at pH8, which previous calibration had shown would elute IAA. The eluate was partitioned three times against equal volumes of diethyl ether at pH3, the dissolved water removed by freezing and the ether then reduced to dryness. The residue was redissolved in a suitable volume of methanol and portions chromatographed on Whatman 3MM filter paper (prewashed with methanol) prior to bioassay and mass spectrometry. The paper chromatograms were loaded (under a stream of nitrogen) with 5g fresh weight tissue equivalents per cm. origin line, together with IAA marker spots, and were equilibrated in the chromatography tank before adding solvent. The chromatograms were developed (isopropanol: ammonia: water:: 8: 1 : 1) in a descending manner for 6-8 hours at room temperature in subdued light. After redistillation, diethyl ether was stored in the dark at $4^{\circ}C$ for as short a time as possible before use.

Estimation of IAA recovery during preliminary purification procedures

(a) PVP column chromatography

5-^{3}H-IAA was applied, in a small volume of buffer (0.1M K_2HPO_4 at pH8), to a PVP column as described in the previous paragraphs (extraction and purification of endogenous IAA). Fractions (5 ml) were collected, dried in scintillation vials, either on a heated plate at $40^{\circ}C$ or in a vacuum desiccator, and then eluted with methanol prior to liquid scintillation counting.

The recoveries of 5-^{3}H-IAA-derived radioactivity in the PVP column eluate (0.7 to 2.7 column volumes), were in the range 66-82% (Table 3).

Table 3

Recovery of radioactivity in PVP column eluate as % of that applied.

Experiment	Column volumes collected	% recovery
1	0.67 - 2.3	72.6
2	0.80 - 2.5	69.6
3	0.80 - 2.7	69.1
4	0.80 - 2.2	65.9 - 82.4

(b) Methanol extraction, ether partition, PVP column chromatography and paper chromatography

Freeze-dried alder nodules were homogenised and 1-^{14}C-IAA was added to the homogenate before overnight extraction. Aliquots of the extract were removed at each stage in the preliminary purification procedure to monitor losses and final recoveries of IAA were 32-38% (Table 4). The levels of recovery of radioactivity (44%, Table 4) from PVP column chromatography in the presence of plant extracts were much lower than those obtained with IAA alone (66-82%), Table 3).

Table 4

Recovery of radioactivity, as % of initial level, at various stages during preliminary purification.

Purification step	Replicate extracts		
	1	2	3
Ether partition (% in ether	97.9	99.1	99.8
(% in aqueous	2.1	0.9	1.2
% of initial radioactivity recovered in ether phase	70.3	72.9	72.0
PVP chromatography			
Recovery from PVP alone	-	43.5	43.8
% of initial radioactivity recovered in ether phase, partitioned from PVP column eluate	-	31.8	31.7
Paper chromatography			
Recovery from paper alone	-	100	100
% of initial radioactivity eluted from paper	38.2	32.3	36.9

Bioassay

The Avena coleoptile straight growth test (Avena sativa var. 'Victory') was used with 15-35 g fresh weight tissue equivalents for each bioassay. The thoroughly dried chromatograms were divided into 20 equal sections and eluted with 2.5 ml 1% sucrose for 1-2½ hours before adding the coleoptile segments (0.5 cm long, cut 0.5 cm from the apex of the 2±3 cm long coleoptile and floated on distilled water for 1-3 hours before use; 10 segments per section of chromatogram). A chromatogram control and IAA standards (0.005- 5 µg) were included in each bioassay. The segments were incubated for 16-24 hours, at 25°C, in darkness under conditions of high humidity and were subsequently measured using the shadowgraph technique. A short cut computation method using ranges (Snedecor and Cochran, 1967) was used at 1% risk ($p = 0.01$) to analyse variance in each bioassay.

Preparation of samples for Gas-chromatography - Mass Spectrometry (GC-MS)

The portion of the chromatogram corresponding to that of the IAA marker spot (detected by Ehrlich's reagent), was cut out and eluted with methanol for 1-3 hours. The eluate was filtered, the residue washed twice, the combined washings reduced to dryness and transferred to a small vial. The vials were stored over phosphorus pentoxide at 4°C until required for analysis. The samples were dissolved in acetonitrile and converted into trimethylsilyl derivatives by the addition of BSTFA (Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire). to produce a reaction mixture BSTFA:CH_3CN:: 5:1, v/v which was incubated at 30°C overnight over silica gel. Derivatives were stored subsequently at 4°C over silica gel. Solvents and reagents as well as sample were water-free to prevent hydrolysis of TMSi derivatives; this problem can be overcome to some extent by the addition of excess silylating reagent.

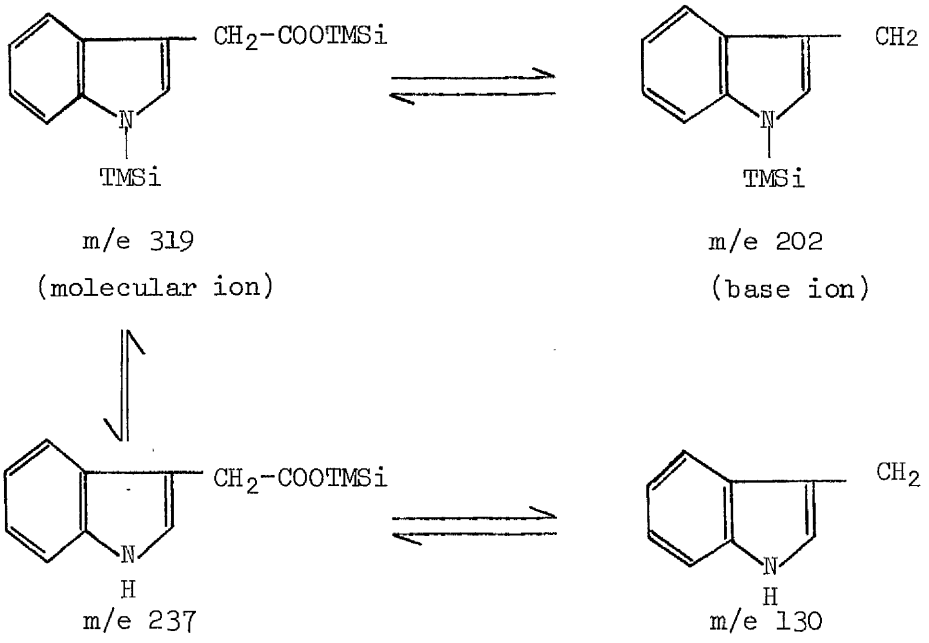
GC-MS

Silylated samples were subjected to gas chromatography using various silicone columns (1% SE-30, 1% OV-101, 1%, 2% SE-33), at temperatures between 160-220°C, with helium as carrier gas at a flow rate of 40-45 ml/min. The gas chromatograph (Pye Series 104) was

coupled through a single stage silicone membrane separator to an AEl MS 30 mass spectrometer. Standards containing 0.02 - 1 µg/µl IAA were used both to calibrate the response of the mass spectrometer and to determine the retention time of the IAA - trimethylsilyl derivative. As the sample eluted from the GC column, that portion corresponding to the position of the IAA trimethylsilyl derivative was directed to the MS for analysis. The MS was used in either the single-ion monitoring mode (S.I.M.) at m/e 202 or in a few later samples, in the multiple-ion monitoring mode (M.I.M.) using m/e 202 and 319. Attempts were also made to obtain full mass spectra from several of the extracts to identify IAA.

Ions produced in the mass spectrometer from the trimethylsilyl derivative of IAA.

Silylation can occur at both the carboxyl and amino group of the IAA molecule.



m/e 319 (molecular ion)

m/e 202 (base ion)

m/e 237

m/e 130

1.2 Results

The results of bioassays and mass spectrometric estimations of endogenous IAA in the extracts obtained using the preliminary purification procedures, are shown in Table 5a,b .

In three of the four bioassays of 'Peralite' - grown Alnus glutinosa, root nodule extracts showed no auxin activity, whereas the parental roots measured on three of these occasions showed 4-10 µg IAA equivalents/kg. fr. wt. tissue. The other bioassay showed the presence of IAA-like activity in the nodule extract (8 µg IAA equivalents /kg. fr. wt.), but levels were low compared to the one determination of nodule material harvested from the field (20 µg IAA equivalents /kg. fr. wt.). The results presented are not corrected for losses which radioactive recovery data suggest were of the order of 70% (Section 1.1, Table 4).

In extracts from 'Peralite'-grown plants of Myrica gale, there was no activity in the IAA zone of root nodules or nodule roots and very little activity (0-5 µg IAA equivalents /kg. fr. wt.) in extracts of the parent roots. Extracts from field material again showed higher activity, the nodules being of very low activity (3 µg IAA equivalents /kg. fr. wt.) and the parental roots much higher (80 µg IAA equivalents /kg. fr. wt.).

In the two nodule extracts of 'Peralite'-grown Vicia faba, the levels of IAA-like activity were higher (8-9 µg IAA equivalents/ kg. fr. wt.) than those of the parental roots (0-2 µg IAA equivalents/ kg. fr. wt.). As was found in extracts of the other two plant species, levels of IAA-like activity were much higher in field-grown material, although here only one determination of a nodule extract was made (40 µg IAA equivalents /kg. fr. wt.).

The results obtained by GC-MS using S.I.M. did not always agree with the bioassay results. With Myrica gale, where only one set of plants was assayed, both methods gave similar results. However, agreement between bioassay and GC-MS S.I.M. results was shown only in extracts of plants from two, out of a total of eight, harvests of Alnus glutinosa and Vicia faba. The GC-MS results were generally much more variable for a given species than those results obtained by bioassay. Possible sources of this variability were:-

Table 5 a.

Estimation of endogenous IAA-like activity in extracts of Alnus glutinosa, Myrica gale and Vicia faba. Extracts were partially purified by solvent partitioning, P V P column chromatography and paper chromatography. Bioassay and GC-MS in the S.I.M. and M.I.M. modes were used for quantitative and qualitative estimations.

Sample	Bioassay		GC-MS		
	Sample size- fresh wt. equivalents (g)	IAA equivalents µg/kg.fr.wt.	Sample size - fresh wt. equivalents (g)	S.I.M. (m/e 202) µg IAA/ kg.fr.wt.	M.I.M. 202 319
Alnus glutinosa					
1. Nodules	30	0	55	2	+ +
Parental roots	35	10 ± 14%	165	2	+ +
2. Nodules	15	0	50	0	- -
Parental roots	35	5 ± 20%	165	0.05	+ +
3. Nodules	30	8 ± 14%		-	N.D. N.D.
Parental roots	30	4 ± 14%		-	N.D. N.D.
4. Nodules	15	0	60	0.5	N.D. N.D.
5. Nodules (a)	15	20 ± 10%	60	just +ve	N.D. N.D.
Myrica gale					
1. Nodules	30	0	125	0	- -
Parental roots	35	5 ± 15%	230	2	+ +
Nodule roots	25	0	80	0	- -
2. Nodules	35	0		N.D.	N.D. N.D.
Parental roots	35	1 ± 9%		N.D.	N.D. N.D.
Nodule roots	20-30	0		N.D.	N.D. N.D.
3. Nodules	30	0		N.D.	N.D. N.D.
Parental roots	35	0		N.D.	N.D. N.D.
Nodule roots	30	0		N.D.	N.D. N.D.
4. Nodules (d) (+ nodule roots)	35	3 ± 6%		N.D.	N.D. N.D.
Associated roots	30	80 ± 12%		N.D.	N.D. N.D.

Table 5 b.

Sample	Bioassay		GC-MS		
	Sample size- fresh wt. equivalents (g)	IAA equivalents µg/kg.fr.wt.	Sample size- fresh wt. equivalents (g)	S.I.M. (m/e 202) µg IAA/ kg.fr.wt.	M.I.M. 202 319
Vicia faba					
1. Nodules	15	8 ± 27%	45	0.5	+ +
Parental roots	35	0	165	0.1	+ +
2. Nodules (b)	35	9 ± 11%	105	10	+ +
Parental roots	35	2 ± 8%	265	just +ve	- +
3. Nodules		N.D.	70	40	N.D. N.D.
Parental roots		N.D.	500	4	N.D. N.D.
4. Nodules (c)	20	40 ± 17%	100	500	N.D. N.D.
5. Non-nodulated roots	30	0		N.D.	N.D. N.D.

(i) - Absence of ion from trace

(ii) + Presence of ion on trace

(iii) N.D. Not determined

(iv) Material was harvested from plants grown in 'Peralite' or water culture except for:-

(a) Field material from coppice near Milngavie c.20 years old.

(b) Field grown material var. 'Exhibition Longpod'.

(c) Field grown material var. 'Masterpiece Green Longpod'.

(d) Field material from Garraries Forest, New Galloway.

(v) The values in the table are not corrected for losses which radioactive recovery data suggest were of the order of 70%.

(vi) Minimum detectable level of IAA (a) by bioassay = 250 ng./bioassay (b) by GC-MS S.I.M. = 0.5 - 1 ng/injection.

(1) Run to run variation in samples of the same extract. Using similar amounts of sample, IAA levels varied in successive determinations in the same series and may have been due to:-

(a) Inaccuracies in the volume of sample injected.

(b) Drift of M.S. response (ion focussing and/or magnetic field strength).

(2) The presence of impurities. Ions of m/e 202, derived from impurities, eluting with IAA-TMSi, could give spurious measurements.

The minimum detectable level of IAA by GC-MS S.I.M. varied, depending upon the sensitivity of tuning on any particular run and was usually of the order of 0.5 - 10 ng per injection (≡ 10-60 ng/ extract).

Estimation of IAA levels could not be made from M.I.M. data (M.I.M. was only attempted in later experiments as facilities became available), since the relative peak heights of the ions m/e 202 and 319 at the elution point of IAA-TMSi in plant extracts, differed from the relative abundance of these ions in standards. This was probably due to interference from impurities, mentioned above. The presence or absence of ions m/e 202 and 319 is indicated in Table 5a,b, but it should be noted that the mass spectra which were obtained did not allow positive identification of IAA due to the high background of interfering ions and the low levels of IAA present.

Gas-liquid chromatography (GLC) of partially purified plant extracts

The variability in IAA levels, estimated from S.I.M. data (Table 5 a,b), and the difficulties described above, both in M.I.M. in samples of extracts and in attempts to obtain mass spectra, suggested that further attention should be given to purification of extracts. A large number of the extracts were analysed separately by GLC to investigate and, if possible, effect further separation in the IAA-TMSi region.

Typical GLC traces for the different types of sample extract from the three species, are shown in Figure 1 . Large numbers of impurity peaks were present in the IAA-TMSi region, confirming the above suspicions regarding the impurity of the extracts. Elution of samples from the column took 1-2 hours, so that some of the problems encountered with S.I.M. could be ascribed to overlap in sample elution from the GC.

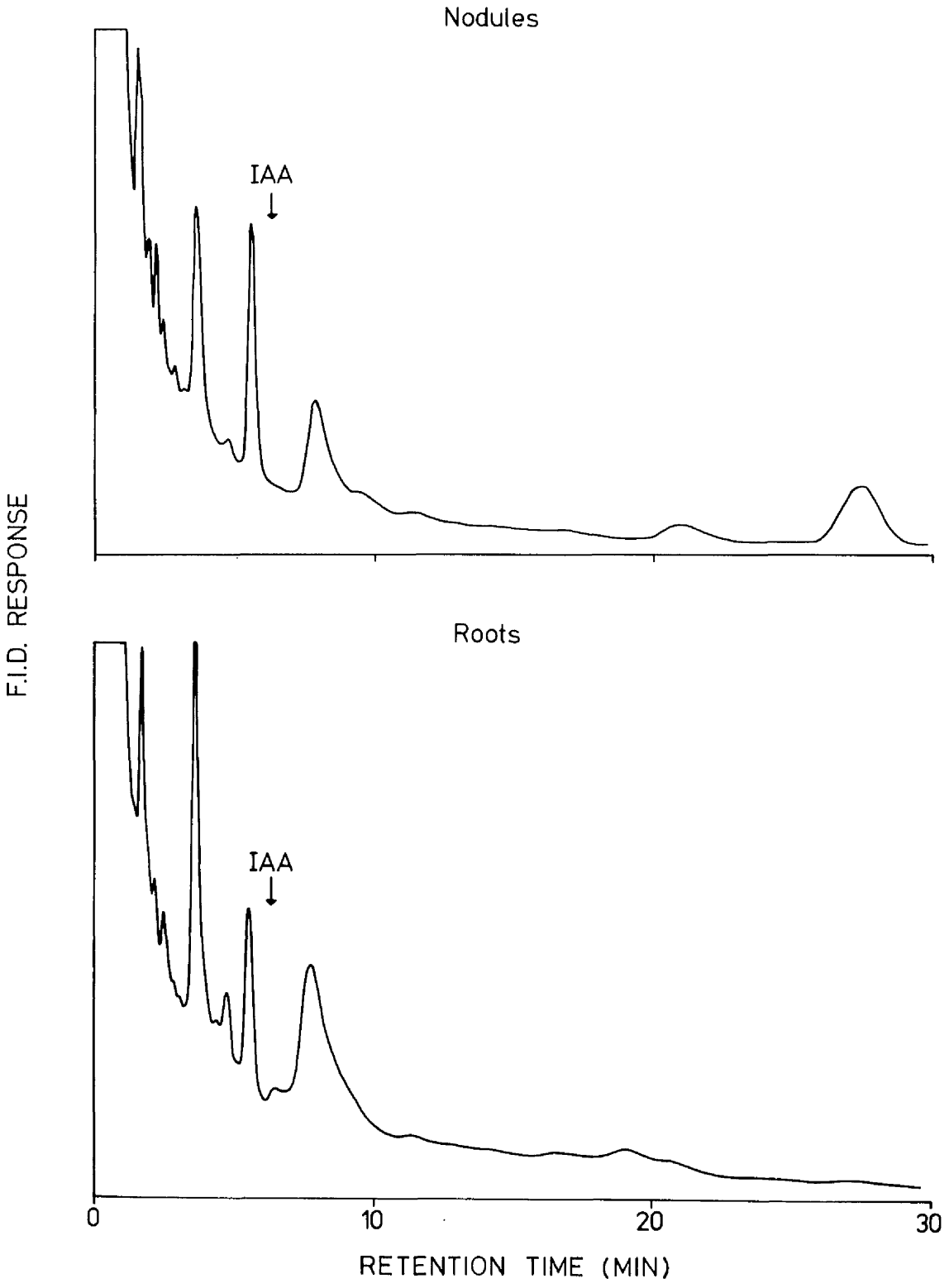
Figure 1

Gas-liquid chromatograms of silylated root nodule and root extracts of *Alnus glutinosa* and root extracts of *Vicia* i Elution point of IAA determined immediately before chromatography of each extract.

Chromatograph:- Pye, series 104.
Column:- 3% OV-17 (Gas Chrom Q, 80/100 mesh, $\frac{1}{4}$" x 7')
1% SE-52 (Gas Chrom Q,100/120 mesh, $\frac{1}{4}$" x 7')
Temperature:- column 160°C, detector 210°C
" 190°C, " 250°C
Carrier gas:- nitrogen, 50-60 ml. min^{-1}.
Detector:- flame - ionisation.

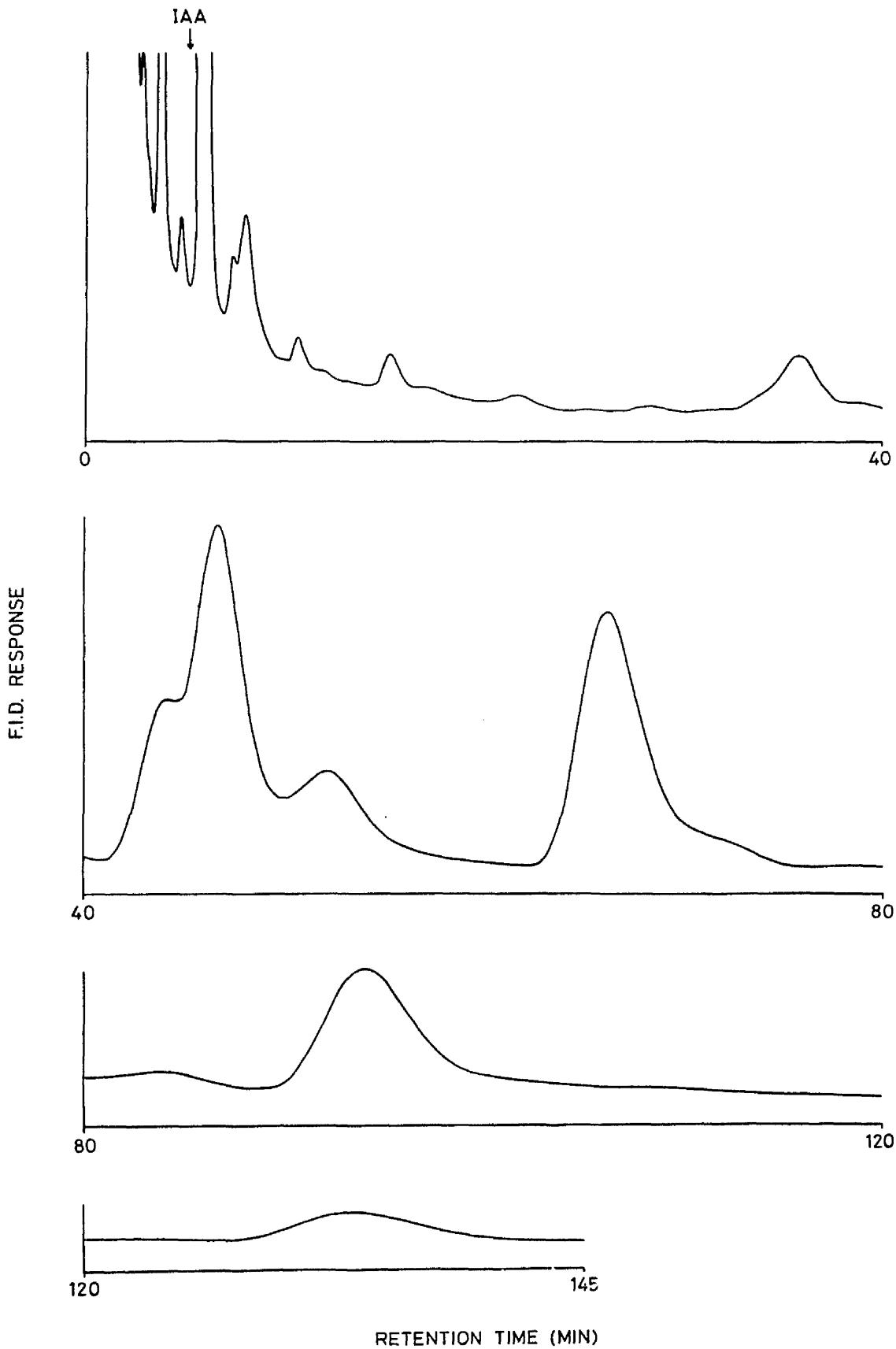
Aliquots (1-5 µℓ) of extracts, comprising 5-15% of the total sample were used for analysis.

IAA-TMSi Standards (2-4 $\mu g.\ell^{-1}$) were dissolved in BSTFA:CH_3CN::1:4 (solutions were yellow-green but showed no loss in GC response or presence of additional impurities). With greater dilution of the BSTFA, all GC response disappeared after a few hours.


Alnus glutinosa
Nodules
IAA
Roots
IAA
F.I.D. RESPONSE
0
10
20
30
RETENTION TIME (MIN)

Vicia faba

Roots



2. Development of purification procedures

The purification methods for endogenous IAA used initially in this investigation, although based upon published procedures with other tissues (Greenwood et al., 1972; Bridges et al., 1973; Elliott and Greenwood, 1974), were not successful when used with nodulated root systems (Section 1). Insufficient sample purity at the GC-MS stage introduced large variations into the estimation of the low levels of IAA in the extracts by single-ion-monitoring (at m/e 202). Positive identification of IAA by GC-MS was unsuccessful for the same reasons. This early work showed the necessity for further purification of nodulated root extracts for critical identification and quantification of IAA levels. Preliminary experiments suggested that the techniques of gel permeation chromatography (Reeve and Crozier, 1976) and preparative high performance liquid chromatography (Reeve et al., 1976), developed at Glasgow and used mainly in the analysis of plant extracts for radioactively-labelled gibberellins, were likely to be useful also for the analysis of acidic and neutral indoles. These techniques have been developed further for the purification of IAA in extracts from the species used in these investigations.

2.1 Extraction of plant material and initial purification

The extraction procedure differed from that used previously (Section 1.1) in several respects:-

(1) In later harvests, 1-^{14}C-IAA was added to the homogenate (plant material in 80% aqueous methanol) before overnight extraction, to monitor losses during the extraction and purification procedures.

(2) PVP purification took the form of a slurry rather than a column. The aqueous methanol filtrate, after reduction to the aqueous phase, was resuspended in 0.1 M K_2 HPO_4 buffer at pH8. Insoluble PVP, suspended in the same buffer, was filtered to remove excess buffer and added to the aqueous phase (1g filtered PVP/2g. fresh weight equivalents nodule tissue and half the rate for root tissue) to form a slurry.

(3) Ether partition was performed after PVP treatment rather than before, to streamline the purification procedure. The slurry with PVP was filtered through a Buchner funnel, washed twice with buffer and the combined filtrates acidified to pH3 and partitioned three times against equal volumes of diethyl ether.

(4) The residue (dried ether extract) was stored over silica gel at -18°C until required for further purification.

2.2 Gel Permeation Chromatography (GPC)

Introduction

The acidic ether soluble extracts, obtained as described in Section 2.1, were usually of high dry weight, requiring further purification using a procedure with high sample capacity such as a suitable GPC system. In this technique, a swollen, non-rigid gel is usually used, in conjunction with an organic solvent, to effect the separation of compounds according to molecular size. Used as a preliminary purification procedure, GPC has the advantage that related compounds, covering a wide range of polarity, are eluted together. Due to the absence of adsorption effects, successive samples can be run without overlap. Theoretical and practical considerations of gel permeation chromatography are detailed in Bombaugh (1971).

In the chromatographic system used for the present investigation, the column was packed with neutral, porous polystyrene beads swollen with tetrahydrofuran to form a non-rigid gel operating within a molecular weight range of 0-1500 (elution is in order of decreasing molecular size). The eluting solvent was tetrahydrofuran, which is an excellent solvent for impure plant extracts, allowing the high sample capacity of the column to be fully utilised.

Chromatographic system.

Two 2.5 x 100 cm glass columns were connected in series and packed with Bio-beads (Bio-Rad Laboratories Ltd., Bromley, Kent); the first column contained 50 cm SX-12 and 50 cm SX-8 and the second column 100 cm SX-4. The columns were operated at room temperature.

Samples with a dry weight up to 1g were dissolved in 2 ml. tetrahydrofuran and introduced onto the column by means of a six-port sample valve fitted with a 2 ml. sample loop. Elution of the samples was with tetrahydrofuran at a flow rate of 2.5 $ml.min^{-1}$, delivered by a micro-metering pump at 5 p.s.i. Before use, tetrahydrofuran was refluxed over cupric chloride for 30 minutes, redistilled and stored in darkness under nitrogen. Compounds eluting from the column were monitored by means of a Fresnel-type differential refractometer linked to a chart recorder.

It has been reported previously for this system (Reeve and Crozier, 1976) that:-

Vo (void or exclusion volume of column) = 350 ml.
(using expanded polystyrene, m.wt. > 1500)
Vt (total volume of column) = 610 ml.
(using methanol, m.wt. = 32)

Sample purification using GPC

In the early experiments, the column was calibrated using radioactively-labelled IAA (5-^{3}H-IAA and 1-^{14}C-IAA, 94-348 x 10^{6} d.p.m.) immediately before samples were run, to check the elution volume of IAA. Later, 1-^{14}C-IAA was added to extracts after homogenisation, to allow endogenous IAA recovery to be estimated, although GPC column eluate was also monitored. In both cases, fractions (10 ml) of column eluate were collected between 450-650 ml. and aliquots (50 µl) dried in scintillation vials in a stream of cold air and then dissolved in methanol prior to liquid scintillation counting. Early calibrations indicated an IAA recovery level of 72% (in 60 mls column eluate, between 520 and 610 mls elution volume), but in later calibrations recovery levels were much reduced (50-54%). This reduction was thought to be due to contamination of the column with charcoal, derived from extracts analysed by other users of the GPC system. In the later extracts, the internal IAA standard indicated recovery levels of 58% and 68% for IAA in nodule and root extracts respectively of *Alnus glutinosa*, and of 42% and 75% for nodule and root extracts respectively of *Myrica gale*.

Acidic ether soluble extracts of plant material, prepared as described in section 2.1, were dissolved in 1 ml. tetrahydrofuran

(e.g. 377 and 745 g. fresh weight equivalents of nodule and root extracts respectively of Alnus glutinosa could be purified per run) and taken up into the sample loop followed by two washes of 0.6 and 0.4 ml solvent. The extracts were applied to the column via the sample valve, the eluate monitored and fractions collected as described previously; fractions corresponding to, or containing labelled IAA were bulked for further purification, reduced to dryness by rotary evaporation and the residue stored over silica gel at - 18°C.

The refractive index profiles, of the column eluates of extracts of each of the three species of plant material under investigation, are shown in figure 2 . Large amounts of impurity were eliminated (as measured by change in refractive index of column eluate) from the IAA elution volume of all the extracts examined.

2.3 Preparative high performance liquid chromatography (preparative HPLC)

Introduction

Of the several types of liquid chromatography available, liquid-liquid (partition) chromatography (LLC) was used in the present preparative HPLC work. In this technique, compounds in the sample partition between a stationary phase coated onto a solid support, and a mobile phase which is pumped through the column. A second type is liquid-solid (adsorption) chromatography (LSC) which was used in the analytical HPLC procedures.

High performance in liquid chromatography has been achieved with the use of small diameter columns and solid supports of small particle diameter, coupled with high mobile phase flow rates (10 times gravity feed rates). The highest resolution and most precise quantification of compounds is achieved using HPLC systems with analytical columns having small diameters, of the order 1-3 mm. Since separation efficiency declines rapidly in an overloaded column, such small diameter columns limit sample size and consequently they are unlikely to be suitable for preparative purposes.

For preparative work, columns of wider diameter are required. Although these offer high sample capacity, they do not behave ideally. Lowering of the column efficiency occurs through so-called 'wall effects',

Figure 2

Gel permeation chromatography. Profiles showing change in refractive index with elution volume during purification of extracts from Alnus glutinosa, Myrica gale and Vicia faba.

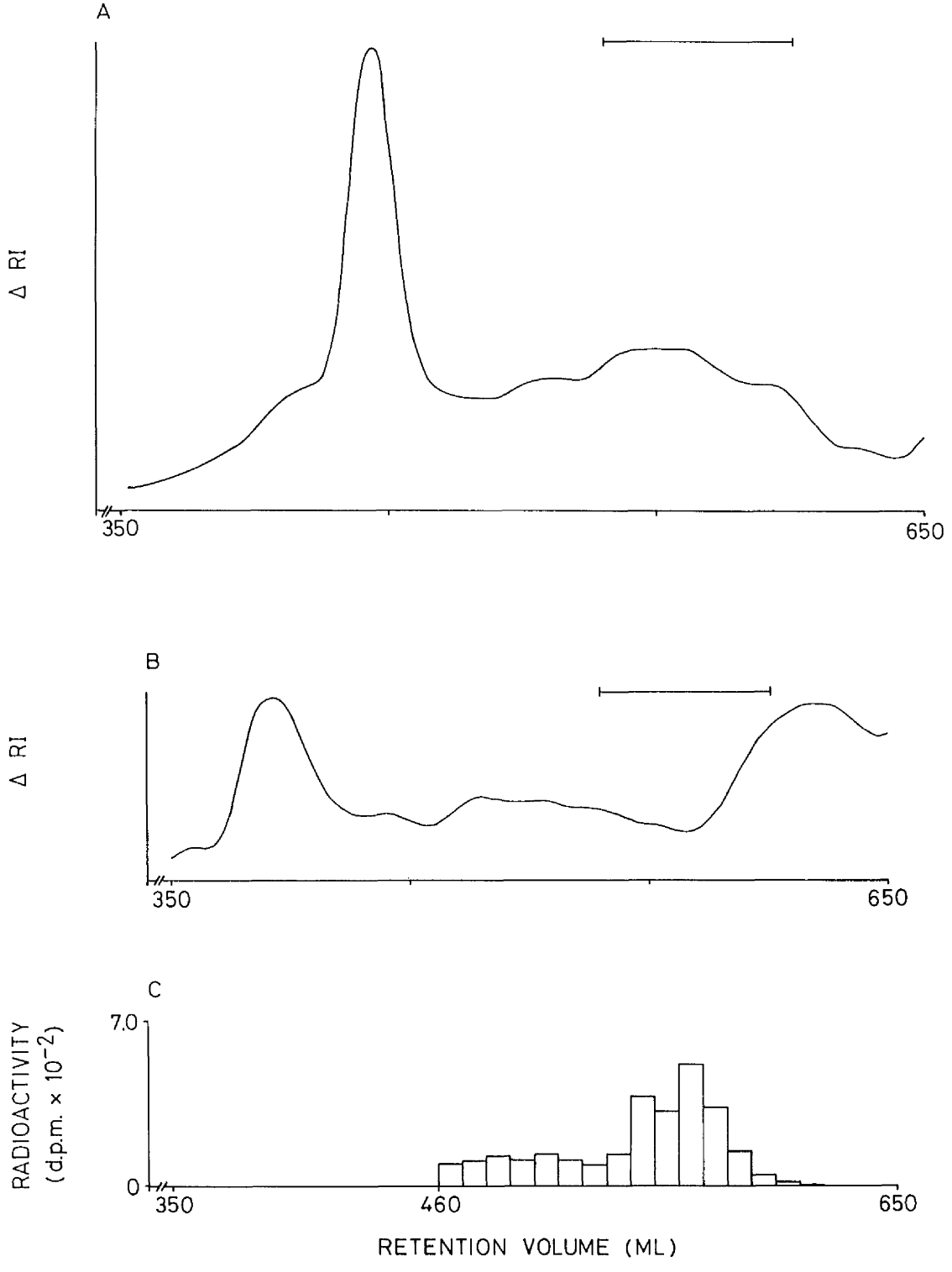
Bars indicate volume of eluate collected for further purification.

A Nodules

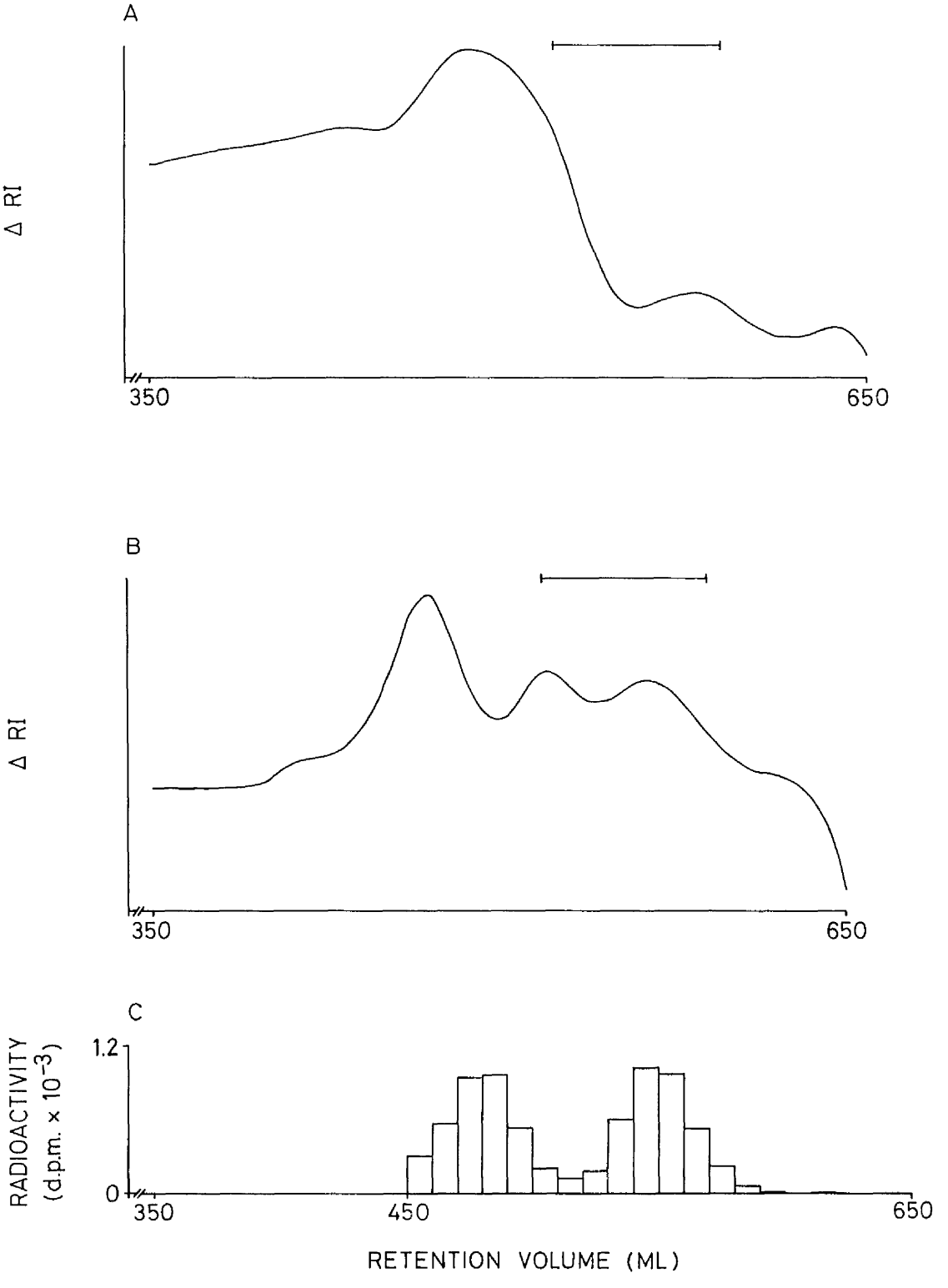
B Roots

C Radioactivity recovered from aliquots (50 µl) of eluate fractions (10 ml) from a nodule extract of the particular species.

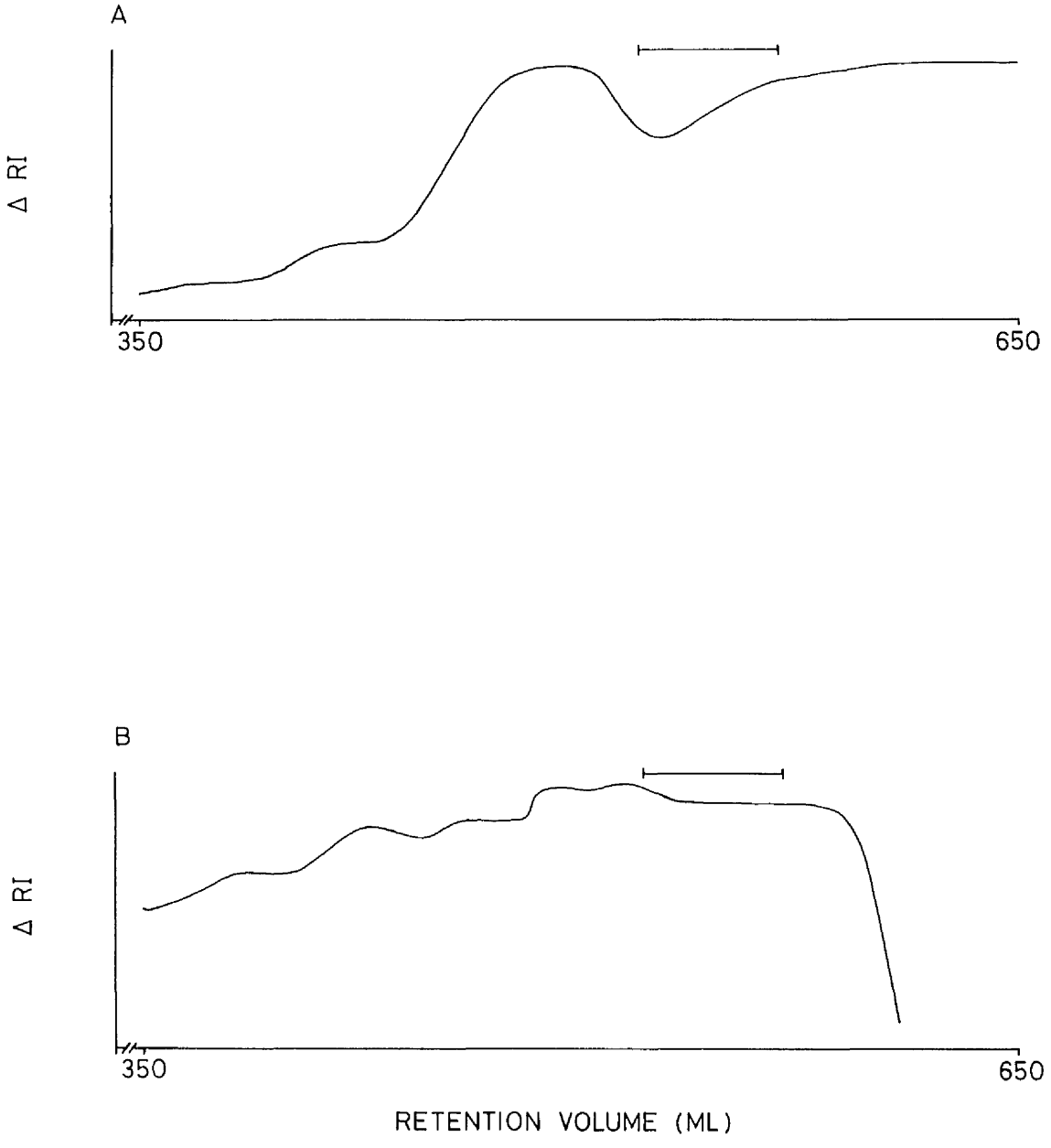
Alnus glutinosa



Myrica gale


A
Δ RI
350
650
B
Δ RI
350
650
C
RADIOACTIVITY
(d.p.m. x 10⁻³)
1.2
0
350
450
650
RETENTION VOLUME (ML)

Vicia faba



whereby sample molecules reach the wall of the column and travel faster than those in the centre. This results in anomalies in peak shape. In narrow-bore columns, transfer between the wall and centre of the column is sufficiently rapid to avoid these effects. With larger diameter columns, greatest column efficiency occurs where sample molecules never reach the disturbed wall area; these are columns of 'infinite-diameter'. The minimum internal diameter to achieve a column of 'infinite-diameter' can be calculated given the length and particle size (Kirkland, 1971). Increased sample capacity can also be achieved by means of higher stationary phase loadings. This will improve column stability, although there is some loss in column efficiency.

A wide variety of compound types, both polar and non-polar, can be separated by LLC due to the diversity of partitioning solvents available for selection of appropriate stationary and mobile phases. LLC is most commonly used with a polar stationary phase and a much less polar mobile phase. This separates the more strongly polar compounds, which are preferentially retained in the polar stationary phase. When non-polar compounds are separated, the phases are reversed ('reversed-phase' LLC).

The mobile phase must fulfil several practical requirements, the most important of which is to achieve maximum resolution in the sample, of all the compounds of interest. The capacity factor, k', describes the retention of a compound during chromatography by the stationary phase. This is a function of the polarity of the sample and the polarities of the stationary and mobile phases.

$$k' = \frac{t_R - t_o}{t_o}$$

t_R = retention time of the compound

t_o = retention time of non-retained compound (i.e. solvent front).

Solvent (i.e. stationary phase and mobile phase) polarity can be quantitatively defined. In LLC one measure commonly employed is the Hildebrand solubility parameter (δ), values of which range from 6 for fluorocarbons (least polar) to 21 for water (most polar). The δ values of those solvents which have been used to develop the chromatographic systems described in this study, are given in Table 6 .

Sample k' values vary with solvent composition and are usually adjusted by altering the composition of the mobile phase, rather than the stationary phase. Although initial selection of the mobile phase is largely empirical, further adjustment can be based on δ values. In LLC, as used here, as the solvent strength of the mobile phase is increased (δ values increase), its polarity increases

Table 6

Values (δ) of the solubility parameter (Hildebrand) for solvents used in the present sample purification procedures (Snyder,1971a)

Solvent	δ	Solvent	δ
Water	21	Dichloromethane	9.6
Methanol	12.9	Tetrahydrofuran	9.1
Dimethylsulphoxide	12.8	Ethyl acetate	8.6
Acetic acid	12.4	Diethyl ether	7.4
Acetonitrile	11.8	Hexane	7.3

and k' values of the particular compound are decreased.

In complex mixtures, when even with optimum solvent strength two or more compounds may be incompletely separated, greater resolution may be achieved by the use of a solvent gradient, in which the strength of the solvent is gradually changed during elution. The k' value is then optimum for each compound as it moves through the column. Further theoretical and practical details of LLC are discussed in Kirkland (1971) and Snyder and Kirkland (1974).

Chromatographic System

Preparative HPLC was utilised in this project for two purposes. Firstly, for further purification of impure extracts following solvent partition and GPC, as a step towards the identification and estimation of endogenous IAA levels. Secondly, to scan extracts for radioactive metabolites of widely differing polarities derived from plants treated with radioactively-labelled IAA. These extracts, which were subjected only to solvent partitioning, were very impure and frequently contained only low levels of radioactivity, necessitating the use of a chromatographic system of reasonably high sample capacity but capable also of resolving a wide range of sample components.

The LLC system described in this project (Figure 3) has been developed from the silica gel partition column of Powell and Tautvydas (1967) to provide a much faster and more efficient separation. A rigid gel such as silica is necessary to sustain solvent programming (and also the high mobile phase flow rates used in HPLC).

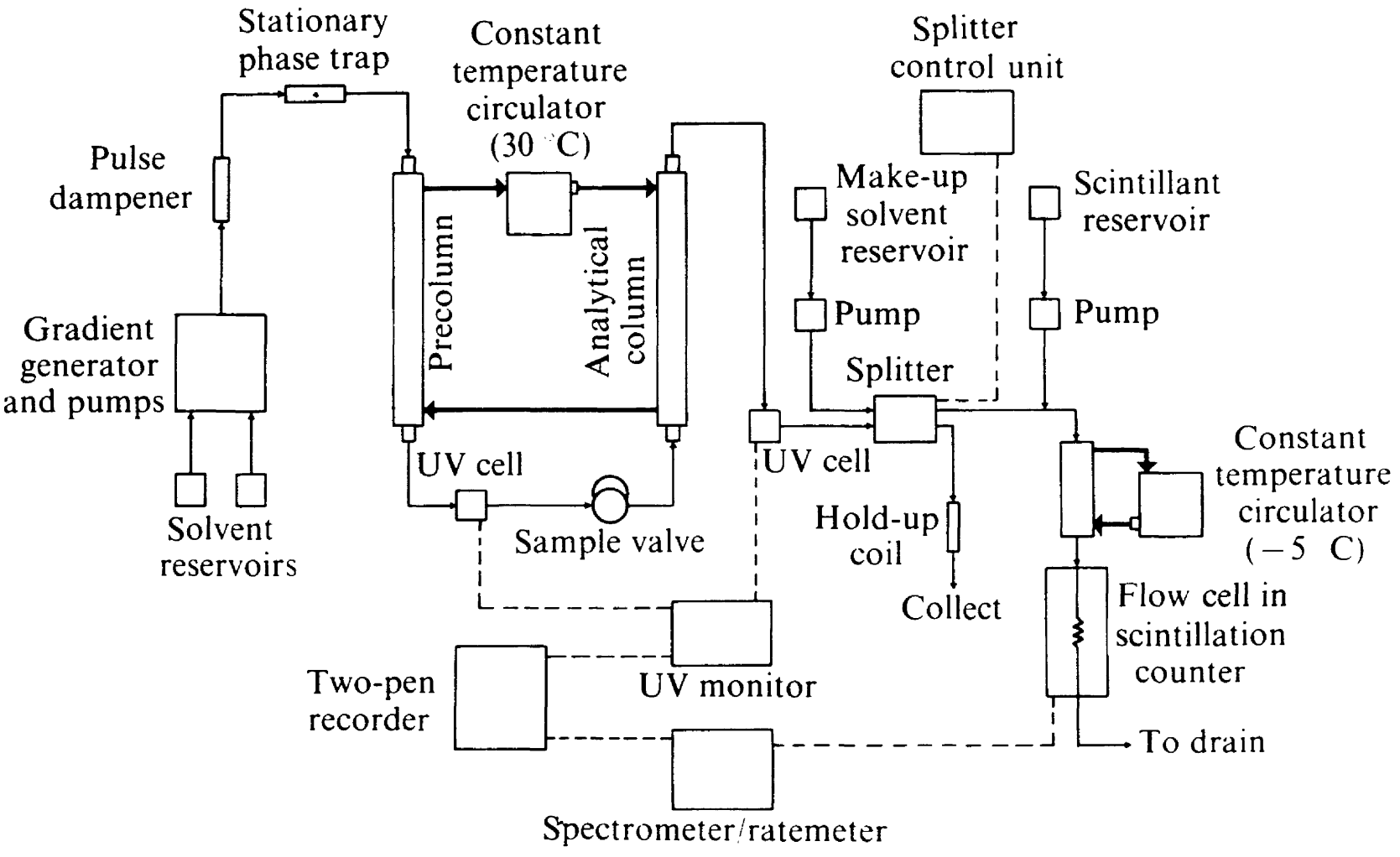
The columns (10 x 450 mm) used for analysis contained either 10 or 20 µm silica gel particles (Partisil 10, 20 - Whatman Ltd., Maidstone, Kent). The silica gel was loaded initially with a stationary phase of 40% (v/w) 0.5 M formic acid. In later work, this was replaced by 40% (v/w) 1.0 M acetic acid because of problems with irreversible changes in the column after prolonged use (condensation reaction products were retained and eventually saturated the column, raising the pH of the stationary phase). The mobile phase was either a gradient of ethyl acetate in hexane, variable between 0-100%, or a constant concentration of ethyl acetate in hexane; these mobile phases were saturated with either 0.5 M formic acid or 1.0 M acetic acid as appropriate. After thorough degassing,the mobile phase was delivered by a dual pump gradient generator (ISCO, model 382 Dialograd, with modified column inlet fittings) at a flow rate of 4.6 ± 0.1 ml. min^{-1}. Depending on the composition of the mobile phase, a column pressure of 140-200 p.s.i. was developed. The mobile phase reached the chromatographic column via a pulse dampening system, a stationary phase trap and a pre-column. Although virtually immiscible, there is always some mutual solubility of the mobile and stationary phases. To prevent the mobile phase gradually removing the stationary phase from the chromatographic column during use, the phases were pre-equilibrated by passing the mobile phase, saturated with stationary phase, through a pre-column. For optimum column stability and consistent sample retention times, the pre-column and chromatographic column were maintained at a constant temperature of 30.0 ± 0.05°C by means of a water jacket.

The high sample capacity of the column enabled individual components up to 10 mg. dry weight, and multicomponent samples up to 100 mg. dry weight to be chromatographed without loss of resolution. Samples (maximum volume 330 µl) were dissolved in column eluate (identical in composition to the mobile phase), taken up into a 330 µl sample loop and were introduced onto the chromatographic column through

Figure 3

The 'preparative' high performance liquid chromatograph with U.V. absorbance and radioactivity monitors.

(Reproduced with kind permission, Reeve and Crozier, 1978).


Stationary phase trap
Constant temperature circulator (30 °C)
Splitter control unit
Pulse dampener
Make-up solvent reservoir
Scintillant reservoir
Precolumn
Analytical column
Gradient generator and pumps
Pump
Pump
Splitter
UV cell
UV cell
Constant temperature circulator (−5 C)
Solvent reservoirs
Sample valve
Hold-up coil
Collect
Flow cell in scintillation counter
Two-pen recorder
UV monitor
To drain
Spectrometer/ratemeter

a six-port sample valve. The characteristics of a column of 'infinite-diameter' were maintained by the use of a specially constructed column inlet fitting (Reeve et al., 1976) to introduce relatively large sample volumes onto the column. This fitting confined the sample to the column centre, by allowing it to enter the column both from the top and from the side of the inlet simultaneously; it was then kept in position by the entry of the mobile phase via the same route.

The chromatographic capacity of the system was utilised fully, both in terms of resolution and of the relatively rapid analysis time, by continuous monitoring of the column eluate. All eluate passed through the flow cell of a U.V. monitor (254 mm) and all or part was then diverted, as appropriate, through the flow call of a radioactivity monitor (Reeve and Crozier, 1977). In studies where radioactive compounds were monitored but not collected, all the eluate was mixed with scintillation fluid delivered from a reservoir by a metering pump at a flow rate of 2.0 ± 0.1 ml. min^{-1}. The mixture then passed through a cooling bath at $0^{o}C$ before entering a spiral glass flow cell of a manual liquid scintillation counter (Laboratory Impex, P.O. Box 14, Twickenham, Middlesex). The signals from the counter were processed by a spectrometer/ratemeter to produce a counting rate proportional to the concentration of radioisotope in the flow cell. For solutes eluting with a k' of 1.7, the minimum level of radioactivity resolved by the monitor, was 3×10^3 d.p.m. for ^{3}H and 1×10^3 d.p.m. for ^{14}C. The U.V. absorbance levels and radioactivity counting rates were simultaneously displayed on a dual channel chart recorder which also integrated the radioactivity trace. Collection of compounds following preparative HPLC was aided, firstly, by the presence of an internal radioactive standard, and secondly by the use of a splitting device, after the U.V. monitor stage, which diverted a preset proportion of column eluate for analysis of radioactivity. This allowed more accurate collection of particular peaks with as few impurities as possible. The original flow rate of the eluate collected,was made up with solvent consisting of equal volumes of ethyl acetate and toluene.

Following chromatography of an extract using a mobile phase of constant composition (e.g. in endogenous work where the compound polarity range of interest was limited), re-equilibration of the column was unnecessary and analyses could be performed continuously

without column deterioration. If a moderate mobile phase gradient (e.g. 40-60% ethyl acetate in hexane in 20 minutes) had been used for the elution of a sample, the column was regenerated by removing the last part of the solvent gradient. This was usually achieved gradually (over about 20 minutes), by reversing the gradient until the original solvent composition had been regained. A severe mobile phase gradient (e.g. 50-100% ethyl acetate in hexane in 65 minutes) required the removal of the mobile phase entirely (by passing a stream of nitrogen through the column). The column was then allowed to re-equilibrate for 15-20 hours at 30°C. This type of gradient was used in the radioactive tracer experiments for a broad scan of each extract, over a wide polarity range.

Further details of the chromatographic system can be found in Reeve et al (1976).

Sample purification using the preparative HPLC

(a) Non-derivatised extracts

From preliminary runs using 5-^{3}H-IAA, a mobile phase composition was selected which would elute IAA at a suitable k' value (approximately 3.0). Rapid elution of all the components of the sample, together with optimum resolution of IAA, was achieved with a gradient of 40-60% ethyl acetate in hexane completed in 20 minutes.

For each sample, the appropriate fractions from GPC were washed into a small conical test tube with ether, and this was then removed in a stream of oxygen-free nitrogen. The residue was redissolved in 330 µl column eluate, taken up into the sample loop and injected onto the column via the sample valve. The column eluate was collected at the predetermined elution point of the radioactive IAA standard, reduced to dryness by rotary evaporation and stored over silica gel at - 18°C. In later experiments, the column eluate was collected more precisely at the IAA elution point, using the splitting device. An internal radioactive marker (1-^{14}C-IAA) was incorporated into the tissue homogenate at the beginning of the extraction procedure, immediately after blending in aqueous methanol. Small aliquots of extract (eg. 2%, 6%) were run initially without the splitter, to determine the size of the aliquot to be split off for assay of radio-

activity when purifying the whole extract.

Although a considerable proportion of U.V. absorbing material was eliminated from the region of interest in all the extracts, a large proportion remained (Figure 4), indicating the necessity for further purification of the samples. This was accomplished by methyl esterification of the column eluate collected at the IAA elution point, followed by further chromatography on preparative HPLC (following paragraphs).

(b) Methyl esterified extracts

The carboxyl group on the side chain of the indole nucleus can be esterified to produce a compound with different retention characteristics, so that using the same chromatographic system, but with different mobile phase conditions, a second separation from impurities can be effected.

Methyl esterification to form methyl indole-3-acetate (IAA-Me) was performed using diazomethane. The efficiency of three methods for methyl esterification with diazomethane was investigated as described below. The progress of esterification using 5-^{3}H-IAA, was followed with the radioactivity monitor of the preparative HPLC.

Preparation of diazomethane

Diazomethane was generated by the action of concentrated alkali on p-toluene-sulphonyl-methylnitrosamide (Nitrosan-Dupont (U.K.) Ltd., Hitchin). The generating flask (2g nitrosan, 7.5 ml ethylene glycol, 5.0 ml saturated KOH and 20 ml generating solvent) was warmed gently in a beaker of hot water and diazomethane distilled over in the generating solvent and condensed in a receiver flask (10 ml generating solvent) surrounded by ice. The flask containing the diazomethane solution was tightly sealed, and stored if necessary for a short time at -18^{o}C.

Methyl esterification of IAA

The three methods for the generation and subsequent reaction of diazomethane with IAA were as follows:-

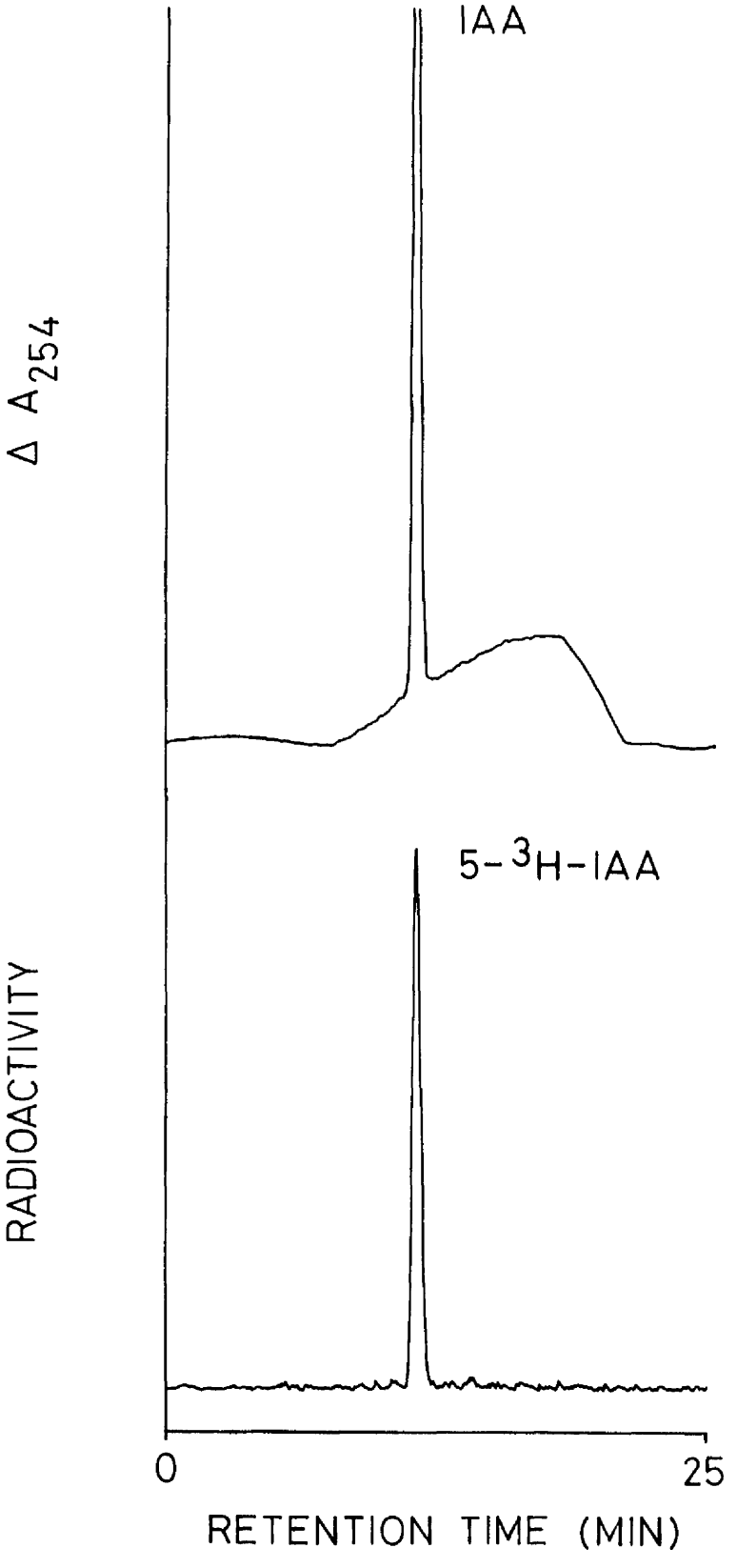
(1) Generation of diazomethane in diethyl ether. Diazomethane was prepared as described above, with diethyl ether as the generating solvent,

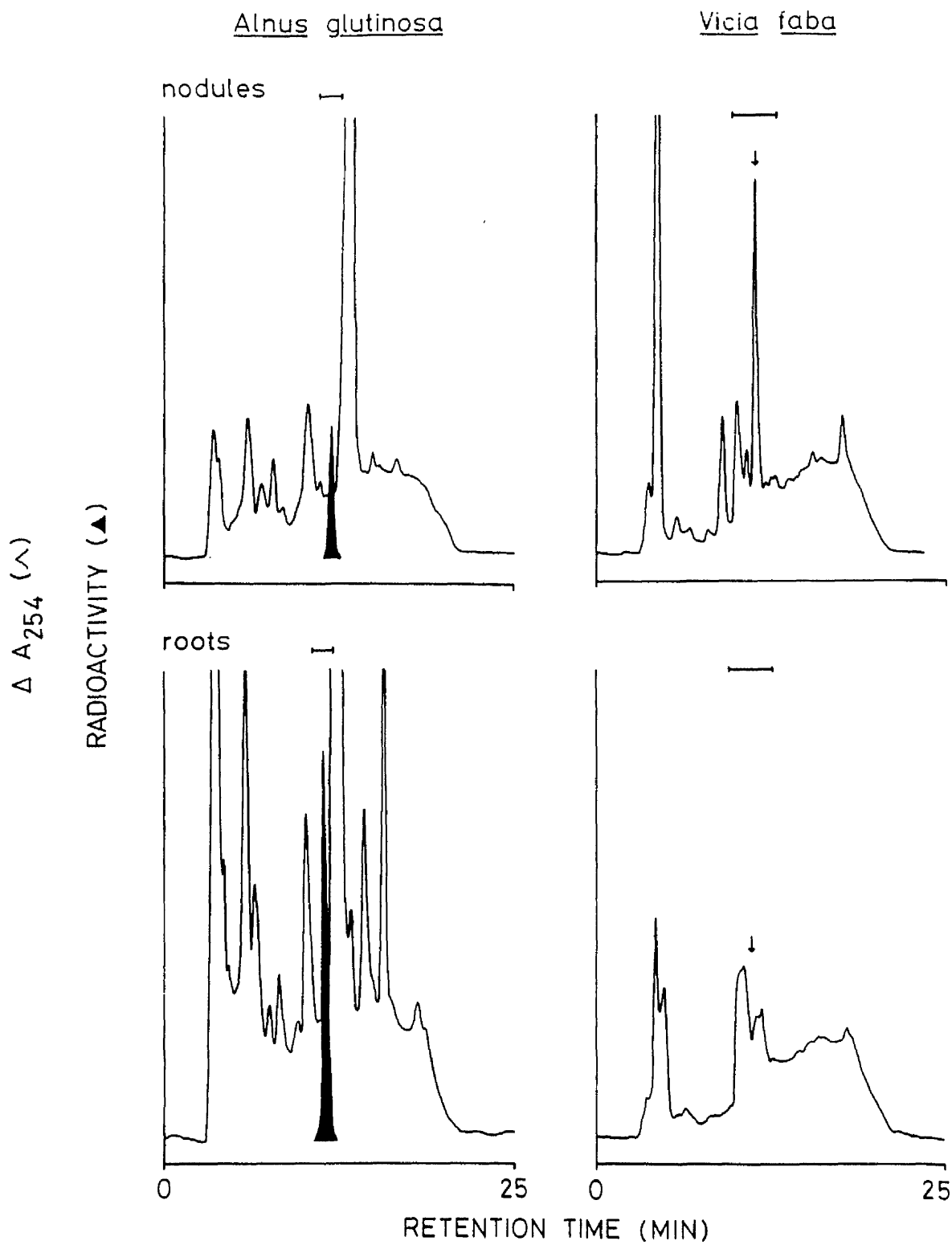
Figure 4

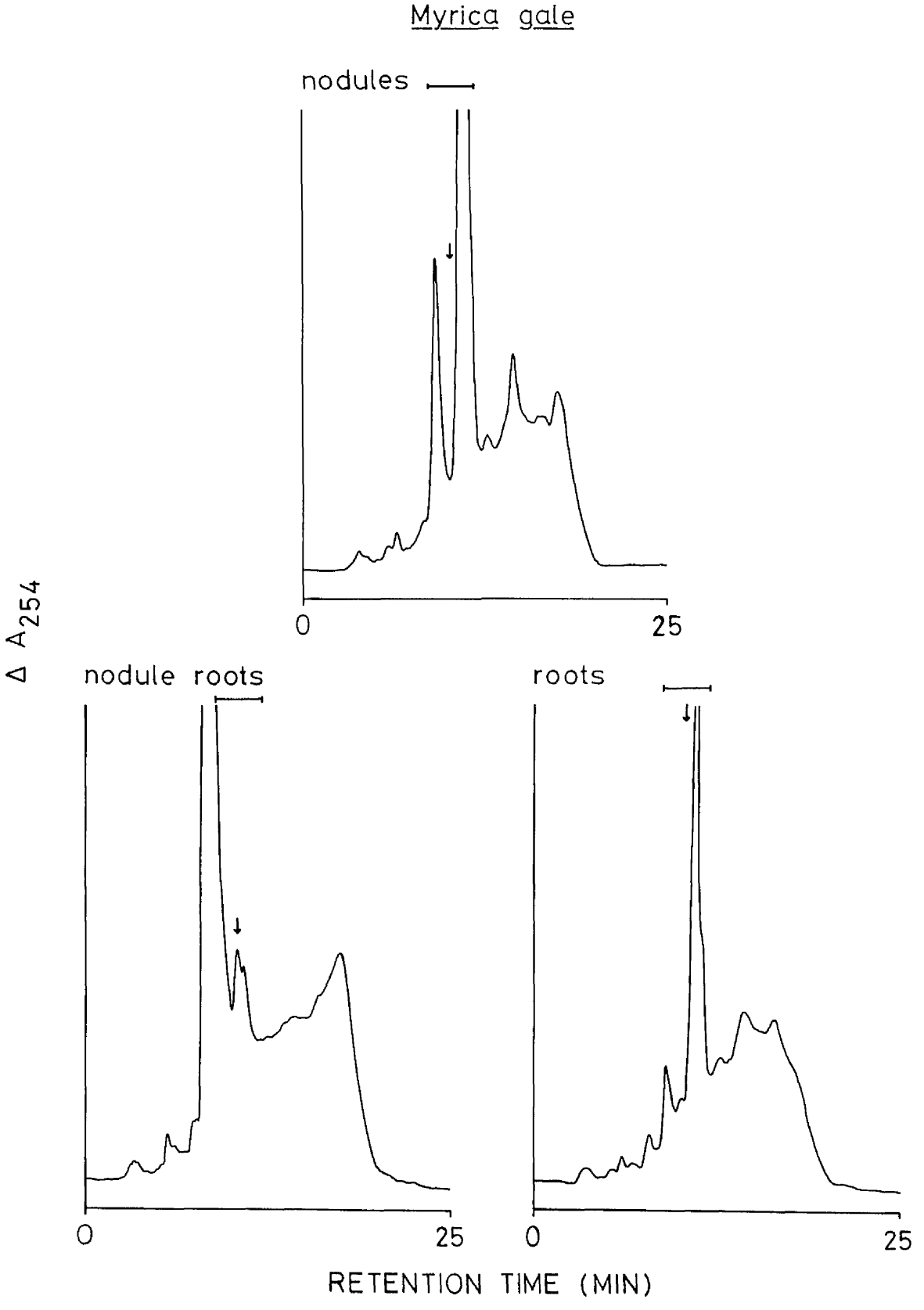
Preparative high performance liquid chromatograms showing the U.V. absorbance and radioactivity of eluates of 5-^{3}H-IAA and of root nodule, nodule root (where applicable) and root extracts of Alnus glutinosa, Vicia faba and Myrica gale.

Arrows represent the elution point of 5-^{3}H-IAA, determined immediately before purification of each extract. Infilled peaks represent the elution point of the internal marker (1-^{14}C-IAA).
Bars indicate volume of eluate collected for further purification.

Column:- Partisil 10 ODS 20 (10 x 450 mm)
Column temperature:- 30.0 ± 0.5°C
Stationary phase:- 0.5 M formic acid
Mobile phase:- gradient of 40-60% ethyl acetate in hexane in 20 minutes
Flow rate:- mobile phase 4.6 ± 0.1 ml. min^{-1}
scintillant 2.0 ± 0.1 ml. min^{-1}


IAA
Δ A254
5-3H-IAA
RADIOACTIVITY
0
25
RETENTION TIME (MIN)


Alnus glutinosa
Vicia faba
nodules
roots
Δ A254 (▲)
RADIOACTIVITY (▲)
0
25
0
25
RETENTION TIME (MIN)


Myrica gale
nodules
nodule roots
roots
Δ A254
0
25
0
25
0
25
RETENTION TIME (MIN)

and was added (1 ml) to dry 5-^{3}H-IAA at room temperature, the reagent being removed immediately after mixing with a stream of oxygen-free nitrogen. Only 26% esterification was achieved. Esterification was then performed in sealed vials at various temperatures and for varying lengths of time, in an attempt to improve conversion of the free acid to its methyl ester. Inconsistent results (up to 63% conversion) were obtained.

(2) Generation of diazomethane in dichloromethane. During tests with diazomethane as generating solvent, it was noticed that decomposition of radioactive IAA began immediately on dilution with dichloromethane which had been stored in the light, and continued for up to 30 minutes after dilution (Table 7). There was no deterioration of radioactive IAA diluted with dichloromethane stored in the dark, or with methanol over similar periods.

Table 7

Comparison of the effect of methanol, and dichloromethane Stored both in the dark and the light, on the decomposition of 5-^{3}H-IAA.

Treatment	% of original radioactivity in form of IAA after treatment
5-^{3}H-IAA + Me OH	100
5-^{3}H-IAA + CH_2Cl_2(light) 0min.	79.3
30 min.	40.4
5-^{3}H-IAA + CH_2Cl_2(dark) 0 min.	100
30 min.	99.3

(3) Generation of diazomethane in diethyl ether with addition of methanol to the reaction mixture. Ethereal diazomethane was prepared as described previously, but 10% methanol was incorporated into the reaction mixture. Methanol is thought to act as a catalyst in the reaction, although excess is inhibitory giving a low rate of esterification (20%, Schlenk and Gellerman, 1960). The reaction was tested

using small aliquots of nodule and root extracts of Alnus glutinosa, which had been collected from the first run on preparative HPLC, and which contained 1-^{14}C-IAA as internal marker. Ethereal diazomethane (1 ml) was added, together with a drop of methanol, to each of the samples in sealed vials, at room temperature. After 30 minutes, no breakdown products were detected in either sample, and the occurrence of a single peak at the retention time for IAA-Me showed 100% methyl esterification of IAA. Because of its freedom from the problems encountered with the other procedures, this technique was adopted routinely for methyl esterification of sample extracts.

Methyl esterification and chromatography of sample extracts

Samples which had been partially purified by the GPC and preparative HPLC procedure already described, were transferred to a 'reacti-vial' (Pierce Chemical Co., Rockford, Illinois, U.S.A.) using a small volume of ethyl acetate, which was then removed with a stream of oxygen-free nitrogen. The ethereal diazomethane solution (1 ml) was added, together with a drop of methanol, to each sample which was stored overnight at -18°C, after which the excess reagent was removed in a stream of oxygen-free nitrogen.

Prior to re-chromatography of the methyl esterified samples on preparative HPLC, methyl esterified 5-^{3}H-IAA was used to determine a suitable mobile phase composition which would elute the ester with a k' value of about 1.5. This was achieved with 30% ethyl acetate in hexane. Use of the standard also allowed the elution point of the IAA methyl ester to be determined precisely. The methyl esterified sample, stored in a 'reacti-vial', was redissolved in 3000µl column eluate, taken up into the sample loop and injected onto the column as before, through the sample valve. Eluate was collected, at the IAA-Me elution point, in a 50 ml round-bottomed flask, and after reduction to dryness, the sample was washed once with ethyl acetate and transferred to a 'reacti-vial'. The ethyl acetate was removed by a stream of oxygen-free nitrogen and the sample stored over silica gel at -18°C.

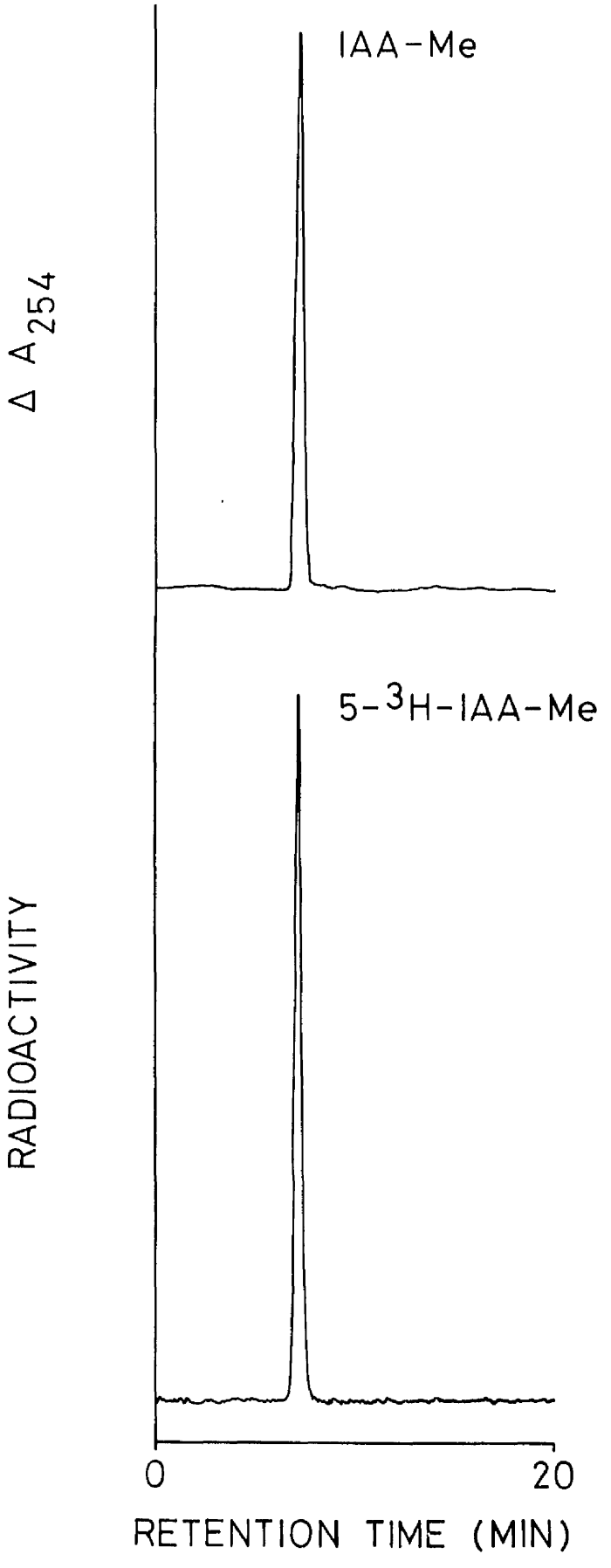
The U.V. absorbance of these HPLC eluates showed further considerable separation of U.V. absorbing material from the IAA-Me region (Figure 5), especially in extracts from Alnus and Vicia.

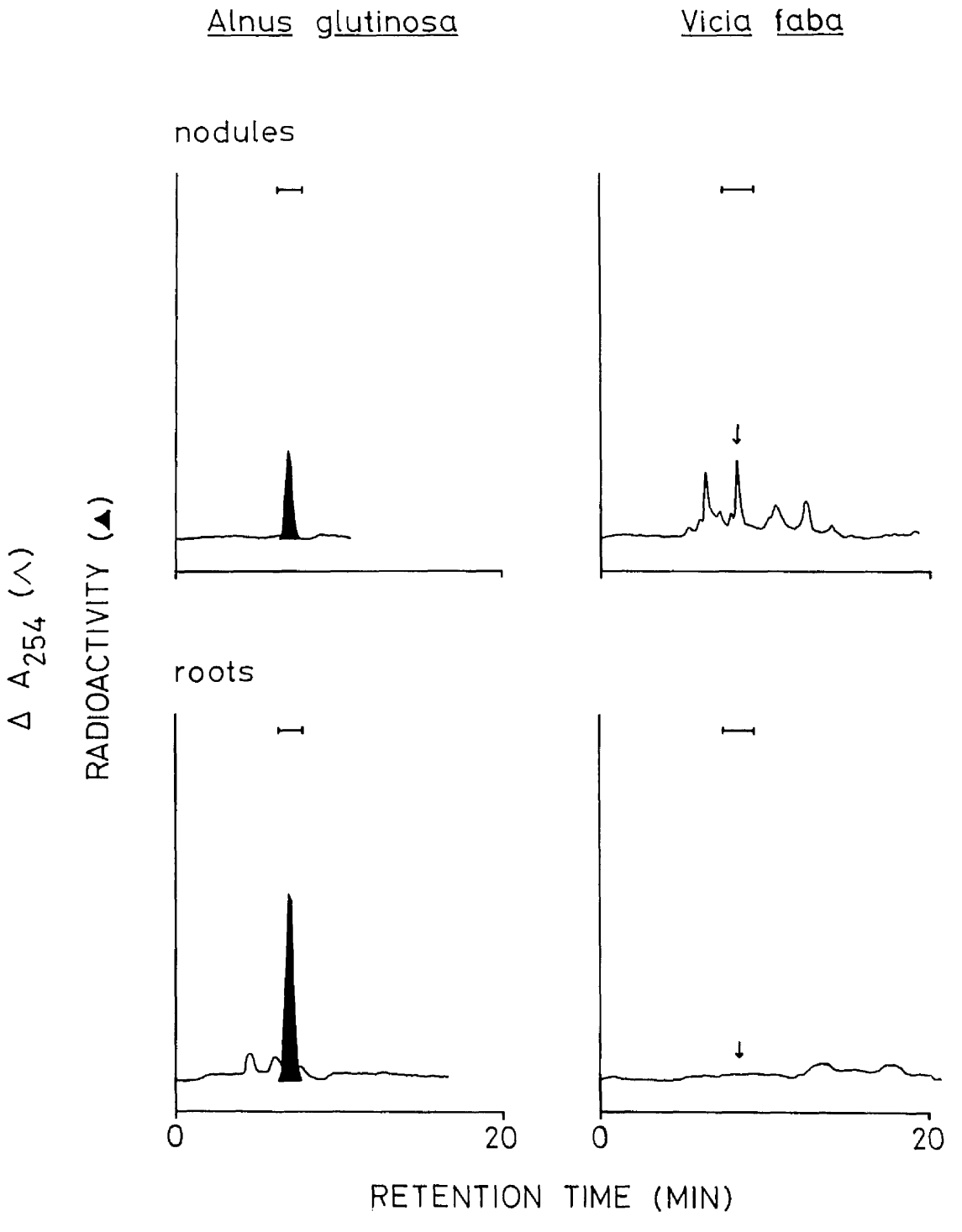
Figure 5

Preparative high performance liquid chromatograms showing the U.V. absorbance and radioactivity of eluates of methylated 5-^{3}H-IAA and of methylated root nodule, nodule root (where applicable) and root extracts of Alnus glutinosa, Vicia faba and Myrica gale.

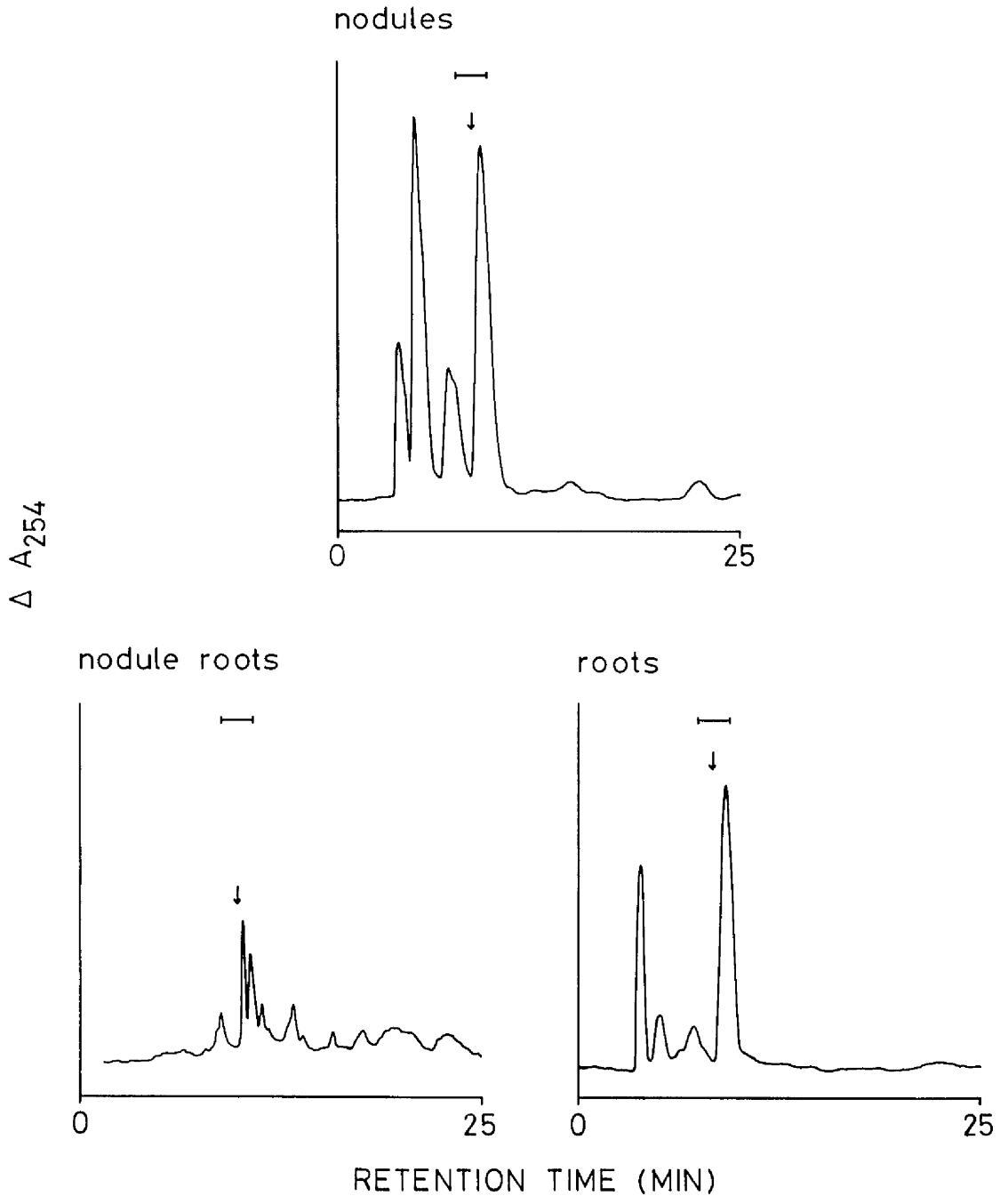
Arrows represent the elution point of methylated 5-^{3}H-IAA, determined immediately before purification of each extract.
Infilled peaks represent the elution point of the methylated internal marker (1-^{14}C-IAA).
Bars indicate volume of eluate collected for further purification.

Column:- Partisil 10 ODS 2 (10 mm × 50 mm)
Column temperature:- 30.0 ± 0.5°C
Stationary phase:- 0.5 M formic acid
Mobile phase:- 30% ethyl acetate in hexane
Flow rate:- mobile phase 4.6 ± 0.1 ml. min^{-1}
scintillant 2.0 ± 0.1 ml. min^{-1}


IAA-Me
Δ A254
5-3H-IAA-Me
RADIOACTIVITY
0
20
RETENTION TIME (MIN)


Alnus glutinosa
Vicia faba
nodules
roots
Δ A254 (∧)
RADIOACTIVITY (▲)
0
20
0
20
RETENTION TIME (MIN)

Myrica gale


nodules
nodule roots
roots
Δ A254
0
25
0
25
0
25
RETENTION TIME (MIN)

However, a large peak remained in nodule and root extracts of Myrica. Further separation of IAA-Me from the remaining impurities, and quantification of the small amounts of endogenous IAA, was achieved by chromatography of the IAA-Me region eluates by analytical HPLC (next Section).

2.4 Analytical high performance liquid chromatography (analytical HPLC)

Introduction

The use of the different separation techniques described above, namely, PVP adsorption, solvent partition, gel permeation chromatography and silica gel partition chromatography on both the free carboxylic acid form of the extract and a derivative (methyl ester), formed the basis of the purification procedure for analysis of IAA in nodulated roots. Additional purification and preliminary quantification of hormone levels was achieved by silica gel adsorption chromatography in an HPLC system.

Conventional, commercially available analytical HPLC apparatus normally incorporate narrow bore columns of 2-5 mm diameter. Their sample capacity is limited, usually reaching a maximum of 500 µg. If these columns are used for the analysis of semi-purified plant extracts, resolution of compounds of interest from a high background of impurities is very difficult, but they can be used successfully for the analysis of compounds of adequate purity, such as those which have been subjected to preparative HPLC.

Liquid-solid (adsorption) chromatography (LSC) using silica gel as a support, is very much akin to thin-layer chromatography although it offers many improvements over that technique. It can be used for samples of intermediate molecular weight (less than 1,000) and those samples which can be separated by TLC can also be separated by LSC. Most types of compounds fall into this category, ranging from non-polar hydrocarbons to fairly polar, water-soluble compounds. High molecular weight samples or ionic lipophobic compounds are not so successfully separated (Snyder, 1971b).

Silica is a polar adsorbent and the main factor in determining the relative adsorption of a compound (i.e. its K' value) is the nature of the functional groups it possesses. Relative adsorption

increases as the polarity and number of these functional groups increase. L S C shows no selectivity between different alkyl group substitution, i.e. between homologues, but does show higher selectivity among certain isomer types, compared to other liquid chromatographic methods. There are two special characteristics of adsorption from solution which confer on L S C its unique retention and selectivity qualities. Firstly, there is competition between sample and solvent (mobile phase) molecules for a place on the adsorbent surface, and secondly there are multiple interactions between functional groups on the sample molecule and corresponding, but firmly fixed sites on the adsorbent surface. Silica is particularly useful in adsorption chromatography for a number of reasons. It is widely available in a variety of particle sizes and shapes, has a high linear capacity (i.e. the sample retention volumes remain constant for large changes in sample size) and produces highly efficient columns, the level of efficiency depending on particle size and geometry.

The water content of the adsorbent plays a critical role in L S C; it increases the linear capacity of the silica and also column efficiency. Its presence decreases adsorbent-catalysed sample reactions and also decreases irreversible sample adsorption, allowing greater recovery of sample components. Relative k' values are strongly affected by the water content of the adsorbent, which must be kept constant for reproducible chromatography. Changes in column temperature have only a small effect on k' values; for every ^{o}C rise in temperature, k' values decrease by about 1%.

Chromatographic strength of a solvent in adsorption chromatography is described by the parameter E^{o} (Snyder, 1968) as opposed to δ (solubility parameter) in partition chromatography.

Table 8

Solvent strength (for adsorption chromatography on alumina) of the solvents used here in analytical HPLC (Snyder, 1971a).

Solvent	E^{o}	
Dimethylsulphoxide	0.6	Strong solvent
Acetonitrile	0.65	↓
Tetrahydrofuran	0.45	
Dichloromethane	0.42	
Hexane	0.01	Weak solvent

Relative solvent strength changes very little from one adsorbent to another.

Elution of a sample with optimum k' values for the compound(s) of interest, requires a mobile phase solvent of suitable strength, the choice of which is aided by reference to their E^{o} values (e.g. Table 8). If k' values are initially too small, the solvent is too strong, and another with a lower E^{o} is substituted. Mobile phase solvent strength can be adjusted further by mixing two solvents in various proportions. Improved separation of particular compounds, once a mobile phase of suitable strength has been obtained, can be achieved by varying the composition of the mobile phase whilst its solvent strength is held approximately constant. The mobile phase solvent(s) are usually of relatively low strength towards the compounds being chromatographed. A much stronger solvent can be added to the mobile phase to obtain the selectivity required. This strong solvent (modifier) tends to concentrate into the adsorbed phase where it interacts slightly with the adsorption sites on the silica surface, thereby altering interactions with the solutes of the sample extract. For a given mobile phase, different modifiers can provide a range of selectivities towards the sample components. To maintain optimum selectivity, not more than 10% of the mobile phase should be composed of modifier.

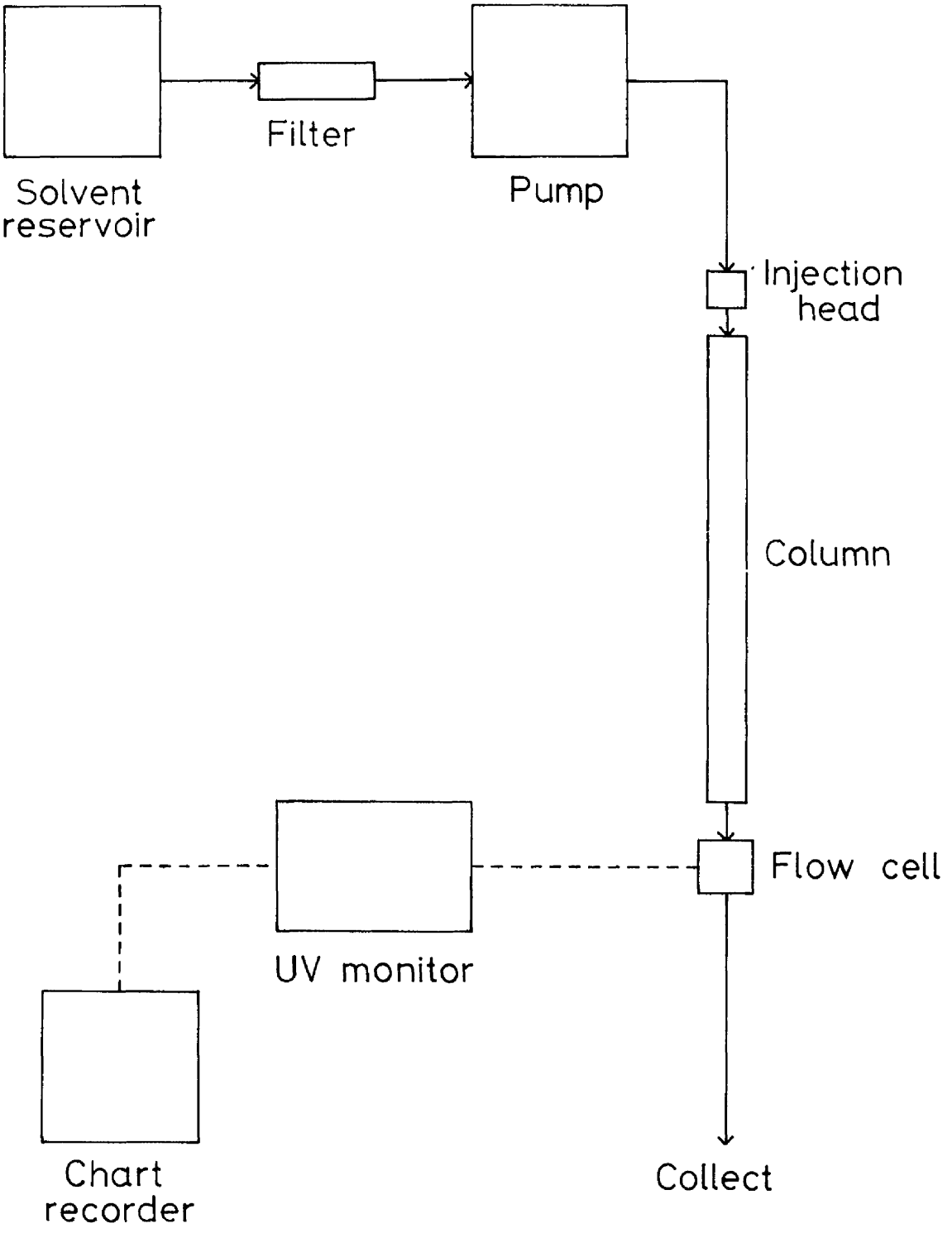
Chromatographic system

The system used was a conventional analytical high-performance liquid chromatograph, illustrated in Figure 6. It comprised a constant pressure pump which delivered the mobile phase to a column (4.6 x 500 mm) prepacked with 10 µm silica gel particles (Partisil 10). A flow rate of 1.5 $ml.min^{-1}$ was achieved using a pressure of approximately 600 p.s.i. The column eluate passed through a U.V. monitor (254 mm) fitted with a 10 µl flow cell. Samples up to 10 µl in volume, were dissolved in column eluate and injected directly onto the column, by interrupting the flow of solvent for a short time. The mobile phase was half saturated with water and thoroughly degassed before use to prevent bubble formation in the eluate.

Figure 6

The analytical high performance liquid chromatograph

(Adapted with kind permission, from Reeve and Crozier, 1978)


Solvent reservoir
Filter
Pump
Injection head
Column
Flow cell
UV monitor
Chart recorder
Collect

Selection of mobile phases for purification of sample extracts

The following procedure was adopted to devise suitable mobile phases for the purification of IAA in sample extracts. Firstly, a weak carrier solvent was selected which gave suitable retention values for IAA. Secondly, modifiers were added to this carrier solvent system to separate IAA from impurities in each of the different plant extracts.

The composition of the weak carrier solvent was determined using 'cold' IAA, methyl esterified as described previously (Section 2.3) and then purified by preparative HPLC. The retention on the silica adsorption column of IAA-Me, was monitored by the U.V. absorbance monitor of the analytical HPLC and solvent composition was altered accordingly. The solvent chosen as carrier was dichloromethane which was diluted initially with equal volumes of hexane to obtain a mobile phase of the appropriate strength. The k' value obtained for IAA-Me with this solvent was too high and was lowered, not by manipulation of the ratio of the two weak solvents of the mobile phase (which would have required changes of at least 25% relative to each other), but by the addition of a stronger solvent. A change of only 0.1% in the concentration of the modifier altered the k' value of IAA-Me. The first modifier used was dimethylsulphoxide which has a much higher E° value than the dichloromethane/hexane carrier (dichloromethane: pentane (50:50), has an E° value of 0.25, Snyder, 1971b). A suitable k' value for IAA-Me (Table 9) was obtained with 1% dimethylsulphoxide in dichloromethane:hexane (50:50).

In order to provide flexibility in purification of the different plant extracts, a second solvent system of differing selectivity was devised. Tetrahydrofuran was chosen as the second modifier and was used at different concentrations, first in dichloromethane alone, and then with dichloromethane diluted in hexane, until a suitable k' value for IAA-Me was obtained (Table 9).

The two mobile phase compositions developed using the modifying solvents, dimethylsulphoxide and tetrahydrofuran were:-

(1) 1% dimethylsulphoxide in dichloromethane:hexane (50:50)
(2) 1% tetrahydrofuran in dichloromethane:hexane (75:25)

Table 9

1AA-Me chromatographed on analytical HPLC. Retention (k' values) with mobile phases of different composition.

	Mobile phase		k' 1AA-Me
*	1% DMSO	50% CH_2Cl_2/50% hexane	2.3 - 2.6
	5% THF	CH_2Cl_2	0.53
	4% "	"	0.67
	3% "	"	0.72 - 0.78
	2.5% "	"	0.81 - 0.83
	1% "	"	1.3
*	1% "	75% CH_2Cl_2/25% hexane	2.1 - 2.4

(i) All the mobile phases were 50% saturated with water

(ii) * The two mobile phases used for purification of extracts.

(iii) There was a small variation in k' values of 1AA-Me in any particular mobile phase with time, but run-to-run variation was extremely small.

Chromatography of samples with analytical HPLC.

Endogenous 1AA levels in sample extracts, purified further by analytical HPLC, were estimated from U.V. absorbance of eluates (lower limit of detection at 254 nm 50 ng 1AA-Me). Calibration curves for U.V. absorbance of the 1AA-Me in the two mobile phase systems are shown in Figure 7 .

Small aliquots (1-2% and then 10%) of the extracts were chromatographed initially, using one of the above mobile phases. If the U.V. absorbance of the eluate showed the 1AA-Me region to be clear of impurity, the remainder of the extract was chromatographed and the

Figure 7

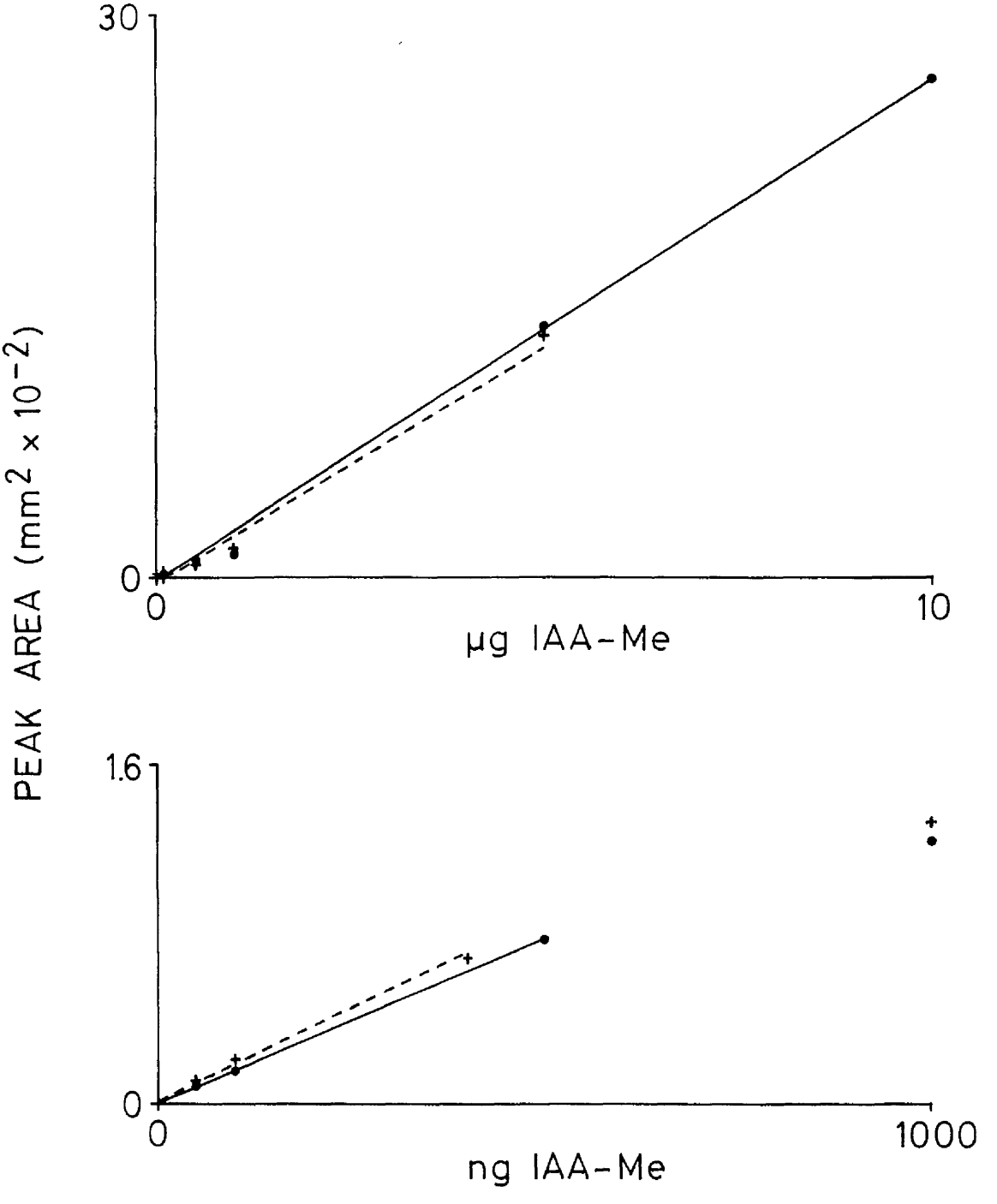
Calibration curve of U.V. detector response at 254 nm to IAA-Me chromatographed separately on an analytical high performance liquid chromatograph using two mobile phases of different composition.

●——● mobile phase composed of 1% dimethyl-sulphoxide in dichloromethane: hexane (50:50).

×------× mobile phase composed of 1% tetrahydrofuran in dichloromethane:hexane (75:25).

Column:- Partisil 10 (4.6 x 500 mm.)
Column temperature:- ambient
Mobile phase flow rate:- 1.5 ml. min^{-1}

IAA-Me standards:- 10 µg IAA-Me.$\mu\ell^{-1}$ methanol
5 µg " " "


30
0
0
10
µg IAA-Me
PEAK AREA (mm^2 x 10^{-2})
1.6
0
0
1000
ng IAA-Me

eluate in the region of IAA-Me collected. Standards of IAA-Me were run before and after each purification run, to allow accurate collection of the IAA-Me region. From the calibration curve and U.V. absorbance of eluate, the levels of presumptive IAA-Me in each extract were estimated. In later work, radioactive IAA was added to the extracts at the beginning of the extraction procedure, as an internal standard, and recovery of IAA was estimated by counting of an aliquot of the presumptive IAA-Me peak.

The mobile phase containing 1% dimethylsulphoxide as modifier, was found to be most suited for the purification of Vicia faba nodule and root extracts (Figure 8) and the mobile phase containing 1% tetrahydrofuran as modifier, to be suitable for the purification of Alnus glutinosa nodule and root extracts (Figure 8). In Myrica gale root extracts, it was found that with both mobile phases, the IAA-Me region either just preceeded, or was on the tail of, a large impurity peak.

Figure 8

Analytical high performance liquid chromatograms showing the U.V. absorbance and radioactivity of eluates of methylated IAA and of methylated root nodule and root extracts of Alnus glutinosa and Vicia faba.

Alnus glutinosa. Extracts chromatographed using a mobile phase of composition:- 1% dimethylsulphoxide in dichloromethane:hexane (50:50).

Vicia faba. Extracts chromatographed using a mobile phase of composition:- 1% tetrahydrofuran in dichloromethane:hexane (75:25).

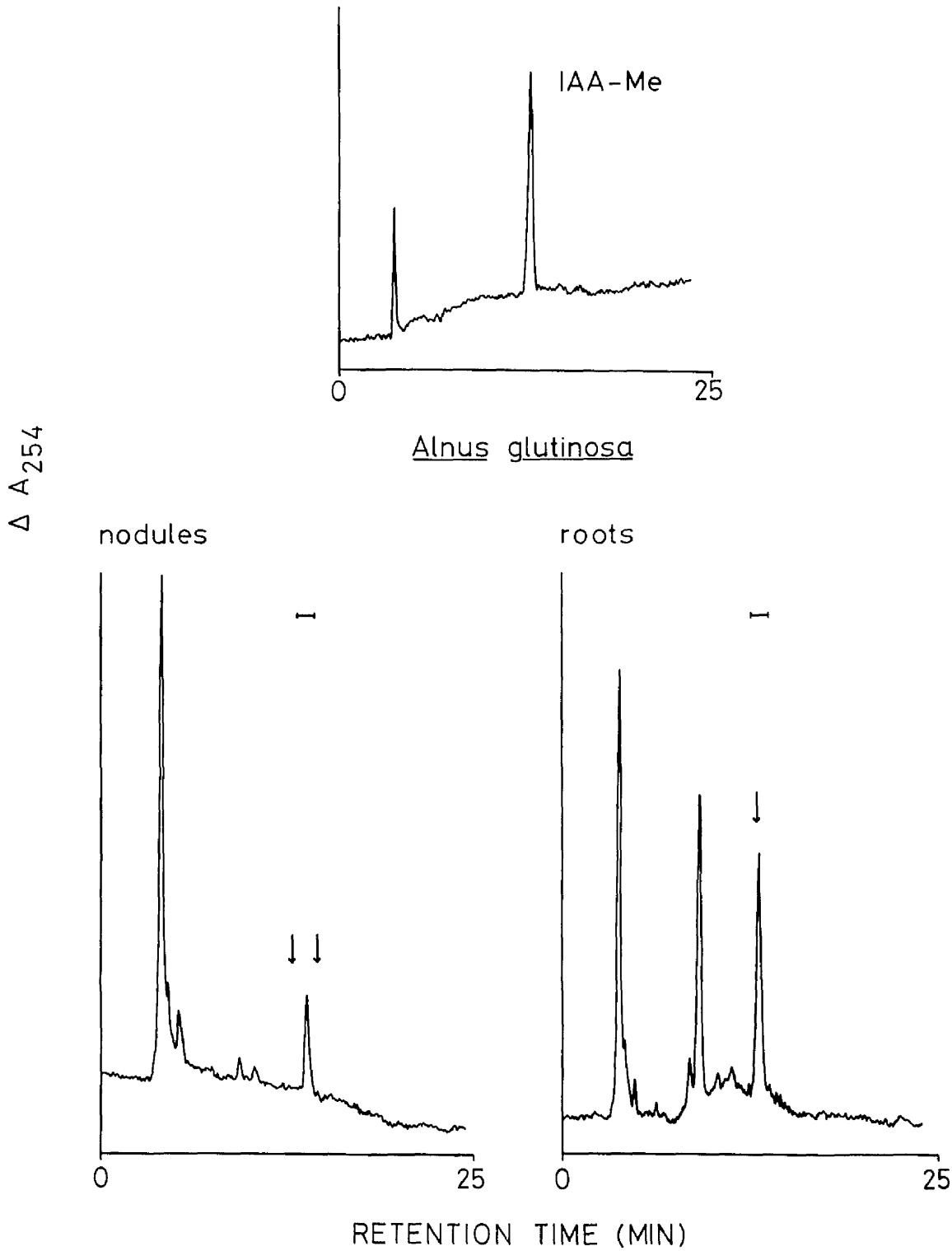
Arrows represent the elution point of methylated IAA determined immediately before purification of each extract.

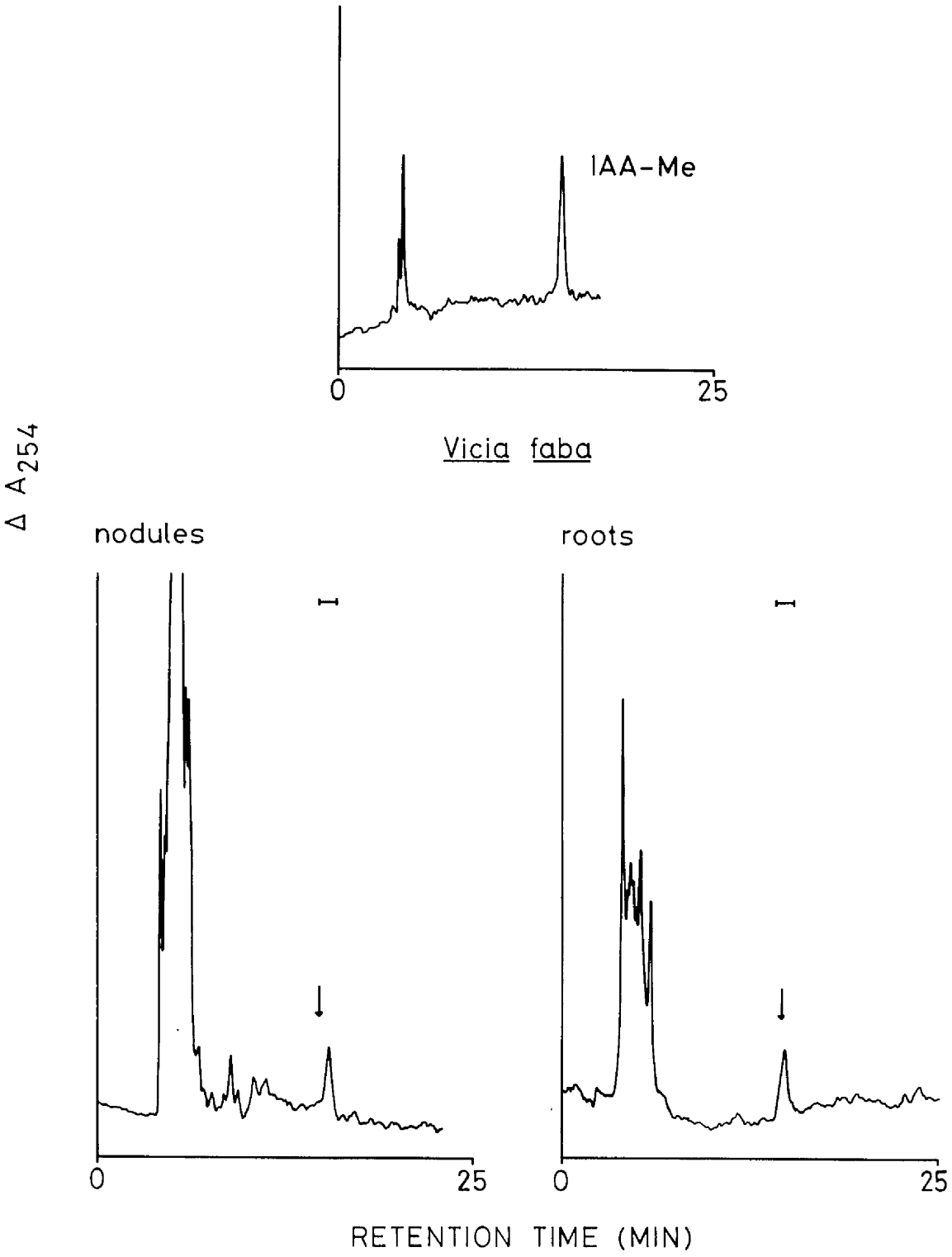
Bars indicate volume of eluate collected.

Column:- Partisil 10 (4.6 x 500 mm.)

Column temperature:- ambient

Mobile phase flow rate:- 1.5 ml. min^{-1}.


IAA-Me
0
25
Alnus glutinosa
Δ A254
nodules
roots
I
I
0
25
0
25
RETENTION TIME (MIN)


IAA-Me
0
25
Vicia faba
Δ A254
nodules
roots
I
I
0
25
0
25
RETENTION TIME (MIN)

2.5 Summary of purification procedures developed for the extraction of endogenous IAA from Alnus glutinosa and Vicia faba root nodules and roots.

Tissue homogenised at high speed (30 sec) in 80% aqueous methanol

↓

1 -^{14}C-IAA added

↓

Extract 16 hours at 4°C (dark)

↓

Buchner filtration

↓

Reduce volume aqueous phase

↓

Slurry with PVP in 0.1 MK_2 HPO_4 at pH8

↓

Filter

↓

Partition 3 times against freshly redistilled diethyl ether at pH3.

↓

GPC

(a) Collect 450-650 ml. in 10 ml. fractions

(b) Take 50µl aliquots for counting

(c) Bulk 530-590 ml (or according to internal marker)

↓

(contd.)

(contd.)

Preparative HPLC

(a) Run extract as free acid in 40-60% ethyl acetate in hexane in 20 minutes

(i) run aliquot (e.g. 2%, 6%)

(ii) run rest, splitting off aliquot to monitor, and collect relevant portion of eluate.

(b) Methylate extract.

Run as methyl ester in 30% ethyl acetate in hexane

(i) run aliquot (e.g. 2%, 6%)

(ii) run rest, splitting off aliquot to monitor, and collect relevant portion of eluate.

Analytical HPLC

(a) (i) Run standard IAA-Me using mobile phase A - *Alnus glutinosa*
B - *Vicia faba*

(ii) run aliquot of extract as a check

(iii) run standard IAA-Me

(iv) run rest of extract and collect relevant portion of eluate.

(b) Measure peak on U.V. absorbance trace at IAA-Me elution point.

(c) Take aliquot of collected peak, count and estimate recovery.

(d) Store sample over silica gel at $-18^{o}C$.

N.B. A - 1% dimethylsulphoxide in dichloromethane:hexane (50:50).
B - 1% tetrahydrofuran in dichloromethane:hexane (75:25)
Both mobile phases 50% saturated with water.

2.6 Results

(a) Estimation of recovery using the purification procedures developed for endogenous IAA.

Addition of 1-^{14}C-IAA as an internal standard to root nodule and root homogenates of Alnus glutinosa prior to overnight homogenisation, allowed losses to be monitored at various stages in the purification process. Final recoveries of IAA were 1-2% (Table 10).

Table 10

Recovery (% of initial radioactivity) at various stages during purification of extracts.

Purification step	% recovery of initial radioactivity	
	Alnus nodule extract	Alnus root extract
In aqueous filtrate after PVP treatment	58.5	-
In ether phase partitioned from aqueous PVP treated filtrate	48.1	41.5
In GPC column eluate at IAA marker position	27.8	28.3
In IAA-Me peak eluted from analytical HPLC	1.1	1.8

Radioactivity recovered after analytical HPLC = 1.6 - 3.2 x 10^4 d.p.m.

The recovery data, while of direct relevance to these particular extractions, serve to indicate the levels of recovery expected with nodulated root extracts of Alnus glutinosa using these purification procedures.

(b) Estimations of endogenous IAA levels

Results for endogenous IAA levels in root nodule and root extracts, estimated from the U.V. absorbance of the presumptive IAA-Me in analytical HPLC eluates, are shown in Table 11.

The levels of IAA extracted in the form of the free acid under the conditions described, are higher in the root nodules than in the parent roots (in Alnus glutinosa approximately 4 times and in Vicia faba extracts 5 times higher, Table 11). The values for Alnus glutinosa root extracts were very similar in experiments 1 and 2 (62 and 61 µg IAA-Me/kg. fr. wt. tissue, respectively).

Table 11

IAA levels in Alnus glutinosa and Vicia faba extracts. Levels measured by U.V. absorbance of analytical HPLC eluates at IAA-Me elution point.

Sample	Sample size - Fresh wt. equivalents (g)	Analytical HPLC U.V. absorbance µg IAA-Me/kg. fr.wt.tissue
Alnus glutinosa		
1. Nodules	170	230
Parental roots	770	62
2. Parental roots	250	61 (1)
Vicia faba		
1. Nodules	61	540 (6)
Parental roots	200	120 (2)

(i) Values in the table are corrected for loss. In Alnus glutinosa Sample 2, and in the Vicia faba sample, corrections for loss were made using recovery data from Alnus glutinosa, sample 1.

(ii) Figures in parentheses are values for IAA-Me/kg.fr.wt.tissue as measured by U.V. absorbance when uncorrected for loss.

3. DISCUSSION

Published estimates of IAA levels in nodulated roots of the species used in these investigations, have all been based on bioassays (except for one study, when spectrofluorimetry was also used). Classical purification procedures (methanol extraction, solvent partition and paper chromatography, with the addition of a DEAE-cellulose column chromatography step for *Vicia faba* roots) were used to separate IAA from other compounds present in tissue extracts. The reproducibility of some of these early estimates was re-examined in the initial work reported here. While *Alnus glutinosa* was the species of primary interest, *Vicia faba* was included for comparison, since the auxin content of the nodules is unknown and it was thought that the apparently lower phenol content of the root system might produce extracts giving less interference in bioassays than the highly phenolic extracts of alder. In contrast, nodules of *Myrica gale*, which were also analysed, although of high phenol content, have been reported not to contain detectable auxin (Silver *et al.*, 1966). Using a classical extraction and purification technique similar to that just described, but with the addition of a PVP column chromatography step to reduce the phenolic content of the extracts, bioassay of *Vicia faba* root extracts (Table 5b) suggested levels of IAA (0-2 µg IAA equivalents/kg.fr.wt.) similar to those reported by Burnett *et al.* (1965) for non-nodulated roots (2-5 µg IAA equivalents/kg.fr.wt.). There are no published values for auxin levels in root nodules of this species, although the levels obtained here by bioassay were higher than those for the roots.

Bioassay results of *Alnus glutinosa* extracts, were the least consistent of the three species studied, possibly reflecting interference from their high content of phenolic compounds. Even after allowance for 70% losses during extraction, when detected, values for root nodule IAA levels were approximately 15-50 times lower for 'Peralite' grown seedlings and 5-20 times lower for the one estimate of field material, than the values obtained by Dullaart (1970), by bioassay and spectrofluorimetry. Although Dullaart obtained significant results from very much smaller samples of tissue (4-5 g fr.wt.) than those assayed in the present studies (30 g fr. wt.), the bioassay was

sensitive to the same level of IAA (about 0.25 μg). Differences in estimates of IAA levels might arise in part from the use of plant material from different sources. Dullaart (1970) always extracted field material, whereas in the investigations reported here, all plants were grown in the glasshouse, in 'Peralite', with the exception of one nodule sample from the field which gave levels closer to, although still much lower than, those obtained by Dullaart. Considerable variability in the hormone levels of field material, collected from different sample sites is to be expected, however, since a variety of environmental and biotic factors could influence hormone accretion by the tissue. For example, the secretion of growth substances, including IAA, by mycorrhizal fungi (Slankis, 1973) might differ between soils and contribute to differences in IAA levels of nodulated root extracts from different localities. The higher levels of IAA extracted from nodules and roots of all three species when these were grown in or harvested from the field, conceivably could be due to contributions from rhizosphere or mycorrhizal micro-organisms.

The absence of significant biological activity from the IAA zone of chromatograms of extracts of nodules from glass-house grown Myrica gale agreed qualitatively with Silver et al.(1966) for Myrica cerifera. However, while these authors were unable to detect significant amounts of auxin in extracts of small, secondary field roots of Myrica cerifera (sample size 40 g fr.wt.), in the present work significant auxin activity of 1-5 μg IAA equivalents/kg. fr. wt. Myrica gale roots was detected in samples of similar size. Silver et al. (1966) reported very high auxin activity (10 mg IAA equivalents/kg.fr.wt.) in non-nodulated young seedling roots ('Peralite' grown) which was about 10^3 the value obtained here, even after taking into account correction for loss. However, as discussed by Dullaart (1970), these very high levels were probably due to the low sensitivity of their bioassay procedure (1.5 mg IAA equivalents/kg.fr.wt.).

The special characteristic of bioassays is their ability to detect biological activity, for which reason they can be of considerable value in the initial analysis of extracts of unknown composition. When the occurrence of the compound of interest, for example in the present work, IAA, is indicated by bioassay, physico-chemical characteristics, for example GC-MS(S.I.M.) can be used for detection

and quantification, to avoid the variability inherent in the use of biological material, as in a bioassay. Several reports of the use of GC-MS to measure IAA levels in relatively crude extracts of plant tissue have been published (see Introduction). Extracts for analysis were obtained using only a partition step, or with the addition of a paper chromatography or TLC step before derivatisation and subsequent GC-MS (Greenwood et al., 1972; Rivier and Pilet, 1974; Caruso et al., 1978; Allen et al., 1979) although some later workers included DEAE-cellulose chromatography (Bridges et al., 1973; Elliott and Greenwood, 1974; Hall and Medlow, 1974; Hillman et al., 1977). In this study, the results obtained with nodulated root extracts, subjected to purification techniques similar to those of the above authors, were not satisfactory (up to 80-fold differences between similar extracts, Table 5a,b). Inconsistency in the IAA levels indicated by GC-MS (S.I.M.) analyses, could have resulted partly from run-to-run variation, which may have involved drift in MS response or inaccuracies in the volume of sample injected. External standardisation of losses was employed in these early studies but much more reliable estimates of variability could have been obtained by the use of an internal standard, such as radioactively-labelled IAA, which was incorporated in later work. An additional source of inconsistency in the analyses, was the impurity of the extracts. Other workers, using GC-MS with minimal preliminary purification, have reported that high backgrounds caused by impurities either made it impossible to obtain mass spectra from the low quantities of IAA present (Rivier and Pilet, 1974), or contaminated the spectra (Allen et al., 1979).

Although GC-MS in the S.I.M. and M.I.M. modes is potentially capable of detecting very small amounts of IAA (S.I.M., 1-5 ng, Hillman et al., 1977; M.I.M., 0.175 - 1 ng, Caruso et al., 1978 and Allen et al., 1979 respectively), just as the auxin bioassay is also very sensitive (Avena coleoptile straight growth test, 2×10^{-5} to 6×10^{-8} M IAA), the potential capacity of both techniques is greatly decreased by the presence of impurities, which interfere with the accuracy and precision of the estimations. The problems encountered in this study are in accordance with the difficulties experienced by the above authors, and GLC of the extracts (Figure 1) suggested that

one of the main sources of variability was indeed the impurity of nodulated root extracts. It was not possible to obtain mass spectra of IAA from these nodulated root extracts, necessary if GC-MS in the S.I.M. mode is to be reliable. Advantage could not be taken of the greater reliability of M.I.M. because the relative abundance of the two ions monitored (m/e 202, 319) differed from those in mass spectra of standards.

Further effort in this project was directed, therefore, towards producing extracts of high purity from the plant species under investigation, so that endogenous IAA levels could be estimated more accurately. After the initial methanol extraction, PVP slurry and ether partition, additional purification methods were introduced based on GPC and HPLC techniques. The value of GPC for purification purposes has been mentioned previously (Section 2.2), although the method is not widely used in plant purification procedures, except as Sephadex chromatography. The techniques described here using HPLC for the analysis of IAA were developed prior to the publication of reports describing its use in IAA purification. Recent reports describing the use of HPLC in the analysis of plant tissue for IAA, have used different separatory mechanisms to those described here, for example, reverse-phase silica gel partition chromatography (Durley, et al., 1978) and ion exchange chomatography (Sweetser and Schwartzfager 1978). In both these studies IAA was measured in the presence of impurities which caused problems either in the confirmation of the identity of IAA or in the loss of detector response. It is likely that more extensive preliminary purification using a greater variety of separation mechanisms, would have improved greatly the effectivity of these techniques and the reliability of the estimates.

The addition of the purification steps developed in this project and summarised in Section 2.5, proved successful in the purification of Alnus glutinosa and Vicia faba extracts, as shown by the progressive reduction in U.V.-absorbing compounds in Figures 4, 5 and 8. With Myrica gale extracts, the additional purification procedures used did not achieve satisfactory separation of IAA from other impurities as described in Section 2.4.; it will be necessary to devise a third mobile phase for the analytical HPLC stage to effect further separation in the IAA-Me region (for example, acetonitrile could be used as a

modifier for the mobile phase, dichloromethane, either diluted or not with hexane).

Because of the considerable time expended in the development of satisfactory purification procedures, only a few results were obtained by U.V. absorbance on analytical HPLC for endogenous IAA levels (Table 11). Those obtained for Alnus glutinosa were still lower (1.5-6.0 times for nodules and 1.5 - 3.5 times for roots) than published data (Dullaart, 1970). There are no published data for root nodules of Vicia faba, but the value suggested previously by bioassay of extracts of non-nodulated Vicia faba roots (Burnett et al., 1965) is much lower (20-60 times) than was found here by U.V. absorbance although similar to that obtained by bioassay. GLC of Vicia faba root extracts showed the presence of high levels of impurities (Figure 1) and it is possible that in both the work of Burnett and the present studies, bioassay activity might have been reduced due to the presence of inhibitors in the impure extracts.

The detection of a U.V. absorbing peak corresponding to the retention time of IAA-Me by means of analytical HPLC (Figure 8) after seven increasingly selective purification stages is not conclusive proof that the peak is IAA-Me. However, additional supportive evidence was provided by co-chromatography with radioactivity derived from 1-^{14}C-IAA, added at the beginning of the extraction procedure and persisting through the separation of the IAA region of the eluate in four separate chromatographic runs. This included two runs in which the free carboxylic acids in the sample were converted to their methyl esters. The probability of correct identification as IAA of the U.V. absorbing peak on HPLC is greatly enhanced by co-chromatography with the radioactive internal marker during a wide variety of different separatory procedures-solvent partitioning, gel permeation chromatography, straight-phase liquid partition chromatography on both non-derivatised and derivatised extracts and liquid adsorption chromatography.

The recovery data obtained during this project, showed that on completion of sample purification culminating in analytical HPLC, between 1-2% of the original IAA present was recovered. Recovery data from the preliminary purification procedures indicated that even between

methanol extraction and ether partition, up to 30% of the IAA could be lost. Mann and Jaworski (1970) also found loss of IAA at every stage during their relatively straightforward extraction and purification procedures; very small amounts of IAA (12 µg) added to sample extracts could be lost altogether. The large losses of IAA which occur during extraction and purification show the importance of standardisation of techniques to allow some correction for loss. Recovery data based on external standardisation procedures can be misleading. For example, in this project recoveries from PVP columns measured using standards were 66-82%, whereas when these were incorporated in samples, recoveries were 43-44%. Reports of erratic losses of IAA during purification (10-70% recoveries of IAA, Bandurski and Schulze, 1977; five-fold differences in recovery of IAA, Little *et al.*, 1978) show the importance, for accurate and precise results, of using internal standards to monitor losses.

Apart from the difficulties of correction for loss during purification, inaccuracies in estimation of free IAA levels might also arise due to the natural occurrence of IAA derivatives (for example, methyl esters, Takahashi *et al.*, 1975), and to the breakdown of indole compounds (for example, Atsumi *et al.*, (1976) advocated measures to avoid conversion of indole-3-pyruvic acid (IPyA) in IPyA rich tissues such as senescent cultured tobacco cells). Hydrolysis of IAA conjugates can also occur. A detailed knowledge of all indole compounds present in an extract would be necessary to reduce errors which might arise from such sources.

Even when it is felt that reliable estimates of IAA levels in a particular tissue have been made, the interpretation of the physiological significance of such estimates is likely to pose numerous difficulties, and the values for the levels of IAA obtained must be viewed with several possibilities in mind:-

(1) Very small changes of IAA levels within a pool of free IAA, may be highly significant in the functioning of the tissue, but may not be detectable within the large mass of tissue extracted. Nodules of *Myrica gale* contain no detectable IAA, so either a very low but physiologically significant level is present, or the hormone is not essential to the functioning of the nodule. The presence of relatively high IAA levels in some root nodule extracts, compared to the parent

roots, could indicate that turnover is slow in the former tissues and that the high levels have no special significance. Alternatively, IAA extracted from parts of the nodules or nodule cells, where normally it may be physiologically unavailable, could contribute to the high levels detected in extracts.

(2) IAA may normally be present in other than a free form in the nodule. In the radiotracer experiments described in Part II of this work, much of the extracted radioactivity partitioning into the aqueous phase may have been due to conjugate formation. Auxin activity, other than IAA, was also found in the bioassay determinations of the acidic ether fraction of both root nodules and roots.

During the course of this present study, it has become obvious that to estimate accurately, and to assess the significance or otherwise of IAA levels in root nodules, it will be necessary to investigate the full auxin complement of the tissue, including any IAA conjugates. Once this has been achieved, correlation of nodule auxin with physiological effects in the host plant, will only be achieved with analytical techniques suitable for quantitative work on much smaller amounts of tissue than those at present necessary. The development and inclusion of techniques to reduce losses of auxin during purification, would help to alleviate this difficulty and also reduce the errors introduced during correction for loss. A useful, alternative approach to analysis of hormone levels in the assessment of the physiological role of auxin in the nodule, might be to use radioactive tracers in studies of IAA pool sizes and turnover kinetics, which could help to indicate the location and rate of IAA metabolism. A major drawback to this approach is the introduction of experimental artefacts due to tissue damage during application of the radiotracer. Preliminary radioactive tracer studies on the fate of IAA applied to nodulated Alnus glutinosa have been conducted, and are described in Part II of this project.

PART II. MOBILITY OF IAA IN ALNUS GLUTINOSA

1. GENERAL METHODS

Application of radioactive IAA and extraction of plant material

Radioactively-labelled IAA (1-^{14}C-IAA, 50-57 mCi/m mol: The Radiochemical Centre. 5-^{3}H-IAA, 23.5-25 Ci/m mol: C.E.A., Gif-sur-Yvette, France) was applied, with minimum damage, to the appropriate plant organ in fine capillary tubes (tip - 0.2mm diameter) drawn from Pasteur pipettes. Prior to use, TLC of 5-^{3}H-IAA revealed an impurity at R_{IAA} 1.5 -1.7 which constituted 11-19% of the total radioactivity (Figure 9 A). The impurity may result from breakdown of IAA during TLC since it was not removed by elution of the IAA peak and rechromatography, and using preparative HPLC the impurity peaks were either smaller or not present (Figure 9 B). At harvest, the plants were divided into parts, weighed and extracted by shaking in 80% aqueous methanol for 12-24 hours, at 4°C in the dark. Extracts were filtered, residues washed twice with aqueous methanol and the combined extracts reduced to the aqueous phase, which was partitioned three times against freshly distilled diethyl ether at pH3. In some experiments, the extract was initially partitioned with diethyl ether at pH8. The ether was dried by freezing overnight followed by filtration, and the filtrate, taken up in a small volume of redistilled methanol was used for further analysis. Aliquots taken at suitable stages during the extraction procedure, were dried and eluted before measurement of recovery of radioactivity.

Solid sample counting for radioactivity

Aliquots (0.1 ml) of extract were dried on stainless steel planchettes under an infra-red lamp, and radioactivity levels were measured with a solid sample gas flow counter (Nuclear Chicago), operated with a gas flow of 2% propane in argon at 7 p.s.i.

Thin-layer radiochromatography

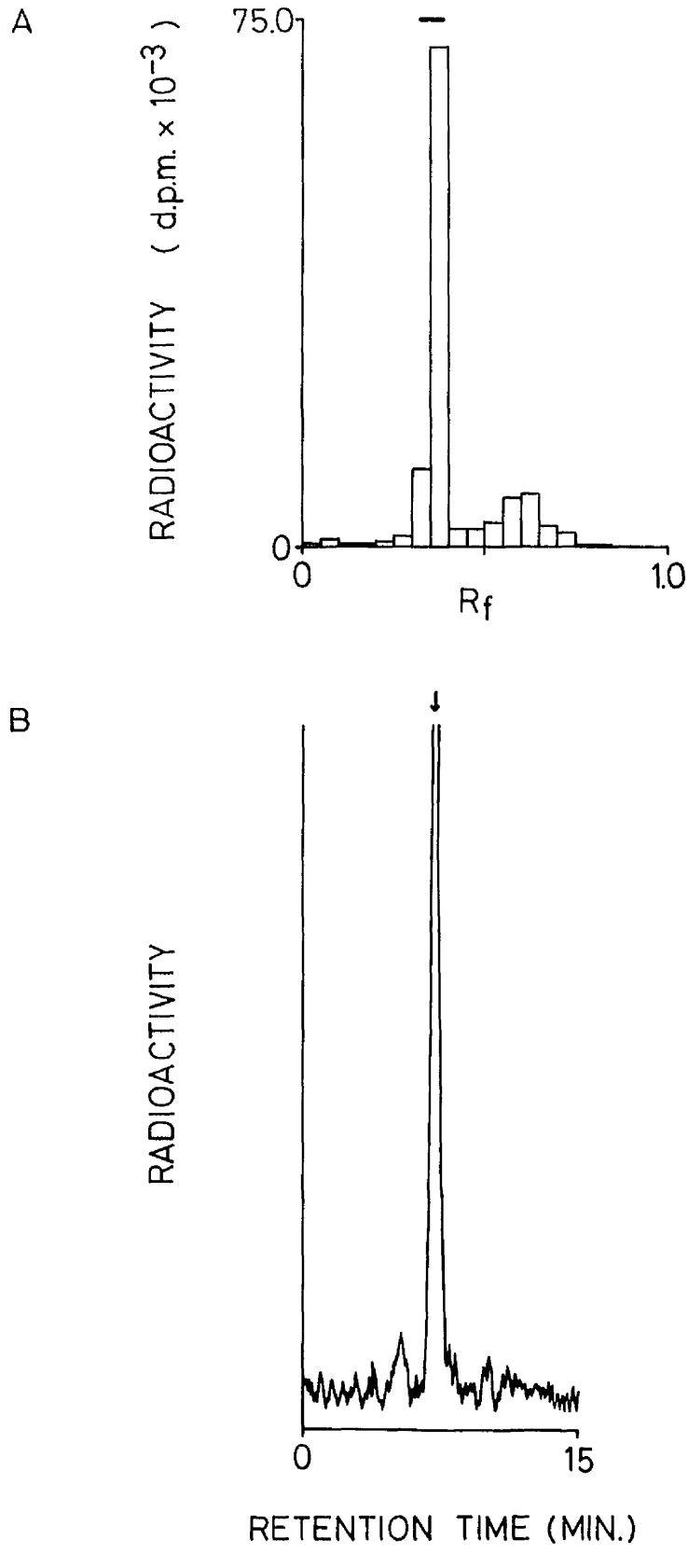
Extracts, together with IAA markers, were chromatographed on

Figure 9

A. Thin-layer radiochromatogram of the stock solution of 5-^{3}H-IAA.
Solvent system:- isopropanol:ammonia:water:: 8:1:1 v|v.

B. Preparative high performance liquid radiochromatogram of the stock solution of 5-^{3}H-IAA (3×10^{4} d.p.m.)

Column:- Partisil 20
Column temperature:- $30 \pm 0.5^{\circ}C$
Stationary phase:- 0.5 M formic acid
Mobile phase:- 60% ethyl acetate in hexane
Flow rate:- mobile phase 4.6 ± 0.1 ml. min^{-1}.
scintillant 2.0 ± 0.1 ml. min^{-1}.


A
75.0
RADIOACTIVITY (d.p.m. × 10⁻³)
0
0
1.0
Rf
B
RADIOACTIVITY
0
15
RETENTION TIME (MIN.)

silica gel plates (0.25mm thick with fluorescent indicator UV 254; Camlab, Cambridge) developed with isopropanol: ammonia: water (8:1:1). The chromatograms were divided into 20, 0.75 cm sections which were scraped into separate plastic scintillation vial inserts. After elution with 0.5 ml methanol for not less than one hour, 5 ml scintillation fluid was added (0.4% w/v PPO, 0.2% w/v dimethyl POPOP in redistilled toluene) and the radioactivity assessed in a liquid scintillation counter (ICN-Tracerlab Comumatic 200). Counts were corrected for background and also for quenching with a quench correction curve, constructed using radioactive hexadecane standards (The Radiochemical Centre, Amersham) and plant extracts, and utilising the automatic external standard channels ratio. The external standard gain, threshold and window settings were checked immediately before each set of counts, to correct for drift.

Liquid scintillation sample oxidation

The residue remaining after aqueous methanol extraction, was dried in an oven for 48 h at 70°C, ground in a mortar and pestle, and either the whole sample, or the maximum permitted aliquot combusted to water and carbon dioxide in a liquid scintillation sample oxidiser (Intertechnique IN4101). For ^{14}C-labelled samples, the scintillant comprised 0.7% w/v PPO in toluene: phenylethylamine: methanol: water (40:33:22:5), and for tritium-labelled samples, 0.5% w/v PPO and 0.03% w/v POPOP in dioxane: toluene (70:30). The wash liquid used after each set of samples and between high and low activity samples, comprised methanol: toluene, 3:2. A memory of 1-4% was associated with the sample oxidiser operation, so that for every group of samples (about 3) with similar activity, a blank sample was run to determine the memory applicable to the next group of samples. As an added precaution, the samples were oxidised in ascending order of activity.

Preparative HPLC of radioactive extracts.

The preparative HPLC system, which was used for the analysis of endogenous IAA in *Alnus glutinosa*, *Vicia faba* and *Myrica gale* root

nodules and roots, adapted from Reeve et al (1976), is described in Part I, Section 2.3. Aliquots of acidic ether fractions, of activity 1 x 10^5 d.p.m. in the case of tritium label, were dissolved in 330 µl column eluate and applied to the column via the sample loop. Cold IAA (60-100 µg) and benzene (1µl) were added to each sample to act as reference points for the radioactive metabolites, benzene being eluted at the solvent front. The sample was eluted with a gradient completed within 40 minutes, of 50-75% ethyl acetate in hexane. The gradient was extended to 100% ethyl acetate when necessary to allow elution of all the radioactivity.

2. MOBILITY OF RADIOACTIVITY FOLLOWING APPLICATION OF 1-^{14}C-IAA to SHOOT TIPS AND TO ROOT NODULES

This experiment was performed in a controlled environment at a temperature of 26°C and a photoperiod of 16 hours. Approximately 2.5 µCi 1-^{14}C-IAA were applied to each plant in 5 µl ethanol (i.e. 5 x 10^{-5} m mol IAA), uptake being completed within about 2 hours. Micropipettes were inserted into the following tissues:-

(a) the apical bud
(b) the top of the stem after removal of the apical bud
(c) a single root nodule or one lobe of a nodule cluster.

For each of (a) and (b), two plants were harvested 24, 48 and 72 hours after application of the label. For (c), two plants were harvested after 24 hours only. The separated parts were extracted in 70% methanol for 2 days at room temperature; aliquots were then counted on a solid sample gas flow counter.

RESULTS

Twenty-four hours after the application of label to the shoot, most radioactivity (62-94%) remained close to the site of application, irrespective of whether label was fed to the apical bud or into the top of the stem after the apical bud had been removed (Table 12 a,b). Very little of the extracted radioactivity was present in the roots

Table 12

Relative distribution of methanol soluble radioactivity. Proportion of radioactivity in separate plant parts as percentage of whole plant (sum of methanol soluble radioactivity in each plant part). 1-^{14}C-IAA applied to different plant organs and plants harvested at various times.

(a) Apical bud

Plant part	% of whole plant methanol soluble radioactivity $\left(\frac{\text{Plant part d.p.m.} \times 100}{\text{Combined plant part d.p.m.}}\right)$					
	24h		48h		72h	
	A	B	A	B	A	B
Fed apical bud	93.5	88.7	86.4	84.6	65.8	76.8
Stem	4.2	5.3	9.1	9.8	31.7	12.6
Leaves	0.9	2.0	3.1	4.6	1.7	7.1
Axillary buds	1.0	0.7	0.2	0.1	0.3	0.8
Roots	1.1	1.1	0.9	0.8	0.4	2.3
Nodules	0.08	0.06	0.2	0.2	0.06	0.5

(b) Stem apex

Plant part	24h		48h		72h	
	A	B	A	B	A	B
Fed stem apex	92.5	62.2	92.6	87.8	82.5	69.6
Stem	3.7	3.6	1.3	6.1	13.7	19.5
Leaves	3.1	31.5	5.1	4.9	3.2	8.5
Axillary buds	0.06	1.1	0.4	0.3	0.5	0.8
Roots	0.7	1.4	0.2	0.8	0.1	0.8
Nodules	0.04	0.06	0.4	0.01	0.06	0.7

(c) Nodule

Plant part	24h	
	A	B
Fed nodule	39.2	84.3
Stem	2.9	1.3
Leaves	2.7	1.1
Axillary buds	0.2	0.3
Roots	54.1	11.7
Nodules	0.3	1.0
Apical bud	0.6	0.3

(i) Sum of methanol soluble radioactivity extracted from each plant part = 1.0 - 8.0 x 10^5 c.p.m.

(0.1-2.3%) or the nodules (0.01-0.7%). However, in one plant, substantial radioactivity (32%) was present in the leaves. There was also little movement, in this case outside the root system, following application of labelled IAA to a nodule (Table 12 c). After 24 hours, radioactivity in the fed nodule comprised 39-84% of the total extracted radioactivity, 12-54% was present in the roots and 0.3-1.0% was detected in other nodules. Only 3.0-6.4% was found in the shoots.

A considerable proportion (66-83%) of the extracted label was present in the fed organ, even 72 hours after application of 1-^{14}C-IAA to the apical bud and stem (Table 12 a,b). Although the proportion of radioactivity extracted from the stem increased with time (from 4-5% to 13-32%), the radioactivity of the nodules and roots showed little change.

3. MOBILITY OF 5-^{3}H-IAA

3.1 Application to apical buds

Glasshouse-grown plants were transferred to a controlled environment cabinet (19°C, 16h photoperiod; 15°C, 8h dark period) 12 days prior to the experiment. Micropipettes containing 6 μCi 5-^{3}H-IAA (i.e. 2.1 x 10^{-7} mmol IAA) in 6 μl 20% aqueous ethanol were applied to the base of the apical buds of 6 plants. Uptake was completed within 4 hours and the plants left for a further 24 hours. The 6 plants were then harvested in pairs and divided into parts which were weighed, extracted into 80% aqueous methanol overnight at room temperature, partitioned into ether and analysed by thin-layer radiochromatography as described previously (see Section 1).

RESULTS

Although methanol soluble radioactivity was detected in all parts of the plant except in the lower leaves, most of the label remained in the fed apical bud (70-80%) or was present in the stem (18-29%) where it may have been located in the upper region adjacent

to the apical bud. The remaining radioactivity, which comprised about 1% of the total extracted into 80% methanol, was distributed mainly between the top 2 leaves and the roots with only 0.02 - 0.05% being detected in the nodules (Table 13).

Retention of radioactivity in the fed apical bud and adjacent tissues, is reflected in the high specific activities of the apical bud and stem. The nodules and roots showed similar specific activity which was very much lower than the fed tissues and about 30-120 times below that of the stem.

When the methanolic extracts were partitioned against ether at pH3, 55-74% of the total radioactivity extracted remained in the aqueous fraction (Table 14). Variation in the proportions of radioactivity which remained in the aqueous fraction of different plant parts may reflect differences in metabolism of the label (Table 15 and Appendix Table 1). The leaves contained the highest proportion (82-100%) of radioactivity in the aqueous fraction, followed by the fed apical bud (69-82%). Much lower levels of radioactivity remained in the aqueous fraction of the nodules (20-64%), stem (21-43%) and roots (22-34%).

The acidic ether fraction accounted for 26-46% of the total radioactivity recovered from each plant (Table 16). Of this, the fed apical bud (48-57%) and the stem (41-51%) contained most of the label and only 0.03-0.09% was located in the nodules and 0.8-1.3% in the roots (Table 16). The high specific radioactivities of the fed apical bud and the stem, again indicate retention of isotope in the fed and adjacent tissues (Table 16). The specific activity of the roots and nodules was similar but up to 140 times lower than that of the stem.

TLC of the stock 5-^{3}H-IAA (Figure 9 A) showed a main peak of radioactivity which co-chromatographed with the 'cold' IAA marker and in addition a small peak near the solvent front. Chromatograms of most of the acidic ether extracts showed that the R_F of the major zones of radioactivity corresponded with those of the stock 5-^{3}H-IAA, and that the position of the 'cold' IAA marker, which was co-chromatographed with each extract, was similar to that of the main peak of radioactivity (Figure 10). The nodules were a notable exception: in two extracts (B and C) the main peak of radioactivity did not

Table 13

Relative distribution and specific activity of methanol soluble radioactivity. Proportion of methanol soluble radioactivity in separate plant parts as percentage of whole plant (sum of methanol soluble radioactivity in each plant part).

Plant part	Methanol soluble radioactivity					
	% of whole plant $\left(\frac{\text{Plant part d.p.m. x 100}}{\text{Combined plant part d.p.m.}}\right)$			Specific activity $\left(\frac{\text{d.p.m./g. fresh weight tissue}}{\text{x } 10^{-5}}\right)$		
	A	B	C	A	B	C
Fed apical bud	80.3	69.6	80.0	630	270	480
Stem	18.3	29.0	19.4	3.2	3.6	3.2
Top 2 leaves	1.0	0.7	0.3	0.6	0.3	0.2
Lower leaves*	-	-	-	-	-	-
Roots	0.4	0.6	0.3	0.05	0.05	0.03
Nodules	0.05	0.03	0.02	0.1	0.03	0.05
	Sum of methanol soluble radioactivity extracted from each plant part (d.p.m. x 10^{-6})					
Whole plant	9.9	6.0	7.7			

(i) Recovery of the applied radioactivity in methanol soluble extracts of each plant was in the range 34-59% (residues not measured).

(ii) *Extract too highly quenched to measure accurately, but of low activity.

Table 14

Distribution of radioactivity between the aqueous and acidic ether fractions of each plant.

Plant	Fractionated radioactivity / Combined radioactivity of fractions x 100		Total radioactivity in combined fractions (d.p.m. x 10^{-6})
	Aqueous	pH3	
A	74.0	26.0	9.9
B	54.5	45.5	6.0
C	70.7	29.3	7.8

Table 15

Distribution of radioactivity between the acidic ether and aqueous fractions of the separated plant parts.

Plant part	Fractionated radioactivity / Combined radioactivity of fractions x 100					
	Aqueous			pH3		
	A	B	C	A	B	C
Fed apical bud	81.5	68.8	80.3	18.5	31.2	19.7
Stem	42.5	20.7	31.5	57.5	79.3	68.5
Top leaves (2)	82.3	82.4	88.5	17.7	17.6	11.5
Lower leaves	100.0	N.D.	100.0	0.0	N.D.	0.0
Roots	34.2	24.7	22.7	65.8	75.3	77.3
Nodules	56.2	19.6	64.2	43.8	80.4	35.8

(i) N.D. - no radioactivity detected in the extract

Table 16

Relative distribution and specific activity of acidic ether soluble radioactivity. Proportion of radioactivity in separate plant parts as percentage of whole plant (sum of acidic ether soluble radioactivity in each plant part).

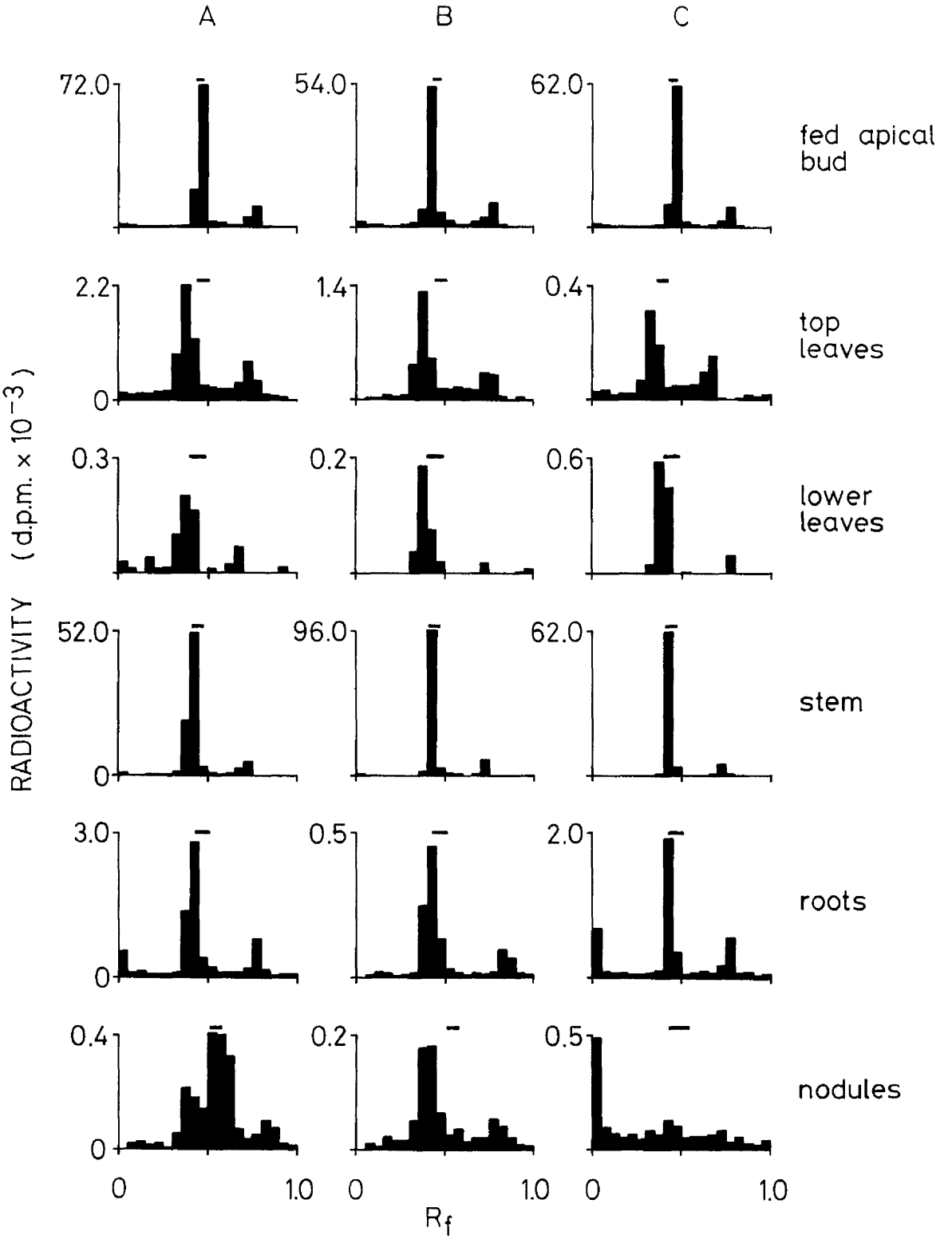
Plant part	Acidic ether soluble radioactivity					
	% of whole plant $\left(\frac{\text{Plant part d.p.m.}}{\text{Whole plant d.p.m.}} \times 100\right)$			Specific activity (d.p.m./g. fresh weight tissue $\times 10^{-5}$)		
	A	B	C	A	B	C
Fed apical bud	57.4	47.8	53.7	117.3	83.4	95.4
Stem	40.6	50.6	45.3	1.8	2.8	2.2
Top 2 leaves	0.7	0.3	0.1	0.1	0.05	0.02
Lower leaves	0.0	N.D.	0.0	0.0	N.D.	0.0
Roots	1.3	1.3	0.8	0.03	0.04	0.02
Nodules	0.09	0.05	0.03	0.05	0.03	0.02
	Sum of acidic ether soluble radioactivity extracted from each plant part (d.p.m.x10^{-6})					
Whole plant	2.6	2.7	2.3			

(i) N.D. - no radioactivity detected in the extract.

Figure 10

Thin-layer radiochromatograms of acidic ether extracts of separated plant parts from plants to which 5-^{3}H-IAA had been applied to the apical bud.

5-^{3}H-IAA (6 μCi - 2.1 x 10^{-7} m mol IAA) was applied to the apical bud of 6 plants which were harvested in pairs (A, B and C) 24 hours after feeding, for extraction and analysis.
Chromatogram solvent system: isopranol:ammonia:water:: 8:1:1 v|v.
TLC marker spots represent the R_F of 'cold' IAA added to and co-chromatographed with the extracts.


A
B
C
72.0
54.0
62.0
fed apical bud
2.2
0
1.4
0.4
top leaves
0.3
0.2
0.6
lower leaves
52.0
96.0
62.0
stem
3.0
0
0.5
2.0
roots
0.4
0
0.2
0.5
nodules
RADIOACTIVITY (d.p.m. × 10⁻³)
0
1.0
0
1.0
0
1.0
Rf

correspond with the IAA marker position and in A there was an additional, less polar peak which corresponded with the main peak of radioactivity in B. The main peak of radioactivity in C was at the origin. Two of the three root extracts also showed activity at the origin.

3.2 Application to root nodules

Plants were grown in water culture in a controlled environment cabinet (photo-period 16h, 19°C; dark period 8h, 15°C), or were transferred to these conditions, from the glasshouse, 12-27 days prior to the experiment. Micropipettes containing 34-58 μCi label (i.e. 1.4 - 2.3 x 10^{-6} mmol IAA) in 5-10 μl 20% aqueous ethanol were inserted into a single root nodule, or into one lobe of a nodule cluster, of three plants in each of four experiments. Uptake of the label varied, but in most plants was completed within 5½ hours, in one quarter of the plants within 12-18 hours but in one plant it was not completed by harvest (see Table 17). The three plants in each experiment were harvested individually 24-26 hours after beginning the application of label, extracts were prepared as described in Section 1 and aliquots of the acidic ether fraction were analysed by TLC and preparative HPLC. The radioactivity of the residue, remaining after aqueous methanol extraction, was measured using liquid scintillation sample oxidation.

Results

Location of radioactivity

Methanol soluble radioactivity was detected in all parts of the alder plant after labelled IAA had been applied via a root nodule (Table 18). The distribution of radioactivity, in the separated plant parts, varied both between the replicate plants in each experiment and between experiments.

Table 17

Rate of absorption of 5-^{3}H-IAA applied by micropipette to a root nodule.

	Time (hours) for absorption of 5-^{3}H-IAA by fed nodule			Volume in which label applied (µl 20% aqueous ethanol)
Plant	A	B	C	
Experiment 1	1.5	1.5	1.5	10
2	5.5	5.5	26	5
3	3.5	16	18	5
4	< 4.0	12	0.2	5

There was no consistent correlation between the time taken for the absorption of label by the fed nodule (Table 17) and the subsequent distribution of radioactivity in the plant. Although most of the methanol soluble radioactivity which was present in each plant (derived by summation of the methanol soluble radioactivity of each plant part) was retained within the fed nodule (24-98%), significant proportions were detected in the roots (0.8-47%), leaves (1-23%) and the stem (0.4-19%). The unfed nodules contained only a small proportion of the total methanol soluble radioactivity (0.05-3.4%), except for one plant in experiment 1. Separate analysis of the bark and wood showed no difference between the levels of radioactivity in the conducting tissues of the stem. Generally, lower levels of radioactivity were detected in the shoot and in the unfed nodules.

The specific radioactivities of methanolic extracts of the separate plant parts (Table 19), although variable, do suggest some accumulation of radioactive metabolites in the apical buds, since in 5 out of 12 plants the specific radioactivity of methanol soluble apical bud extracts was as great as,or greater than that of the other plant parts (excluding the fed nodule). The bark and wood were both of similar specific activity, confirming that there were no differences

Table 18

Relative distribution of methanol soluble radioactivity. Proportion of radioactivity in separate plant parts as percentage of whole plant (sum of methanol soluble radioactivity of each plant part).

Plant part	% of whole plant methanol soluble radioactivity $\left(\frac{\text{plant part d.p.m.}}{\text{combined plant part d.p.m.}} \times 100\right)$					
	EXPERIMENT 1			EXPERIMENT 2		
	A	B	C	A	B	C
Fed nodule	23.5	78.3	56.3	96.4	88.7	97.7
Roots	47.0	16.5	37.5	0.9	2.6	0.8
Leaves	6.2	2.7	4.6	1.2	5.8	1.0
Stem	5.7	1.7	1.4	1.4	2.1	0.4
Nodules	17.4	0.8	0.2	0.05	0.7	0.05
Apical bud	0.2	0.01	0.06	0.05	0.03	0.02

	EXPERIMENT 3			Experiment 4		
	A	B	C	A	B	C
Fed nodule	90.1	82.0	50.1	32.6	58.2	71.6
Roots	5.1	5.5	15.1	31.3	16.4	7.4
Leaves	3.4	8.9	15.2	23.0	15.0	16.6
Stem - bark	1.3	3.4	19.3	4.4	3.9	1.6
Stem - wood				5.0	3.9	2.4
Nodules	0.1	0.2	0.2	3.4	2.6	0.3
Apical bud	0.1	0.1	0.004	0.2	0.2	0.2

Sum of methanol soluble radioactivity extracted from each plant part (d.p.m. x 10^{-6})

EXPERIMENT		1	2	3	4
Whole plant	A	12.5	25.9	21.5	46.9
	B	10.4	23.1	28.1	40.9
	C	13.7	26.7	33.8	28.6

(i) Recovery of applied radioactivity from each plant (residues included):-

EXPERIMENT 1 17-19% ; EXPERIMENT 2 53 - 68%

EXPERIMENT 3 37-43% ; EXPERIMENT 4 39 - 59% .

Table 19

The specific radioactivity of methanol soluble extracts of the separated plant parts.

Plant part	Specific activity (d.p.m./g. fresh weight tissue x 10^{-5})					
	EXPERIMENT 1			EXPERIMENT 2		
	A	B	C	A	B	C
Fed nodule	2100	2700	3840	15600	3600	4400
Roots	16	3.8	7.8	0.3	0.5	0.2
Leaves	1.8	0.7	1.6	1.1	3.8	0.6
Stem	3.6	0.8	0.7	1.4	1.5	0.3
Nodules	77	2.9	1.3	1.7	0.8	0.2
Apical bud	2.1	0.2	1.2	0.4	4.5	0.5

	EXPERIMENT 3			EXPERIMENT 4		
	A	B	C	A	B	C
Fed nodule	190	920	230	660	2200	1300
Roots	0.2	0.7	18	24	18	2.1
Leaves	0.04	0.02	0.6	8.4	4.8	2.8
Stem - bark	0.02	0.3	0.8	10.1	6.8	2.1
- wood				9.7	5.3	2.4
Nodules	0.2	0.04	0.03	17	7.9	0.7
Apical bud	1.8	1.8	0.1	3.4	5.9	3.7

in the distribution of methanol soluble radioactivity between the conducting tissues of the stem.

The nature of the methanol soluble radioactivity was investigated further, to determine whether there were differences in metabolism of the translocated label in, or en route to, the various plant parts. Initially, the aqueous phase of the methanolic extracts of the separated plant parts was partitioned against ether at pH3 (and pH8 in experiment 1), and this produced four fractions, which between them, contained the total radioactivity of each plant part (Table 20 and Appendix Table 2 a,b). The variability of these results, observed between plants, both within and between experiments, may have obscured differences in the partitioning of the plant organ radioactivity. However, in all extracts, a major part of the radioactivity remained in the aqueous fraction (25-83%).

Any IAA present in the plant tissues would partition into the acidic ether fraction. The relative distribution of this radioactivity between the plant parts is shown as a percentage of that in the whole plant (derived by summation of the acidic ether soluble radioactivity of each plant part), in Table 21 . Highest levels were present in the fed nodules (26-80%), with decreasing activity in the roots (3-46%), leaves (1-19%), stem (0.9-41%) and unfed nodules (0.04-10%). The bark and the wood, which were separately analysed, both contained similar levels of acidic ether soluble radioactivity.

Calculation of the specific radioactivity of the acidic ether fraction of the plant parts, showed that the roots contained the highest concentration of isotope in experiments 1,3 and 4, in 7 out of 9 plants (excluding the fed nodule). In experiment 2, however, where movement of radioactivity from the fed nodule was much more limited, the highest specific radioactivity of any plant part (excluding the fed nodule) was shown by the unfed nodules, followed by the leaves and the stem, whereas the roots showed the lowest specific activity (6-50 times lower than the unfed nodules, Table 22).

Table 20

Distribution, following solvent partitioning, of the radioactivity of separated plant parts between aqueous, basic ether, acidic ether and residual fractions.

Plant part	% of plant part radioactivity partitioned into four fractions. (Fractionated radioactivity x 100 / Combined radioactivity of fractions)							
	Aqueous				pH8			
Experiment	1	2	3	4	1	2	3	4
Fed nodule	83.1	59.1	49.2	53.2	4.8			
Roots	71.4	61.2	24.9	55.5	8.0			
Leaves	44.4	34.4	47.1	72.0	1.4			
Stem - bark	51.9	52.1	35.5	71.6	4.4			
- wood				68.8				
Nodules	46.2	36.7	34.4	39.4	17.6			
Apical bud	30.2	-	-	-	0.2			

	pH3				Residue			
Experiment	1	2	3	4	1	2	3	4
Fed nodule	9.9	0.3	11.9	14.4	2.0	40.6	38.9	32.4
Roots	13.3	1.5	54.3	21.5	7.3	37.3	22.8	23.0
Leaves	2.7	1.7	9.9	7.4	51.5	63.8	43.0	20.6
Stem - bark	8.3	1.5	30.9	12.2	35.2	46.5	33.6	16.1
- wood				12.4				18.8
Nodules	3.6	4.8	6.9	7.1	32.7	58.5	58.7	53.5
Apical bud	0.4	-	-	-	69.1	-	-	-

(i) - radioactivity level of extract too low for analysis.

(ii) Data are means from 3 replicate plants per experiment (see Appendix Table 2a,b).

(iii) Solvent partitioning at pH8 was conducted only in experiment 1.

Table 21

Relative distribution of acidic ether soluble radioactivity. Proportion of radioactivity in separate plant parts as percentage of whole plant (sum of acidic ether soluble radioactivity of each plant part).

Plant part	% of whole plant acidic ether soluble radioactivity $\left\{\frac{\text{Plant part d.p.m.}}{\text{Combined plant part d.p.m.}} \times 100\right\}$					
	EXPERIMENT 1			EXPERIMENT 2		
	A	B	C	A	B	C
Fed nodule	37.7	74.9	63.9	80.1	63.8	77.7
Roots	45.5	18.8	33.0	2.8	3.3	7.0
Leaves	1.0	3.1	2.1	9.0	19.0	13.0
Stem	5.4	2.3	0.9	6.9	9.6	1.4
Nodules	10.4	1.0	0.1	1.2	4.2	1.0
Apical bud	0.04	0.0	0.01	-	-	-

	EXPERIMENT 3			EXPERIMENT 4		
	A	B	C	A	B	C
Fed nodule	79.7	71.8	25.8	25.6	69.4	77.4
Roots	17.3	17.1	22.1	41.6	16.9	11.5
Leaves	1.4	6.6	10.8	17.5	7.3	7.9
Stem - bark	1.6	4.3	41.1	4.9	2.8	1.4
- wood				5.8	3.0	1.7
Nodules	0.04	0.2	0.1	4.7	0.7	0.2

Sum of acidic ether soluble radioactivity extracted from each plant part (d.p.m. x 10^{-6})

EXPERIMENT	1	2	3	4
A	1.1	0.2	5.3	6.5
Whole plant B	0.8	0.1	6.0	11
C	1.2	0.1	12	4.9

(i) - radioactivity level of extract too low for analysis.

Table 22

The specific radioactivity of acidic ether soluble extracts of the separated plant parts.

Plant part	Specific activity (d.p.m./g. fresh weight tissue x 10^{-5})					
	EXPERIMENT 1			EXPERIMENT 2		
	A	B	C	A	B	C
Fed nodule	300	190	400	100	15	16
Roots	1.4	0.3	0.6	0.006	0.004	0.009
Leaves	0.03	0.06	0.06	0.07	0.07	0.03
Stem	0.3	0.08	0.04	0.05	0.04	0.005
Nodules	4.1	0.3	0.06	0.08	0.2	0.05
Apical bud	0.03	0.0	0.03	-	-	-

	EXPERIMENT 3			EXPERIMENT 4		
	A	B	C	A	B	C
Fed nodule	150	370	140	100	990	340
Roots	0.9	1.2	12	9.8	6.9	0.8
Leaves	0.04	0.2	0.5	1.3	0.9	0.3
Stem - bark	0.1	0.3	5.3	2.2	1.9	0.5
- wood				2.2	1.6	0.4
Nodules	0.02	0.07	0.07	4.6	0.8	0.1

(i) - radioactivity level of extract too low for analysis.

Analysis of acidic ether soluble radioactivity

(a) Thin-layer chromatography

Thin-layer chromatograms, showing the distribution of radioactivity in the acidic ether fraction of the different plant tissues in the four experiments, formed two groups (Figure 11). In group I (chromatograms from experiment 1), the R_F values of the major zones of radioactivity corresponded with those of the applied 5-^{3}H-IAA solution, and the position of the 'cold' IAA marker was similar to that of the main peak of radioactivity. Chromatograms of extracts from experiments 2, 3 and 4, differed from those of group I, in that a second group of radioactive peaks, with higher R_F values, was much more prominent.

In most of the extracts, the 'cold' IAA marker corresponded with the first group of radioactive peaks. Although the overall pattern of distribution of radioactivity on chromatograms of extracts of replicate plant parts was similar, the relative proportions of the radioactive peaks varied; this was particularly noticeable in experiment 2. Chromatograms of a number of plant part extracts were atypical in several respects, namely:

(1) Root extracts:- Chromatograms of extracts were similar to that of the applied label except for 2B, 2C and 4C in which the second group of radioactive peaks was prominent or predominant.

(2) Leaf extracts:- The IAA marker in chromatograms of extracts from plants 3A and 3B was of higher R_F value than usual.

(3) Fed nodule extracts:- All chromatograms showed a peak of activity at the origin.

(4) Unfed nodule extracts:- The chromatogram of the extract from plant 3A suggested a greater level of metabolism of the label.

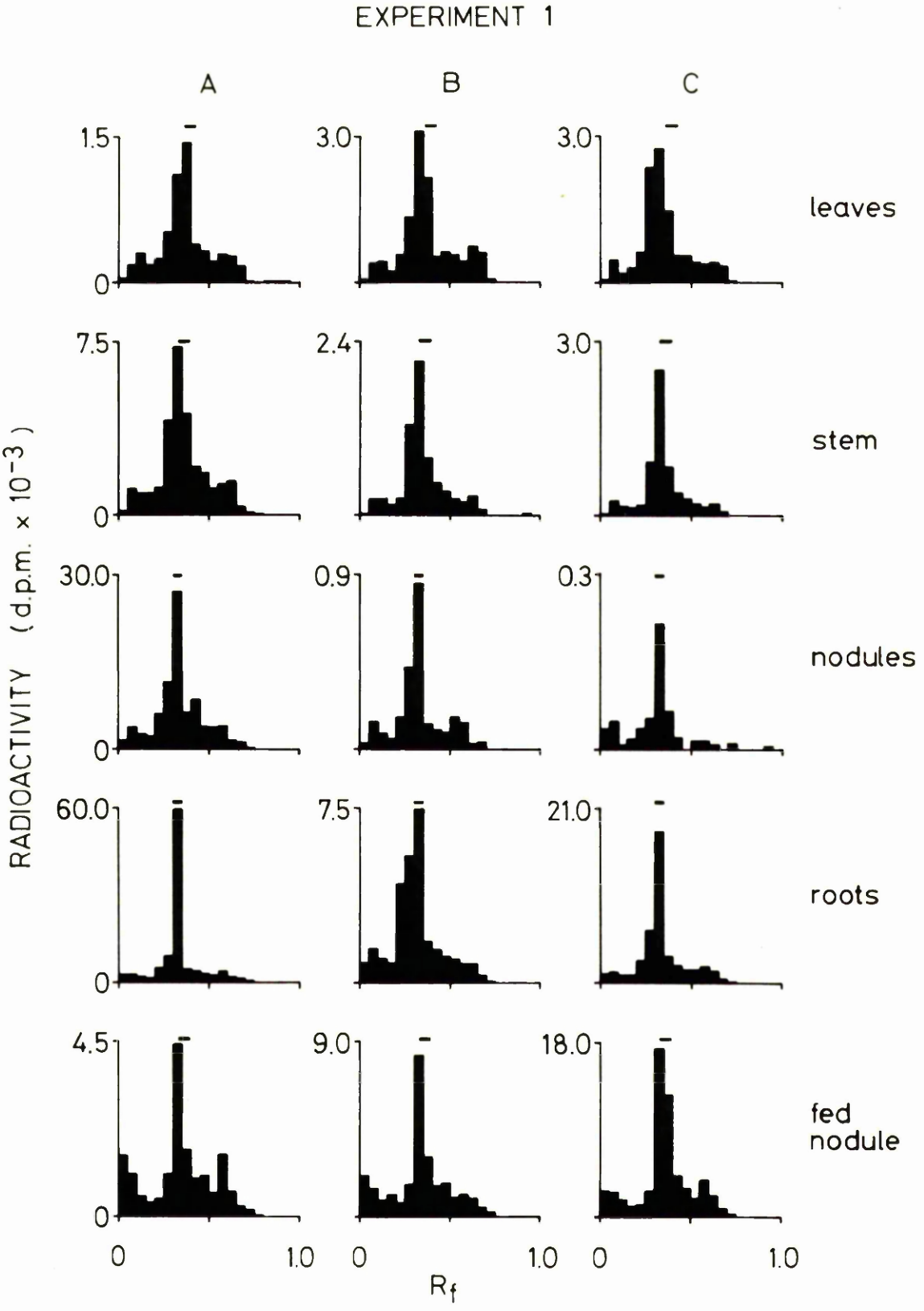
There were no obvious differences in the distribution of radioactivity on chromatograms of the bark and wood, when these were analysed separately in experiment 4.

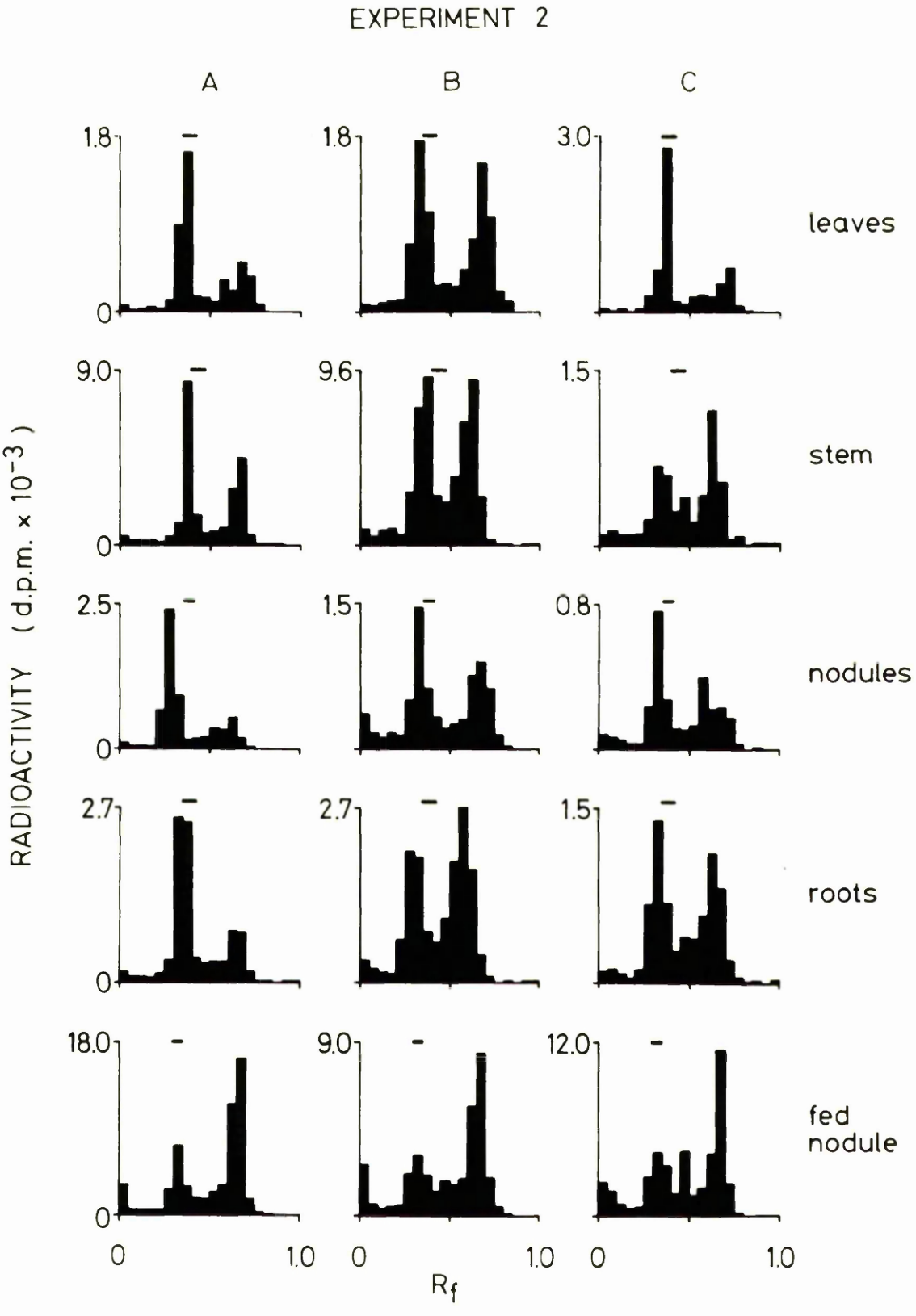
The proportion of eluted radioactivity present at the position of the 'cold' IAA marker in each of the extracts, is shown in Table 23 . In group I, the range of radioactivity present

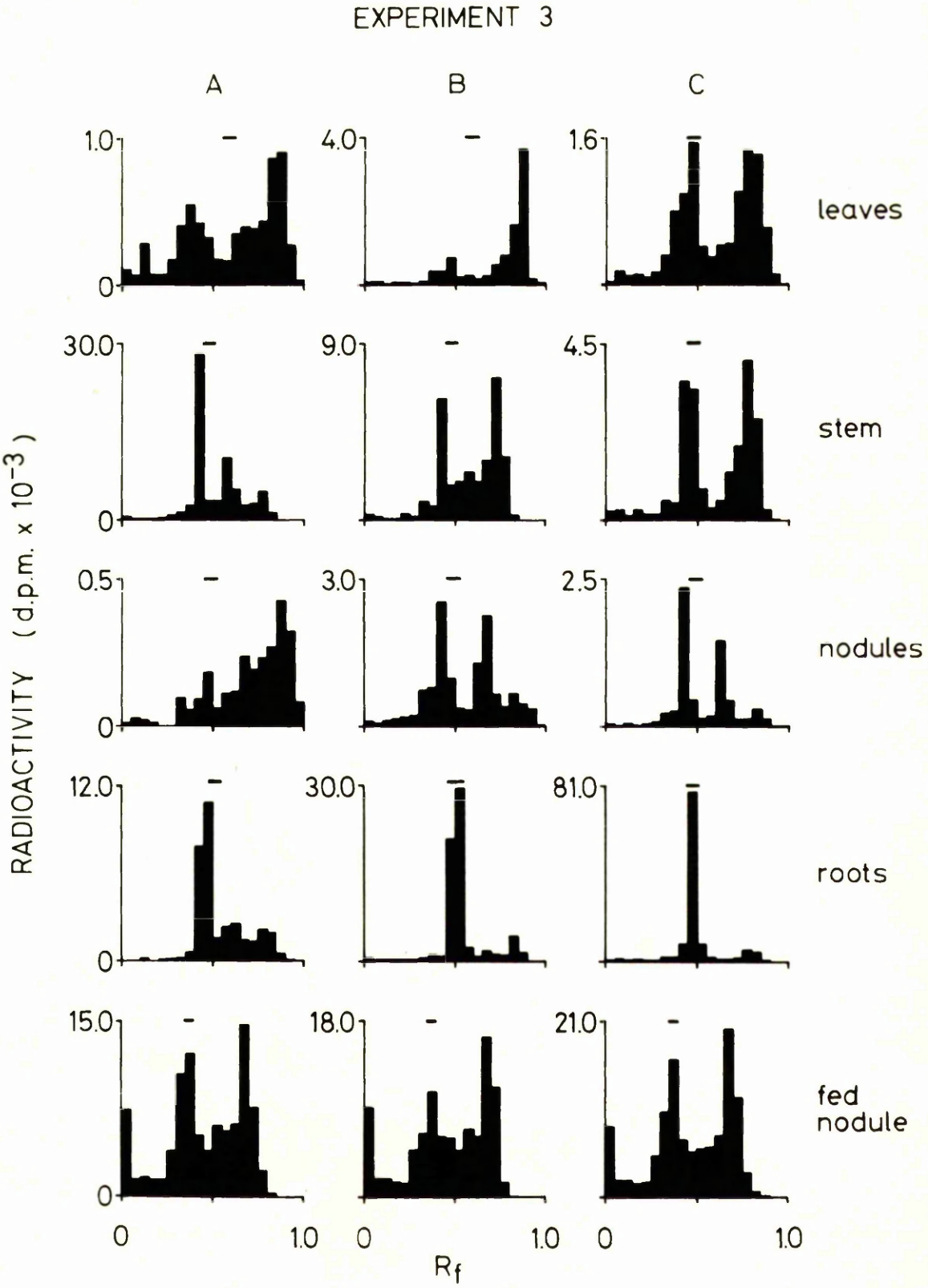
Figure 11

Thin-layer radiochromatograms of acidic ether extracts of plants fed 5-^{3}H-IAA via a root nodule.

5-^{3}H-IAA (34-58 μCi - 1.4 - 2.3 x 10^{-6} mmol IAA) was applied to a root nodule on each of three plants in four separate experiments. Plants (A, B and C in each experiment) were harvested individually 24-26 hours after feeding, and divided into parts for extraction and analysis.
Chromatogram solvent system: isopropanol:ammonia: water:: 8: 1 : 1 v|v.
TLC marker spots represent the R_F of 'cold' IAA chromatographed alongside the extracts.


EXPERIMENT 1
A
B
C
1.5
0
3.0
0
3.0
0
leaves
7.5
0
2.4
0
3.0
0
stem
30.0
0
0.9
0
0.3
0
nodules
60.0
0
7.5
0
21.0
0
roots
4.5
0
9.0
0
18.0
0
fed nodule
RADIOACTIVITY (d.p.m. x 10⁻³)
0
1.0
0
1.0
0
1.0
Rf


EXPERIMENT 2
A
B
C
1.8
0
1.8
0
3.0
0
leaves
9.0
0
9.6
0
1.5
0
stem
2.5
0
1.5
0
0.8
0
nodules
2.7
0
2.7
0
1.5
0
roots
18.0
0
9.0
0
12.0
0
fed nodule
0
1.0
0
1.0
0
1.0
R_f
RADIOACTIVITY (d.p.m. x 10^{-3})


EXPERIMENT 3
A
B
C
1.0
0
4.0
1.6
leaves
30.0
9.0
4.5
stem
0.5
3.0
2.5
nodules
12.0
30.0
81.0
roots
15.0
18.0
21.0
fed nodule
RADIOACTIVITY (d.p.m. x 10^{-3})
0
1.0
R_f

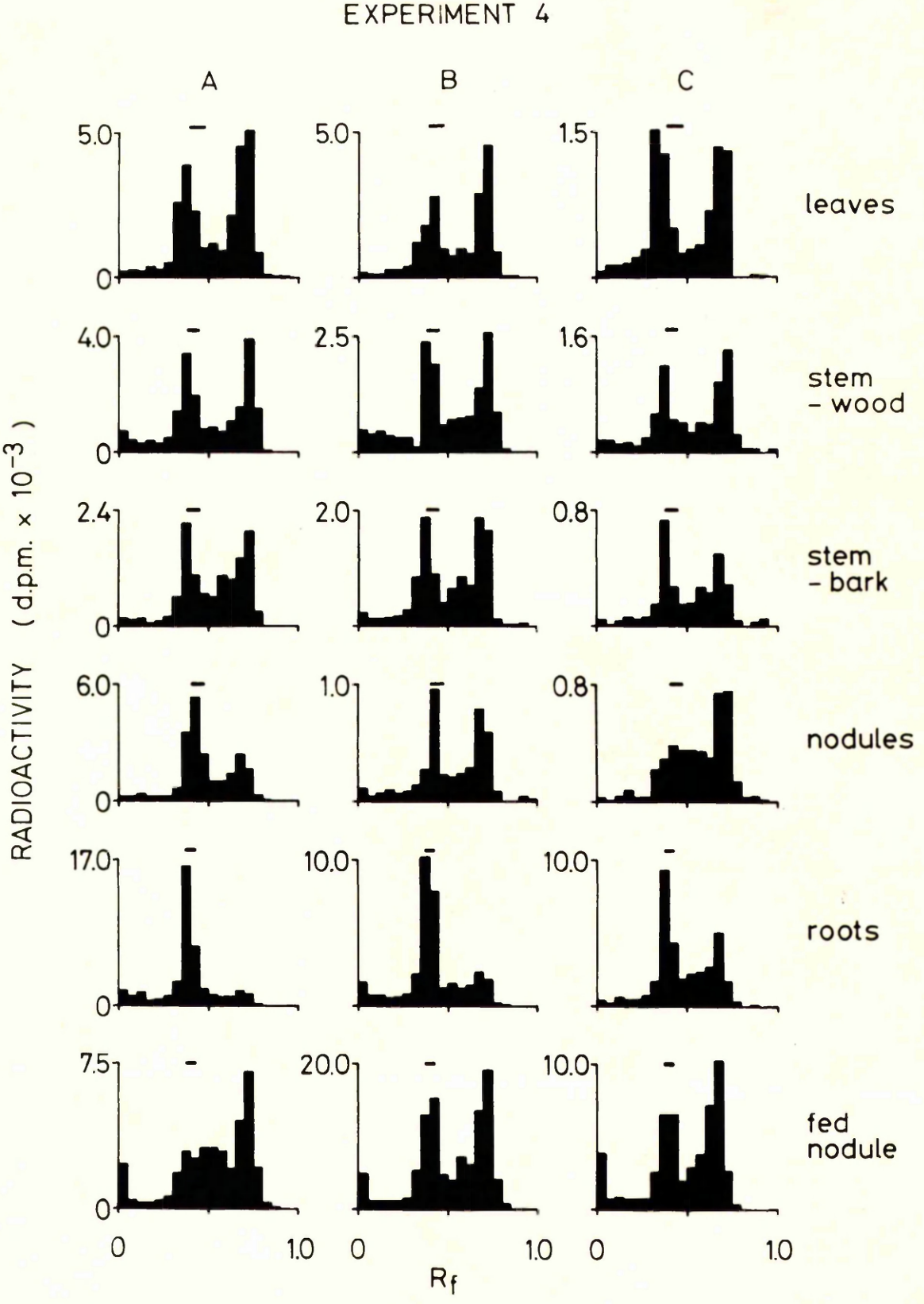

EXPERIMENT 4
A
B
C
5.0
0
5.0
0
1.5
0
leaves
4.0
0
2.5
0
1.6
0
stem
- wood
2.4
0
2.0
0
0.8
0
stem
- bark
6.0
0
1.0
0
0.8
0
nodules
17.0
0
10.0
0
10.0
0
roots
7.5
0
20.0
0
10.0
0
fed
nodule
0
1.0
0
1.0
0
1.0
Rf
RADIOACTIVITY (d.p.m. x 10⁻³)

Table 23

Proportion of eluted radioactivity present at the position of the 'cold' IAA marker in extracts of the separated plant parts. Radioactivity was measured by liquid scintillation counting of eluates from silica gel thin-layer chromatography plates.

	% radioactivity $\frac{\text{IAA region d.p.m.}}{\text{Total eluted sample d.p.m.}}$ x 100											
Experiment	1			2			3			4		
Plant	A	B	C	A	B	C	A	B	C	A	B	C
Fed nodule	37.3	43.4	51.8	22.5	21.1	24.1	24.9	17.8	23.3	16.3	27.6	23.3
Roots	70.1	51.7	63.6	59.9	22.5	33.3	36.8	60.0	75.7	56.3	51.7	39.8
Leaves	44.6	44.9	38.9	57.2	35.1	57.5	14.3	13.1	28.4	27.1	20.8	23.8
Stem - bark	41.0	45.2	53.2	45.9	28.0	24.9	50.9	29.5	37.2	33.6	28.7	36.4
- wood										31.0	34.0	28.3
Nodules	53.1	57.0	60.1	55.5	38.7	39.4	13.2	30.4	45.6	52.6	33.0	24.5

Source of variance	Analysis of variance				
	Sum of squares	Degrees of freedom	Mean squares	Variance ratio (F)	Significance
Between plant parts	11726	4	2932	4.4	Significant at p = 0.05
Between experiments	8994	3	2998	4.5	Significant at p = 0.05
Residual	7915	12	660		
Total	28635	19			

in the IAA region was between 37 and 70%, whereas in group II the range was between 13 and 76%. Root extracts contained the highest proportion of radioactivity in the IAA region, in group I with a range of 52-70% and in group II with a range of 23-76% .

(b) High-performance liquid chromatography

Greater resolution of the range of radioactive metabolites present in the acidic ether soluble plant extracts, was sought by additional analysis using preparative HPLC (Figure 12). With the exception of IAA, the nature of the radioactive peaks eluted from the HPLC column has not yet been established, although some indication of their possible identity can be obtained from a comparison with the retention times of standards, run on a similar gradient (Figure 13). Analyses of related samples were occasionally separated by a few months because of limitations on the availability of the HPLC. To establish the nature of changes in elution patterns which might occur with time due both to changes in the extracts during prolonged storage, and to variation in HPLC column performance, two extracts of fed nodules from experiment 2 ([i], plants A and B, figure 12) were re-chromatographed 18 months after the initial analyses ([ii] , plants A and B, Figure 12). The number and retention times of the radioactive peaks were not greatly altered, although there was a slight deterioration of column performance which led to some broadening of the peaks and loss of resolution.

A multi-component peak was present at the IAA elution point in each sample run; comparison of the retention times of the component peaks in different extracts, suggested the presence of at least five compounds. However, the inclusion of a 'cold' IAA marker in each sample run, enabled the IAA elution point to be distinguished from the other components of the complex.

The proportion of radioactivity which eluted with the IAA marker is shown, for each sample, in Table 24 . Comparison with Table 23 , shows that the proportion of eluted radioactivity at the IAA marker position after TLC analysis was 2-8 times higher than that after preparative HPLC analysis. Of all the extracts, the roots again contained the largest proportion of eluted radioactivity (3-69%) corresponding to IAA, although there was

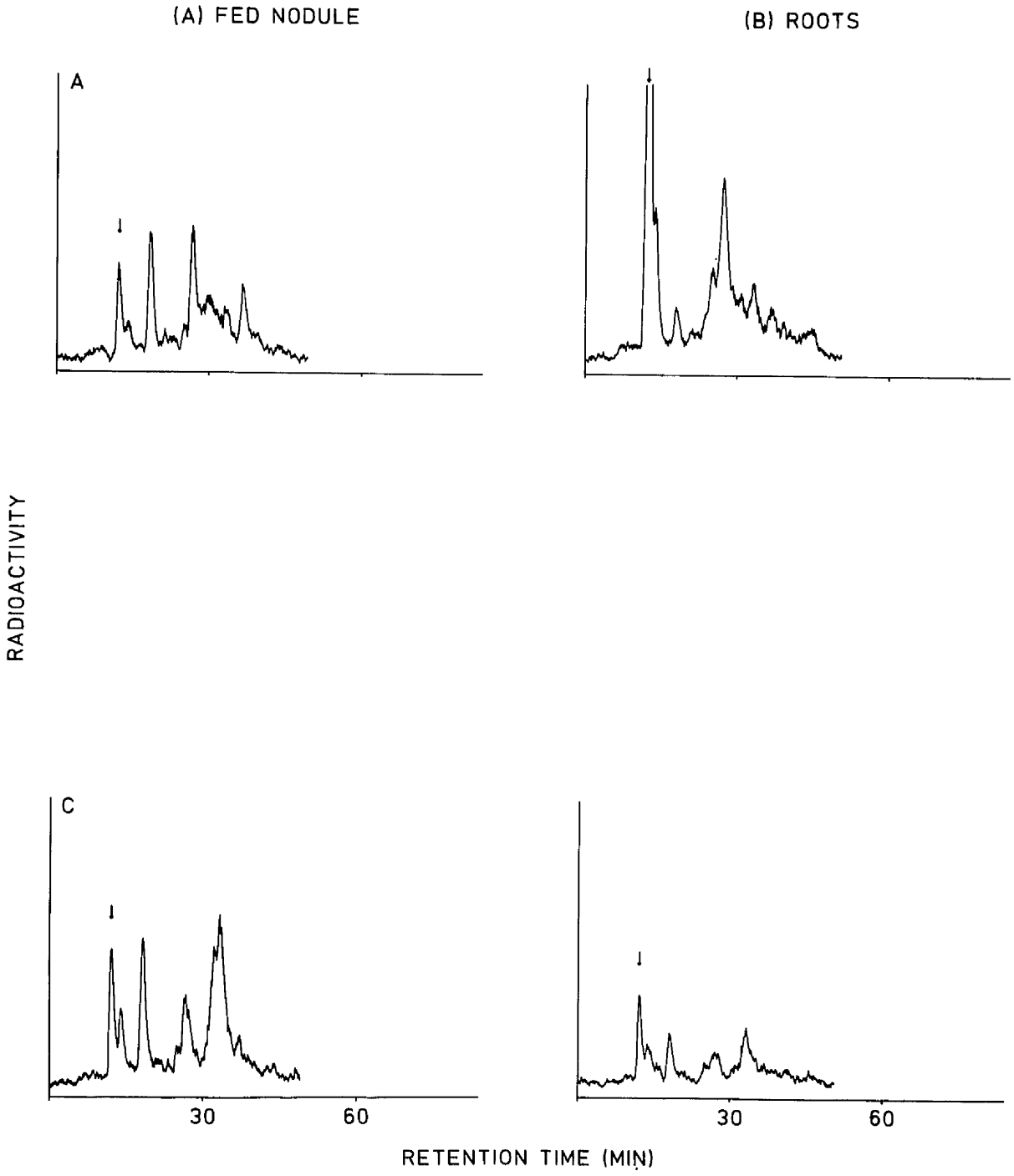
Figure 12

Preparative high performance liquid radiochromatograms of acidic ether extracts of plants fed 5-^{3}H-IAA via a root nodule.

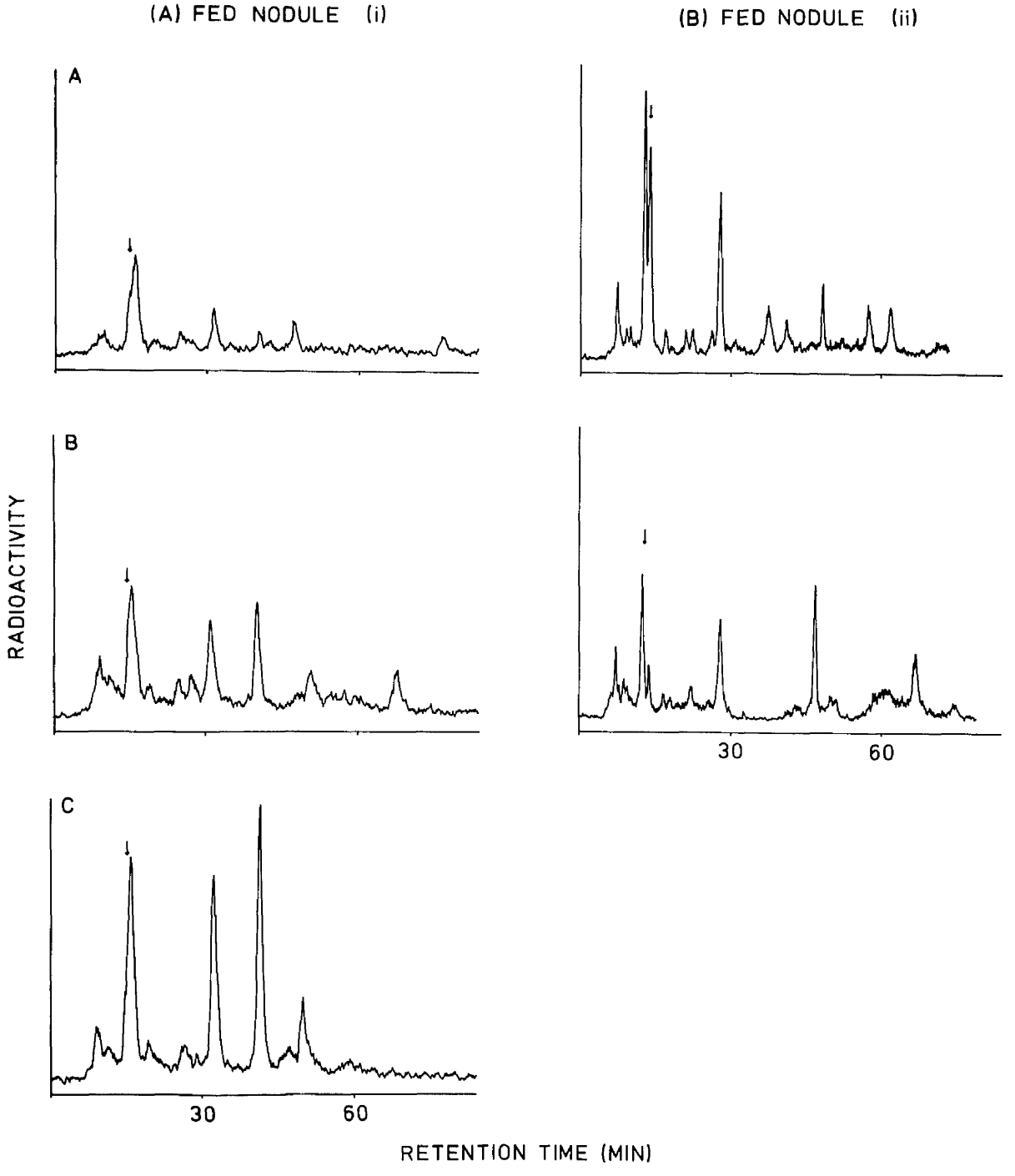
5-^{3}H-IAA (34 - 58 μCi — 1.4 - 2.3 x 10^{-6} mmol IAA) was applied to a root nodule on each of three plants in four separate experiments. Plants (A,B and C in each experiment) were harvested individually 24-26 hours after feeding and divided into parts for extraction and analysis.
Arrows represent the elution point of 'cold' IAA added to and co-chromatographed with the extracts.

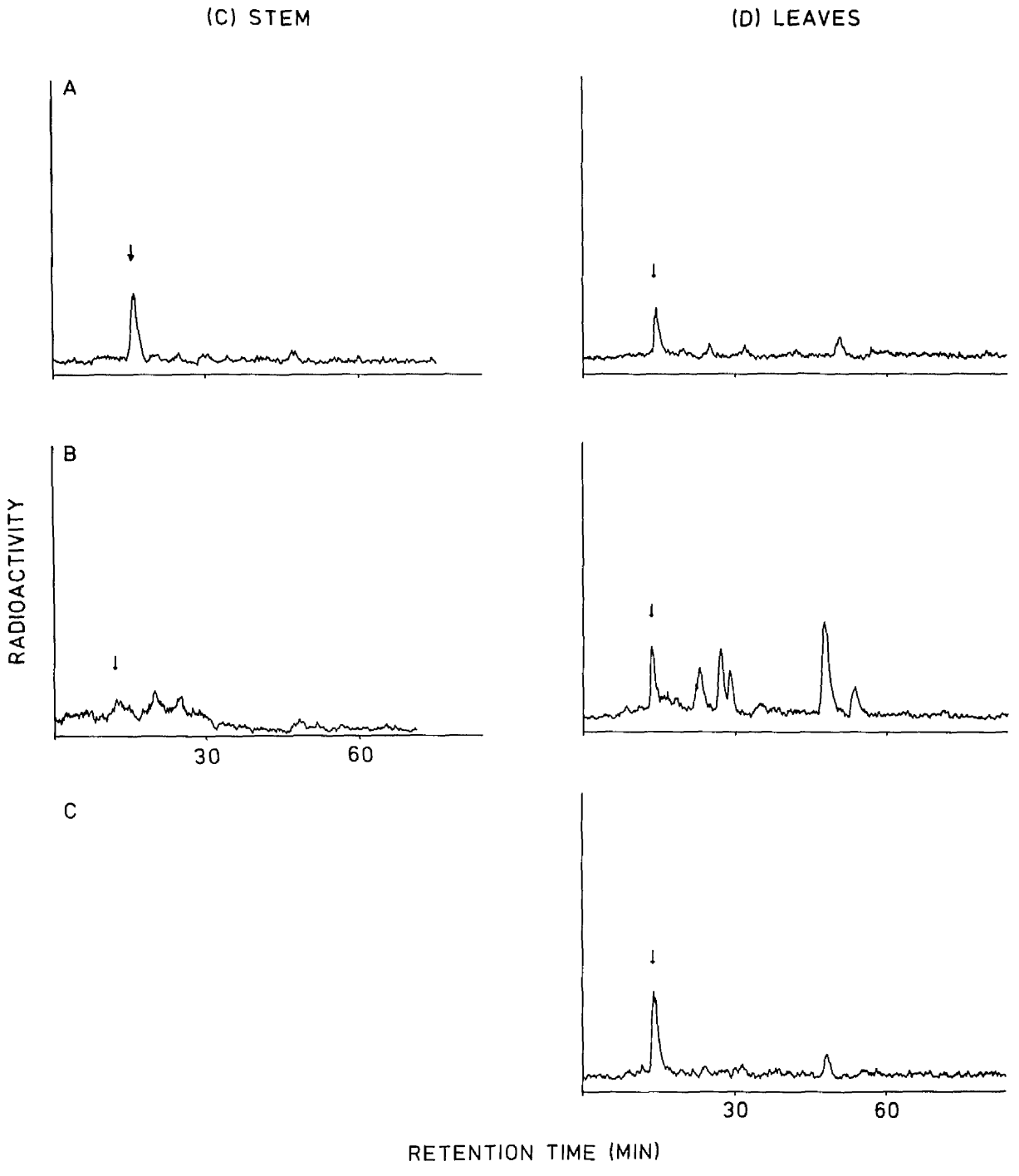
Column:- Partisil 10 or 20
Column temperature:- 30 ± 0.5°C
Stationary phase:- 40% 0.5 M formic acid or 40% 1M acetic acid gra
Mobile phase:- Gradient of 50-75% ethyl acetate in hexane in 40 minutes, extended when required to 100% ethyl acetate in hexane
Flow rate:- Mobile phase 4.6 ± 0.1 ml.min^{-1}.
scintillant 2.0 ± 0.1 ml.min^{-1}.

EXPERIMENT 1

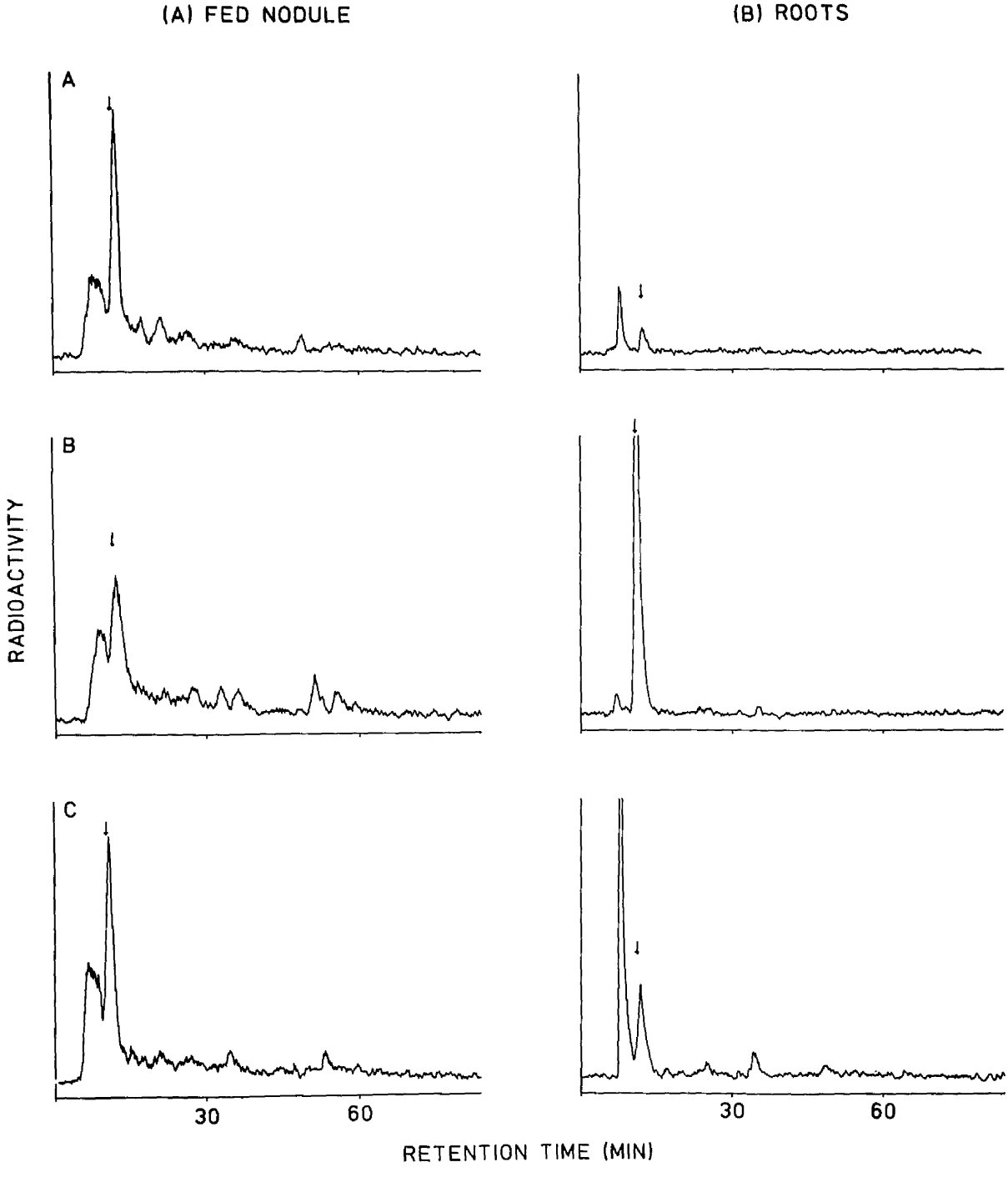


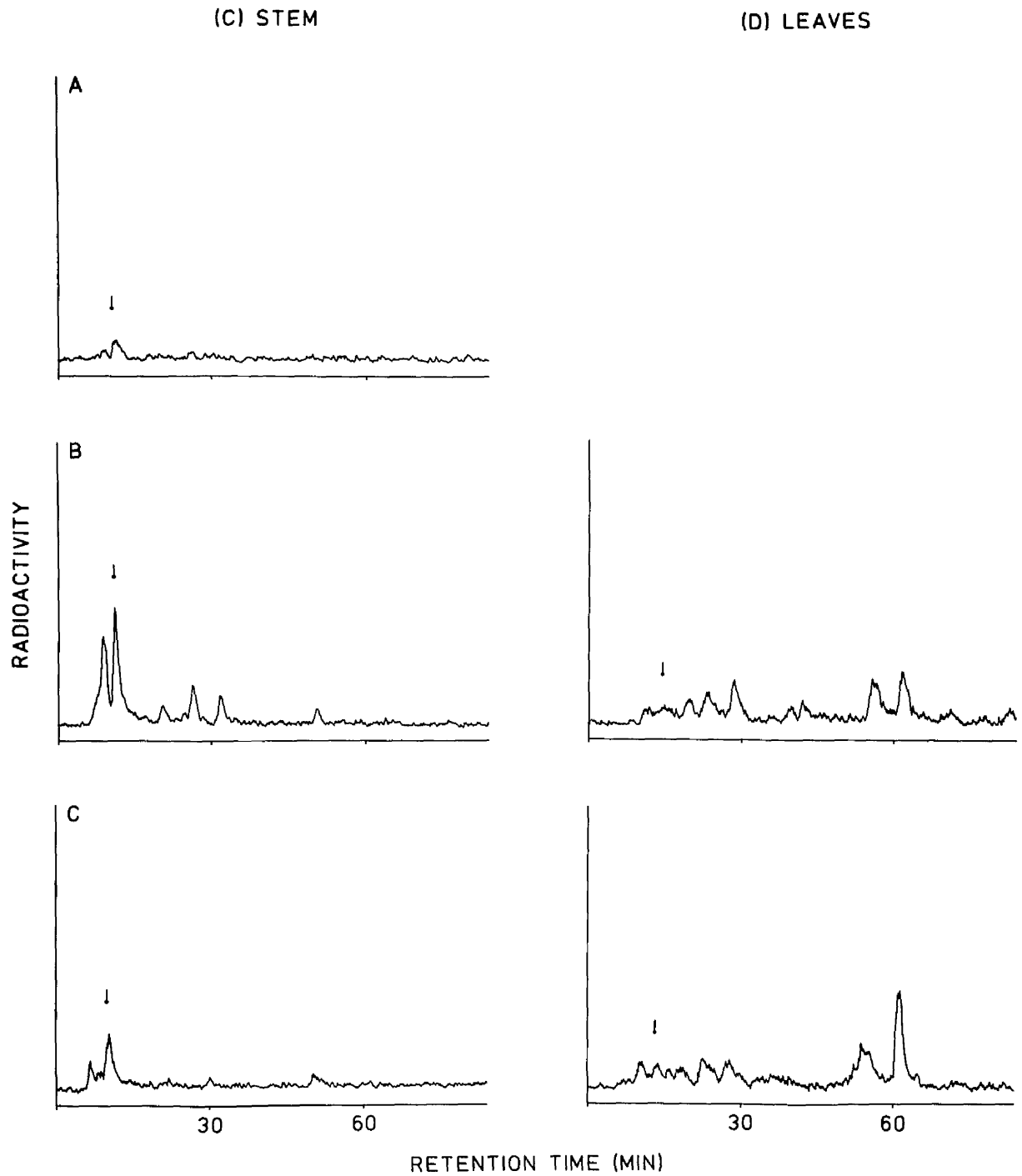
EXPERIMENT 2



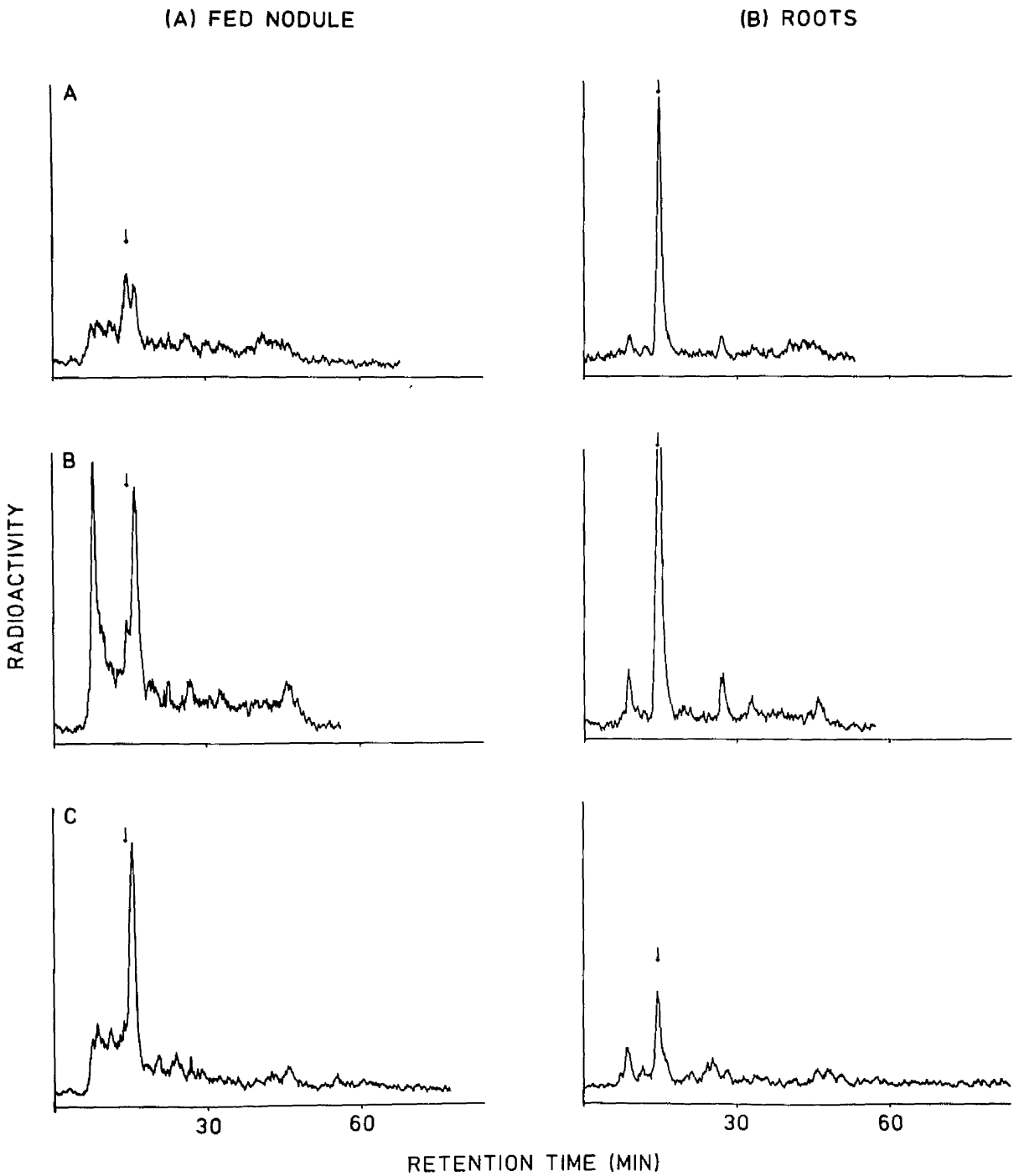

(C) STEM
(D) LEAVES
A
B
C
RADIOACTIVITY
30
60
30
60
RETENTION TIME (MIN)

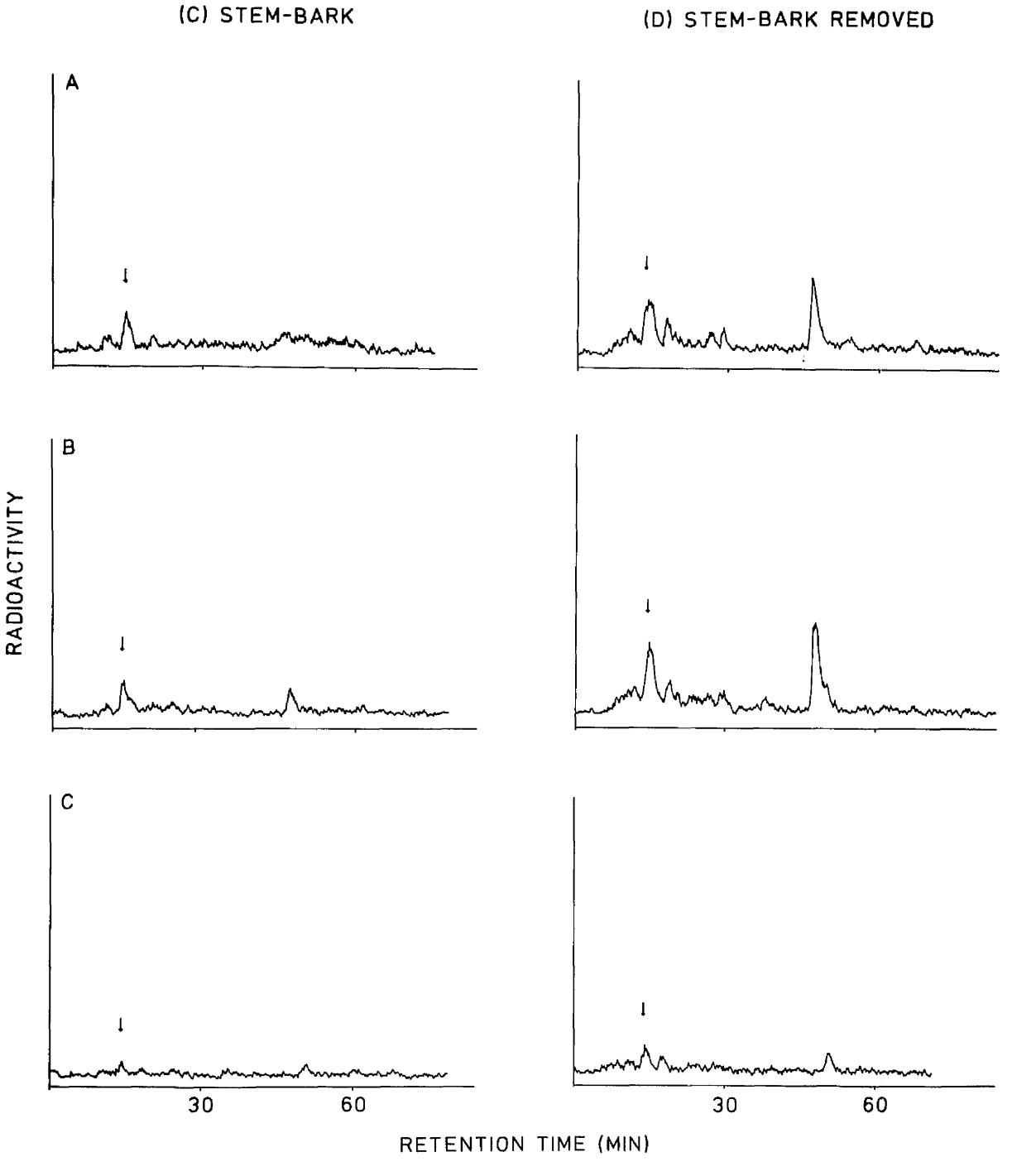
EXPERIMENT 3




(C) STEM
(D) LEAVES
A
B
C
RADIOACTIVITY
30
60
30
60
RETENTION TIME (MIN)

EXPERIMENT 4




(C) STEM-BARK
(D) STEM-BARK REMOVED
A
B
C
RADIOACTIVITY
30
60
30
60
RETENTION TIME (MIN)

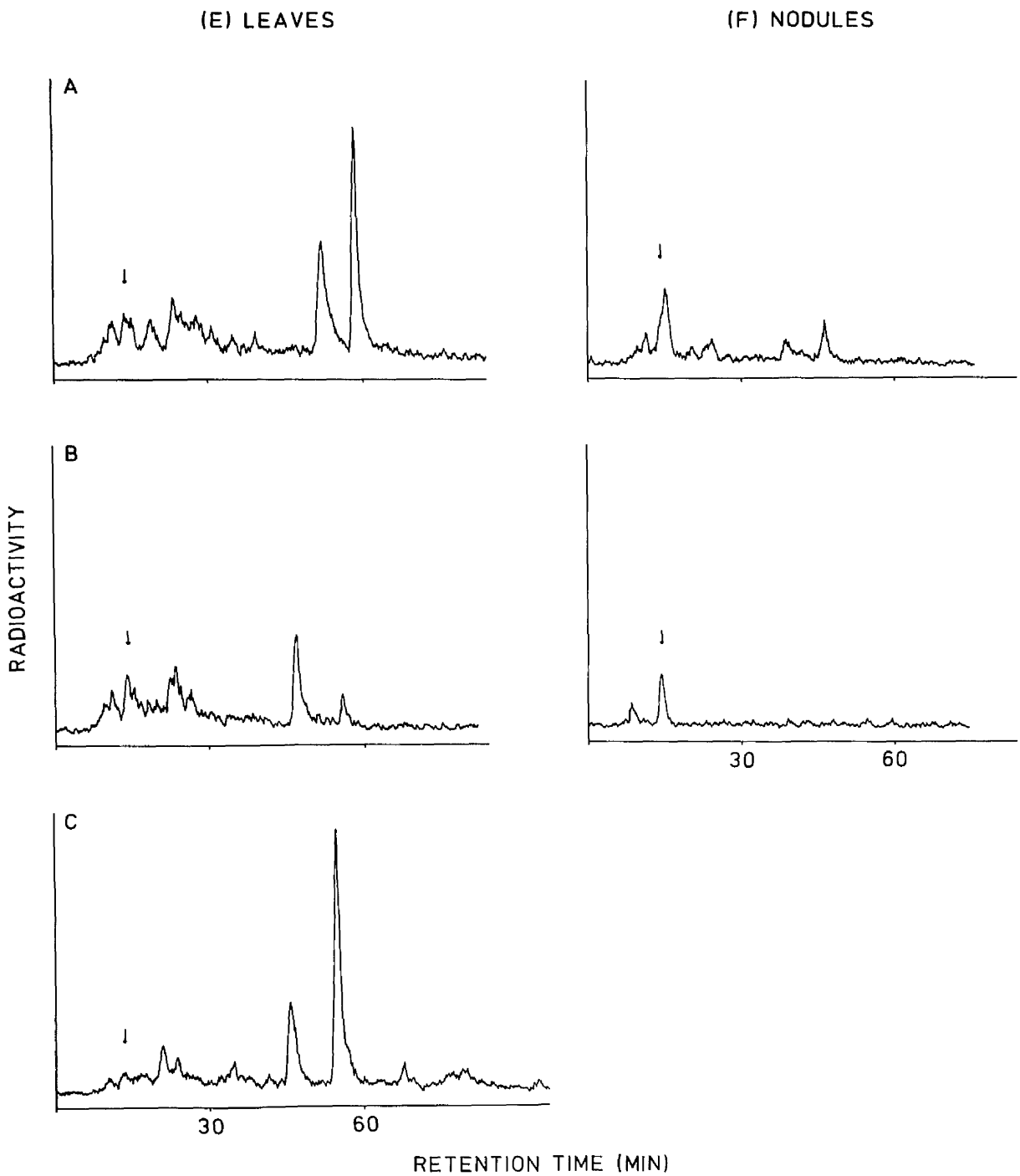

(E) LEAVES
(F) NODULES
A
B
C
RADIOACTIVITY
30
60
30
60
RETENTION TIME (MIN)

Figure 13

Preparative high performance liquid chromatogram of acidic and neutral indoles as detected by U.V. absorbance.

Column: - Partisil 20
Column temperature:- 30 ± 0.5°C
Stationary phase:- 0.5 M formic acid
Mobile phase:- gradient of 50-75% ethyl acetate in hexane in 40 minutes
Flow rate:- mobile phase 4.6 ± 0.1 ml. min^{-1}
scintillant 2.0 ± 0.1 ml. min^{-1}
Sample:- indole - 3 - acetonitrile (1AN)
indole - 3 - acetic acid (1AA)
indole - 3 - carboxylic acid (1CA)
indole - 3 - lactic acid (1LA)

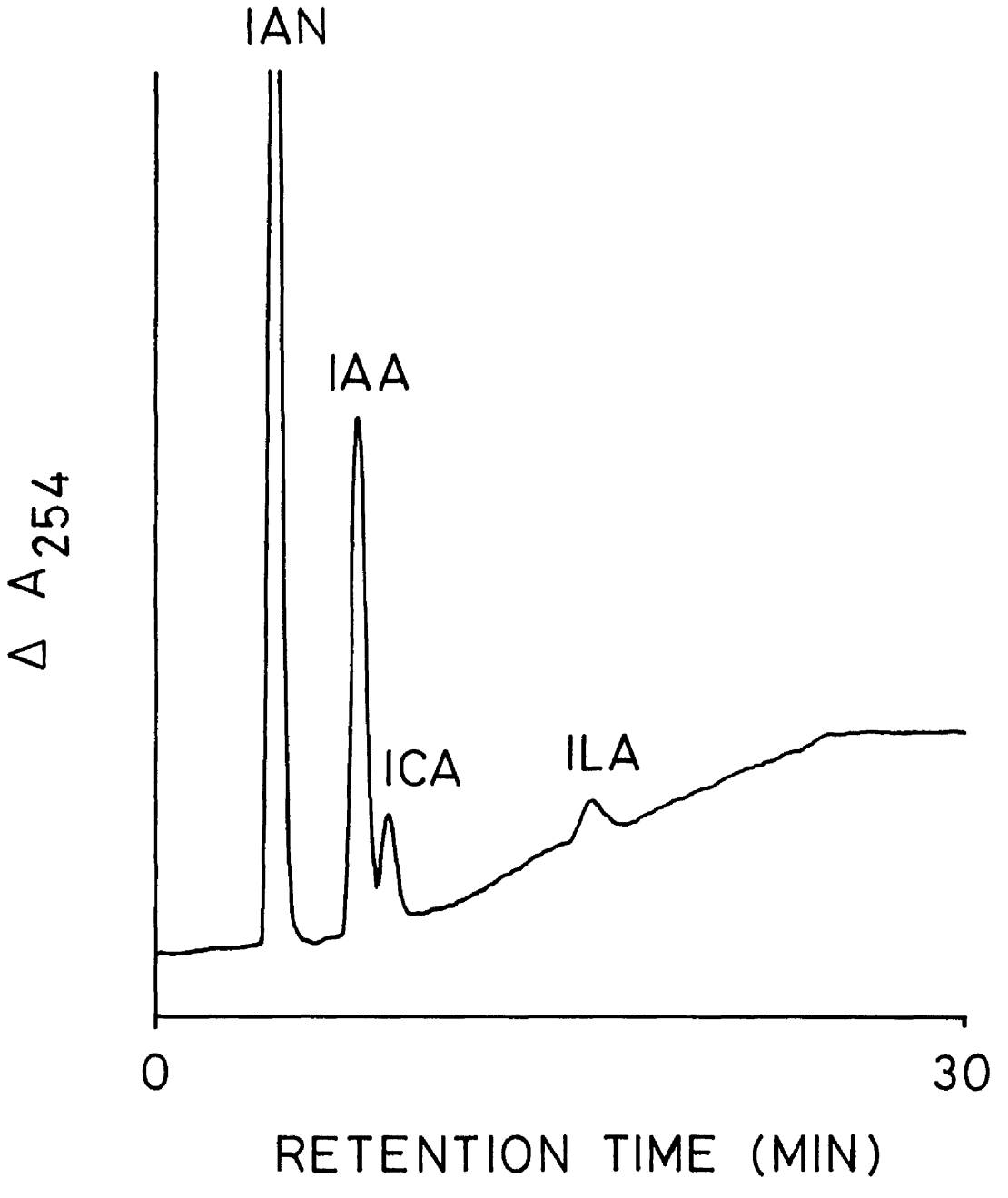

IAN
IAA
ICA
ILA
Δ A254
0
30
RETENTION TIME (MIN)

Table 24

Proportion of eluted radioactivity present at the position of the 'cold' IAA marker in extracts of the separated plant parts. Radioactivity was measured by continuous monitoring of HPLC eluates.

% radioactivity:- $\frac{\text{IAA region d.p.m.}}{\text{Total eluted sample d.p.m.}} \times 100$

Experiment	1			2			3			4		
Plant	A	B	C	A	B	C	A	B	C	A	B	C
Fed nodule	8.3	-	10.2	7.3	3.4	2.9	5.6	5.0	4.7	9.7	5.8	3.5
Roots	26.0	-	16.6	-	-	-	11.1	68.5	3.4	51.4	49.8	17.9
Leaves	-	-	-	5.0	2.9	12.3	-	1.5	1.9	2.4	5.7	1.3
Stem - bark)	-	-	-	14.9	5.6	-	4.3	7.2	6.5	6.7	4.2	7.6
- wood)										5.1	6.1	5.4
Nodules	-	-	-	-	1.9	-	-	-	-	(a) 4.6[ii] (b) 6.9	(a)22.7[ii] (b)37.6	-

(i) - Radioactivity level of extract too low for analysis.

(ii) Duplicate analyses of extract.

considerable variation between the extracts. The leaf extracts contained the least amount of IAA-like activity (1-12%). Least variation between extracts occurred in the fed nodules (3-10%). Stem extracts had a range of IAA-like activity of 4-15%; separate analysis of bark and wood revealed no differences between the stem tissues. The unfed nodules were of generally low activity, which prevented HPLC analysis of all but three extracts. These gave variable results which showed two extracts with low (2-7%) IAA-like radioactivity and one extract with a much higher level (23-38%).

The HPLC radioactivity monitor traces suggest the presence in all extracts of a considerable number of radioactive metabolites of both low and high polarity compared to IAA. Although the relative proportions and the nature of the radioactive peaks varied between extracts of the same part from different plants, many of the peaks were common to at least several extracts and also to extracts of other plant components. The main features of the peaks observed were:

(1) Leaf extracts:- Peaks of higher polarity than IAA were a particular feature of leaf extracts, especially in the extract of plant 2B and the extracts from experiments 3 and 4. The retention times of the largest of the polar peaks varied between 35 - 41 min., but some smaller peaks had elution times of 60 min. The HPLC traces suggested extensive metabolism compared with other plant parts.

(2) Fed nodule extracts:- Radioactivity traces of extracts from experiment 2 (in which the rate of uptake of ^{3}H-5-IAA into the nodule and its subsequent movement to the rest of the plant was slow), showed peaks of high polarity, some of which eluted only after 50 min. Much polar radioactivity was also present in the extensively metabolised extracts from experiment 1. Peaks of lower polarity than IAA were apparent in extracts from experiment 4.

(3) Root extracts:- Highly polar radioactive peaks were present in extracts from experiments 1 and 4, although TLC analysis of extracts from experiment 1 had shown no large peaks other than in the IAA region. Low polarity radioactivity was most obvious in

extracts from experiment 4; none was present in extracts from experiment 1.

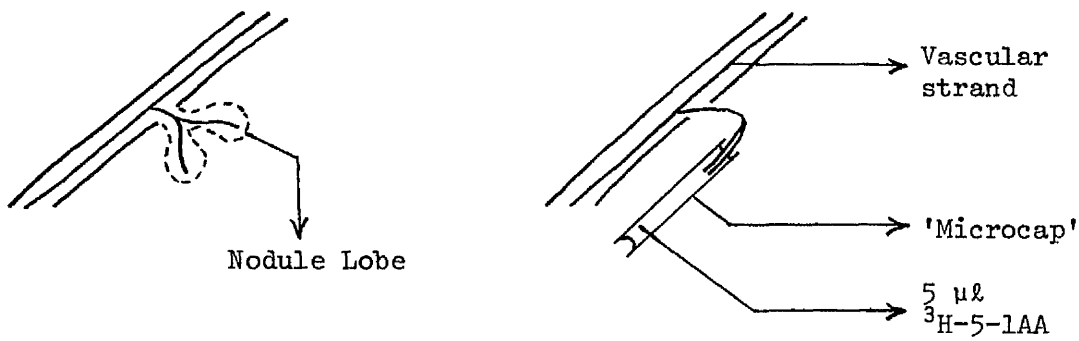
(4) Stem extracts:- Separate analysis of bark and wood produced similar profiles of eluted radioactivity, although the low activity of the extracts may have masked small differences. Radioactive peaks of lower polarity than IAA were present in extracts from experiment 4.

(5) Unfed nodule extracts:- These were of low activity and although some low and high polarity radioactivity was evident, they did not show extensive metabolism compared to other plant parts.

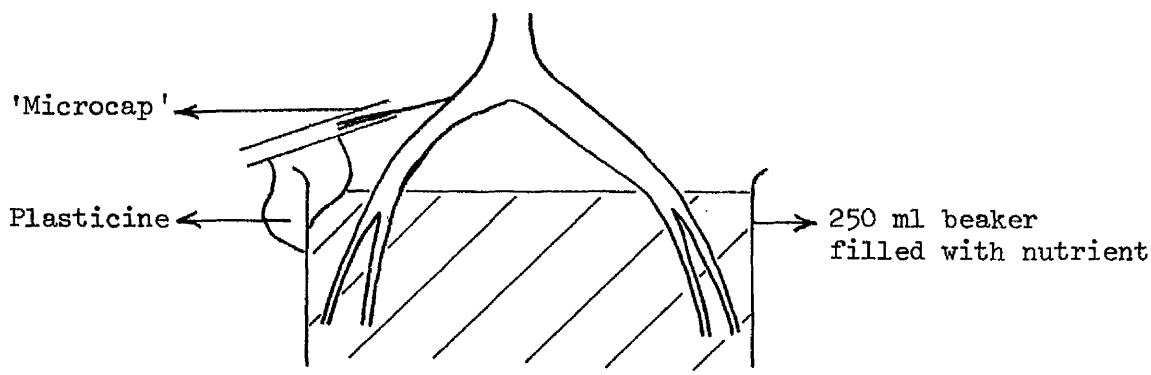
3.3 Application to vascular strands of root nodules

Glasshouse-grown plants were transferred to a controlled environment cabinet (19°C, 16h photoperiod; 15°C, 8 h dark period) 14 days prior to the experiment or were grown in water culture. 20 µCi 5-^{3}H-IAA (i.e. 8 x 10^{-5}mmol IAA) in 5 µl 20% aqueous ethanol were applied to each of three plants in two separate experiments.

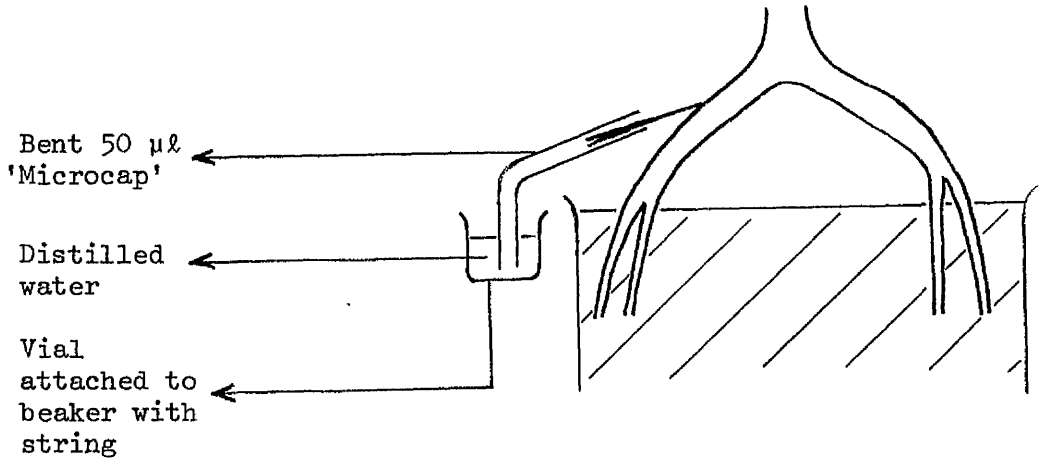
Exposure of Vascular Strand



Application of Label



Chase



The nodule lobes were gently crushed and the tissues external to the stele were carefully removed with forceps to expose the vascular strand(s) as far as its connection with the root. A section of 50 µℓ pipette ('Microcap', Drummond Scientific Co., U.S.A.) was filled with distilled water and placed over the exposed vascular strands. When nodule vascular strands of all three plants were exposed, the mocropipette containing radioactive label was placed in position and the distilled water-filled mocropipette replaced when the label had been taken up.

Uptake of the label was complete within 2½ hours in all plants. The three plants in each experiment were harvested separately, 24 hours after the start of the application of label, and plant parts were extracted as described in Section 1. Aliquots of the acidic ether fraction were analysed by TLC and preparative HPLC and the residues remaining after aqueous methanol extraction were assessed for radioactivity by liquid scintillation sample oxidation, as described in Section 1.

Results

Location of radioactivity

Methanol soluble radioactivity was detected in all parts of the alder plant following application of labelled IAA to the vascular

strand of a root nodule (Table 25). A major part of the methanol soluble radioactivity of each plant (sum of methanol soluble radioactivity in each of plant parts) remained in the fed tissue (22-77%) but a substantial proportion was detected in the roots (16-44%) and the leaves (3-36%), with a smaller amount in the stem (1-14%). No differences were detected in the radioactivity of the conducting tissues of the stem following separate analysis of the bark and wood. Low levels of radioactivity were detected also in the unfed nodules (0.3 - 2.0%) and in the apical bud (0.05 - 0.5%).

The specific radioactivities of the separated plant parts (Table 26) showed a much more uniform distribution of isotope concentration throughout the plant than is apparent from Table 25 . Although the greatest concentration of radioactivity in 3 of the 6 plants was in the roots (except for the fed vascular strand), in 2 of the remaining 3 plants, the apical bud showed the highest specific radioactivity. The unfed nodules also increased in importance as recipients of radioactive label although their specific activity was always lower than the roots (1-20 times). The separated bark and wood of the stem were both of similar specific activity.

The nature of the methanol soluble radioactivity was investigated further to reveal differences which may have resulted from metabolism of the translocated label, in or en route to the various plant parts. Initially, the aqueous phase of the methanolic extracts was partitioned against ether at pH3 (and pH8 in experiment 1) giving rise to three (or four) fractions, amongst which the total radioactivity of each plant part was distributed (Table 27 and Appendix Table 3). The differences in the partitioning of the radioactivity of extracts of the bark and wood of the stem, analysed separately in experiment 2, are a notable feature of this data. Compared to other plant parts, the aqueous fraction of the bark extracts contained the highest proportion of radioactivity (79%) and the wood the lowest (37%). Conversely, the proportion of radioactivity in the acidic ether fraction of bark extracts (10%) was low (although similar to that of other plant parts) and that in wood extracts high (50%). The residual fraction of both these stem tissues contained a similar level of radioactivity.

Table 25

Relative distribution of methanol soluble radioactivity. Radioactivity in separate plant parts as percentage of whole plant (sum of methanol soluble radioactivity of each plant part).

Plant part	% of whole plant methanol soluble radioactivity $\left(\frac{\text{Plant part d.p.m.}}{\text{Combined plant part d.p.m.}} \times 100 \right)$		
	A	B	C
Experiment 1			
Fed vascular strand	65.3	22.0	76.6
Roots	16.4	44.4	18.5
Leaves	11.0	16.6	3.3
Stem	5.8	14.2	1.2
Nodules	1.0	2.3	0.3
Apical bud	0.2	0.5	0.09
Experiment 2			
Fed vascular strand	37.6	29.4	36.4
Roots	40.8	26.6	18.2
Leaves	17.0	31.0	36.3
Stem - bark	1.8	5.3	3.4
- wood	2.4	7.1	4.1
Nodules	0.4	0.6	1.5
Apical bud	0.05	0.05	0.1

Sum of methanol soluble radioactivity extracted from each plant part (d.p.m. x 10^{-6})

EXPERIMENT 1			EXPERIMENT 2		
A	B	C	A	B	C
11.9	15.0	11.7	25.9	39.7	52.6

Table 26

The specific methanol soluble radioactivity of the separate plant parts.

Plant part	Specific activity (d.p.m./g. fresh weight tissue x 10^{-5})		
	A	B	C
Experiment 1			
Fed vascular strand	2800	1900	2200
Roots	4.1	15	4.8
Leaves	4.0	8.7	1.1
Stem	4.6	16	1.1
Nodules	3.9	12	1.5
Apical bud	7.2	17	2.6
Experiment 2			
Fed vascular strand	3500	3000	8700
Roots	21	22	24
Leaves	3.2	14	26
Stem - bark	3.2	15	14
- wood	2.8	13	12
Nodules	1.0	4.2	13
Apical bud	2.4	3.8	9.3

Table 27

Distribution, following solvent partitioning, of the radioactivity of separated plant parts between aqueous, basic ether, acidic ether and residual fractions.

Plant part	% of plant part radioactivity partitioned into four fractions. Fractionated radioactivity x 100 / Combined radioactivity of fractions							
	Aqueous		pH8		pH3		Residue	
Experiment	1	2	1	2	1	2	1	2
Fed vascular strand	62.3	47.1	7.4		7.7	11.8	22.3	41.2
Roots	43.0	58.5	2.7		13.4	2.6	40.9	38.9
Leaves	54.6	68.7	3.3		7.1	12.2	35.1	19.1
Stem - bark	42.9	78.7	6.4		6.2	9.5	44.4	11.7
- wood		37.1				49.8		13.1
Nodules	67.5	56.7	1.8		5.4	4.9	25.3	38.4

(i) Solvent partitioning at pH8 was performed only in experiment 1

(ii) Data are means from 3 replicate plants per experiment (see Appendix Table 3).

The relative distribution between the plant parts of the radioactivity in the acidic ether fraction, into which IAA would partition, is examined further in Table 28 . By expressing the acidic ether soluble radioactivity of the separate plant parts as a percentage of that in the whole plant (derived by summation of the acidic ether soluble radioactivity of each plant part), it can be shown that in four out of six plants, most radioactivity was present in the fed tissue and roots (62-98%) and in the remaining two plants (experiment 2), most radioactivity was detected in the stem (41-44%). The leaves contained a significant proportion (2-29%) of the total acidic ether soluble radioactivity, and the nodules contained the smallest proportion (0.1 - 2.3%). Separate analysis of the bark and stem in experiment 2, showed that the bark contained much less acidic ether soluble radioactivity (1-5%) than the wood (27-39%).

The specific acidic ether soluble radioactivity of the plant parts showed considerable variability between experiments 1 and 2 (Table 29). The roots showed the highest specific activity (excluding the fed tissue) in experiment 1, whereas in experiment 2, excluding the fed tissue, the specific activity of the wood was highest in all three plants. The unfed nodules were generally of low specific activity, having in three of the six plants, specific activities lower than that of any other plant part.

Analysis of acidic ether soluble radioactivity

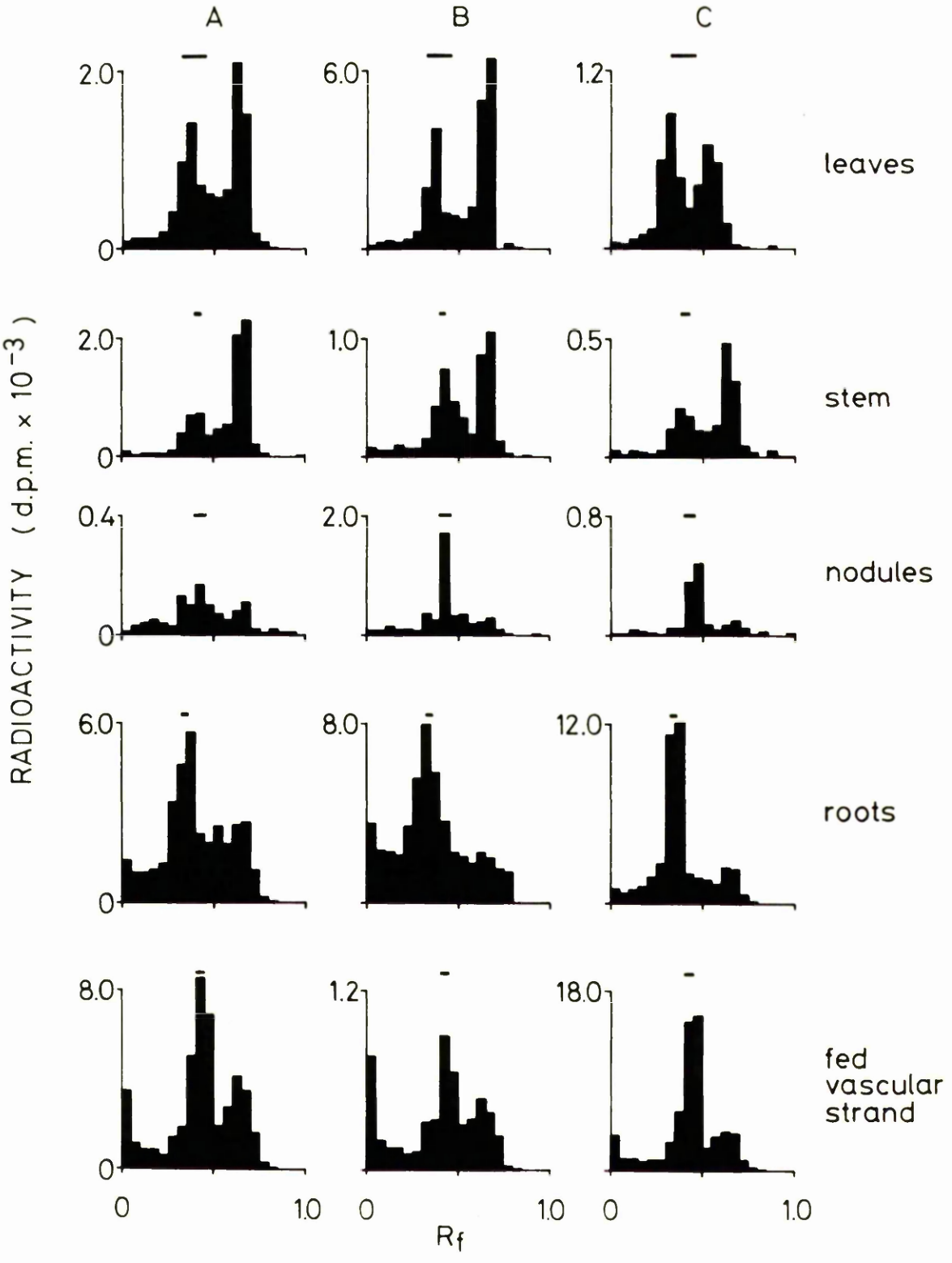
(a) Thin-layer chromatography

All chromatograms showed two groups of radioactive peaks, except those of two nodule and two root extracts in experiment 1, and two root extracts in experiment 2 (Figure 14). The first group of peaks, which corresponded to the 'cold' IAA marker, was predominant in all root extracts, and in extracts of the fed vascular strands and nodules from experiment 1. The second group of radioactive peaks was predominant or prominent in all leaf and stem extracts and in the nodule extracts from experiment 2. Chromatograms of certain plant part extracts were atypical in certain respects, namely:

Figure 14

Thin-layer radiochromatograms of acidic ether extracts of plants fed 5-^{3}H-IAA via the vascular strand of a root nodule.

5-^{3}H-IAA (20 μCi - 8 x 10^{-5} mmol) was applied to the vascular strand of a root nodule on each of three plants in two separate experiments. Plants (A, B and C in each experiment) were harvested individually 24 hours after feeding and divided into parts for extraction and analysis.
Chromatogram solvent system: isopropanol:ammonia: water:: 8: 1 : 1 v|v.
TLC marker spots represent the R_F of 'cold' IAA chromatographed alongside the extracts.


EXPERIMENT 1
A
B
C
2.0
0
6.0
1.2
leaves
2.0
1.0
0.5
stem
0.4
2.0
0.8
nodules
6.0
8.0
12.0
roots
8.0
1.2
18.0
fed vascular strand
RADIOACTIVITY (d.p.m. x 10^{-3})
0
1.0
R_f

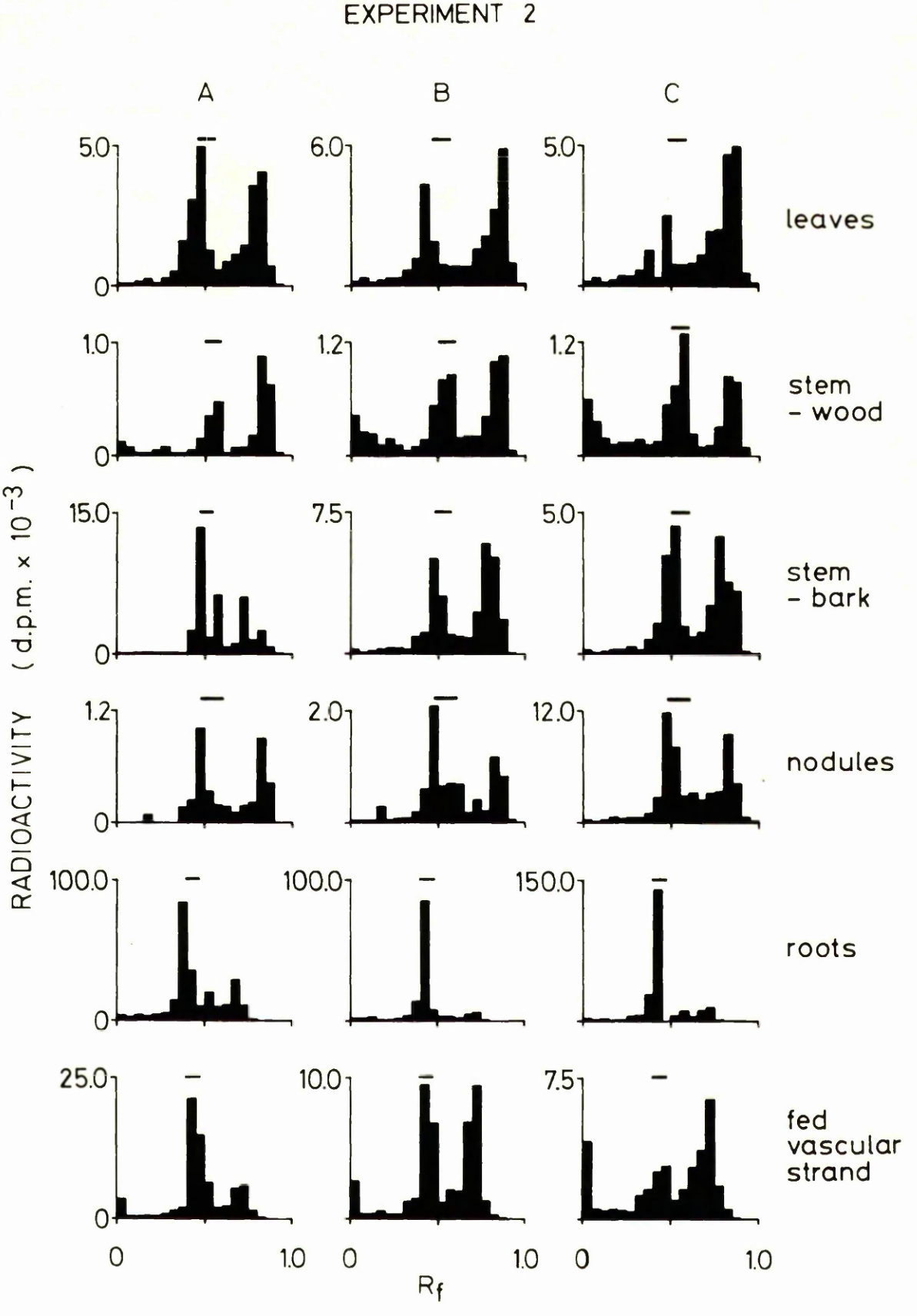

EXPERIMENT 2
A
B
C
5.0
0
6.0
0
5.0
0
leaves
1.0
0
1.2
0
1.2
0
stem
- wood
15.0
0
7.5
0
5.0
0
stem
- bark
1.2
0
2.0
0
12.0
0
nodules
100.0
0
100.0
0
150.0
0
roots
25.0
0
10.0
0
7.5
0
fed
vascular
strand
0
1.0
0
1.0
0
1.0
R_f
RADIOACTIVITY (d.p.m. × 10^{-3})

Table 28

Relative distribution of acidic ether soluble radioactivity. Proportion of radioactivity in separate plant parts as percentage of whole plant (sum of acidic ether soluble radioactivity of each plant part).

Plant part	% of whole plant acidic ether soluble radioactivity $\left(\frac{\text{Plant part d.p.m.}}{\text{Whole plant d.p.m.}} \times 100\right)$		
	A	B	C
Experiment 1			
Fed vascular strand	46.4	8.9	70.1
Roots	24.6	68.8	27.4
Leaves	24.9	6.3	1.5
Stem	3.9	14.9	0.7
Nodules	0.3	1.1	0.3
Experiment 2			
Fed vascular strand	60.1	29.1	20.4
Roots	1.5	5.6	5.0
Leaves	10.1	24.0	28.6
Stem - bark	1.0	3.8	5.2
- wood	27.2	37.2	38.6
Nodules	0.1	0.3	2.3

Sum of acidic ether soluble radioactivity extracted from each plant part (d.p.m. x 10^{-6})

EXPERIMENT 1			EXPERIMENT 2		
A	B	C	A	B	C
1.5	1.3	1.7	6.6	7.0	5.4

Table 29

The specific acidic ether soluble radioactivity of the separated plant parts.

Plant part	Specific activity (d.p.m./g. fresh weight tissue x 10^{-5})		
Experiment 1			
Fed vascular strand	240	70	49
Roots	0.8	2.1	1.0
Leaves	1.1	0.3	0.07
Stem	0.4	1.5	0.1
Nodules	0.2	0.5	0.2
Experiment 2			
Fed vascular strand	1400	520	500
Roots	0.2	0.8	0.7
Leaves	0.5	1.9	2.1
Stem - bark	0.4	1.9	2.2
- wood	8.0	12	12
Nodules	0.09	0.3	2.1

(1) Root extracts: - Two extracts in experiment 1 showed a peak of activity at the origin.

(2) Fed vascular strand extracts: - In experiment 2, the first group of radioactive peaks was predominant only in the extract from plant A. All chromatograms showed a peak of radioactivity at the origin.

The distribution of radioactivity was similar on chromatograms of the bark and wood when these were analysed separately in experiment 2, with the exception of a peak of radioactivity at the origin in two wood extracts.

The proportion of eluted radioactivity present at the position of the 'cold' IAA marker in each of the extracts is shown in Table 30 . The root extracts of experiment 2 contained the highest proportion of IAA-like radioactivity (52-74%) , although in experiment 1 the range was lower (28-54%) because of the greater number of metabolite peaks. The leaf extracts in both experiments showed a low proportion (18-40%) of radioactivity in the IAA region. Similar proportions of IAA-like radioactivity were present in the unfed nodule (35-57%) and fed vascular strand extracts (35-59%).

(b) <u>High performance liquid chromatography</u>

General points relating to preparative HPLC analyses are discussed at the beginning of the relevant results paragraphs in section 3.2.

The radioactivity co-eluting with the 'cold' IAA marker on preparative HPLC is shown for each sample run in Table 31 . The levels of radioactivity in the IAA-region were generally much lower (up to 9 times) than those obtained from TLC analysis. The root extracts contained the highest level of radioactivity associated with the IAA marker in both experiments (15-62%) ,whereas the leaf extracts had the lowest levels, except for plant 2A (which was generally dissimilar to the two other replicates in experiment 2). The bark and wood, analysed separately in experiment 2, had similar levels of IAA-like radioactivity.

Radioactive peaks of both lower and higher polarity, in relation

Table 30

Proportion of eluted radioactivity present at the position of the 'cold' IAA marker in extracts of the separated plant parts. Radioactivity was measured by liquid scintillation counting of eluates from silica gel thin-layer chromatography plates.

% radioactivity:- $\frac{\text{IAA region d.p.m.}}{\text{Total eluted sample d.p.m.}} \times 100$						
Experiment	1			2		
Plant	A	B	C	A	B	C
Fed vascular strand	44.3	34.9	57.5	58.6	37.1	18.0
Roots	29.3	28.4	53.8	51.5	74.4	70.4
Leaves	24.3	25.4	30.5	39.8	28.5	18.4
Stem - bark	17.3	23.1	20.6	55.7	30.6	37.8
- wood				29.3	34.9	39.9
Nodules	34.5	51.5	57.0	42.1	49.6	46.2

Analysis of variance					
Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio(F)	Significance
Between experiments	7925	4	1981	2.0	Not significant
Between plant parts	1355	1	1355	1.4	Not significant
Residual	3955	4	989		
Total	13235	9			

Table 31

Proportion of eluted radioactivity present at the position of the 'cold' IAA marker in extracts of the separated plant parts. Radioactivity was measured by continuous monitoring of the HPLC eluates.

% Radioactivity:-	$\frac{\text{IAA region d.p.m.}}{\text{Total eluted sample d.p.m.}} \times 100$					
Experiment	1			2		
Plant	A	B	C	A	B	C
Fed vascular strand	4.6	3.2	-	22.3	16.1	6.1
Roots	39.3	39.9	14.8	15.9	62.4	36.0
Leaves	2.7	-	-	16.7	1.9	2.1
Stem - bark	-	-	-	21.1	3.6	3.2
- wood	-	-	-	9.4	4.1	4.3
Nodules	-	-	-	-	-	-

(1) - Radioactivity level of extract too low for analysis.

to IAA, were detected in all extracts (Figure 15). The main features of these peaks in the different extracts were:

(1) Leaf extracts: - A large number of peaks were resolved, spread over a wide polarity range. Peaks of much higher polarity than IAA were particularly prominent.

(2) Fed vascular strand extracts: - Extracts from experiment 1 contained prominent peaks which were much more polar than IAA, as well as other peaks covering a wide range of polarity.

(3) Stem extracts: - Prominent peaks of much higher polarity than IAA were present in extracts from experiment 2; these were eluted up to 52 minutes from the injection point. A similar distribution of radioactive peaks was exhibited by extracts of the bark and wood, when these were analysed separately in experiment 2.

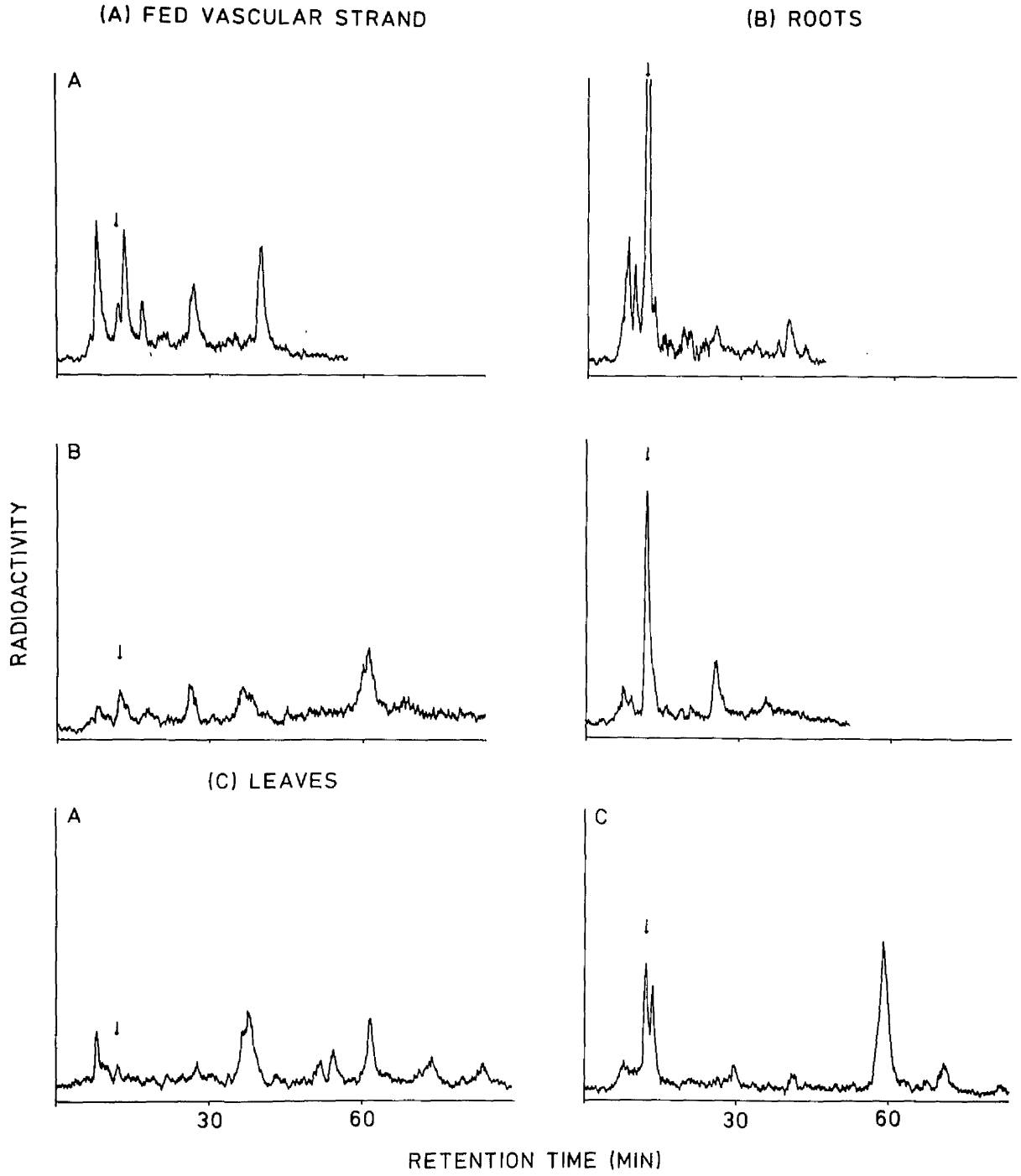
Figure 15

Preparative high performance liquid radiochromatograms of acidic ether extracts of plants fed 5-^{3}H-IAA via the vascular strand of a root nodule.

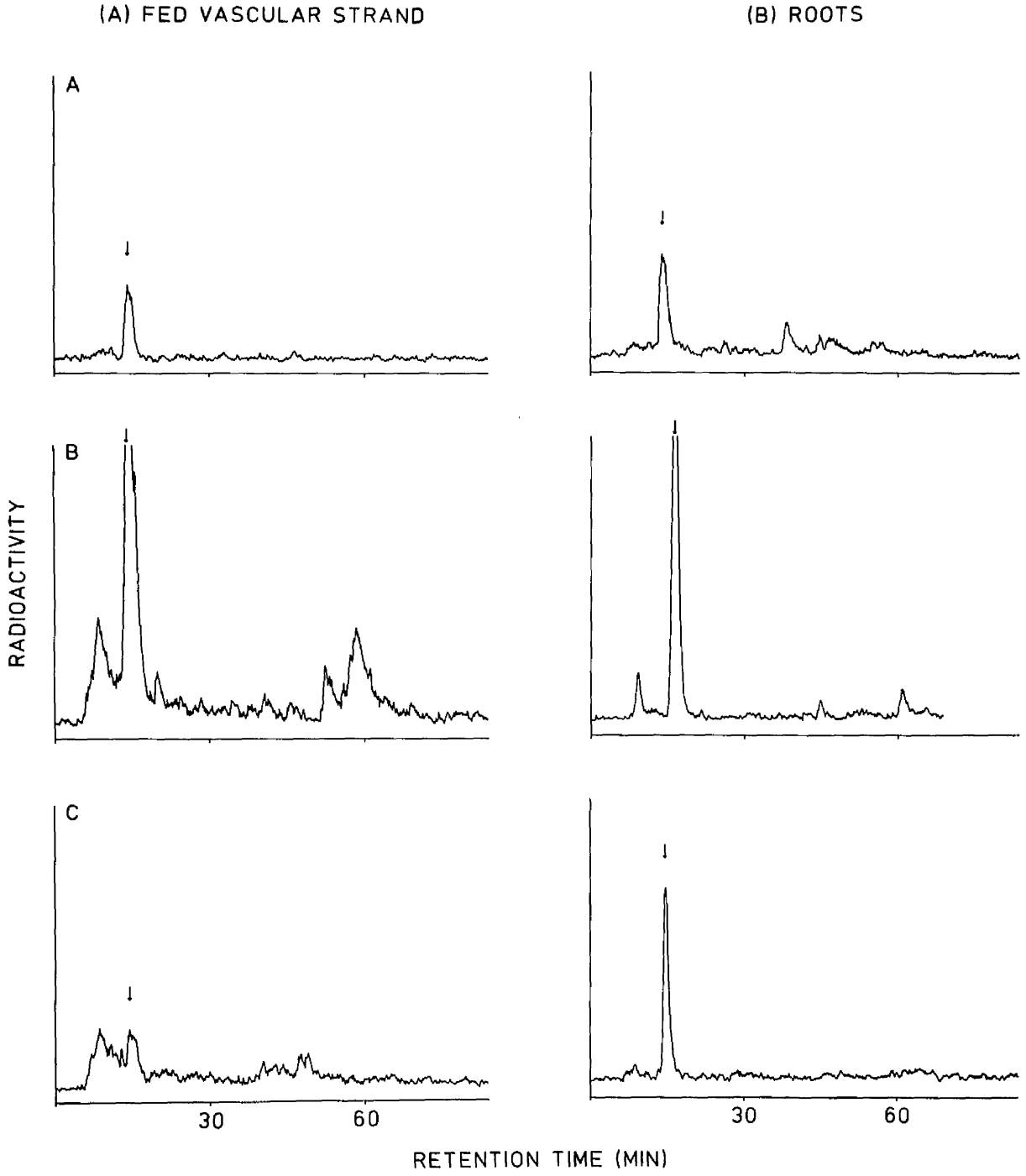
5-^{3}H-IAA (20 μCi - 5 x 10^{-5} mmol) was applied to the vascular strand of a root nodule on each of three plants in two separate experiments. Plants (A, B and C in each experiment) were harvested individually 24 hours after feeding and divided into parts for extraction and analysis.
Arrows represent the elution point of 'cold' IAA added to, and co-chromatographed with, the extracts.

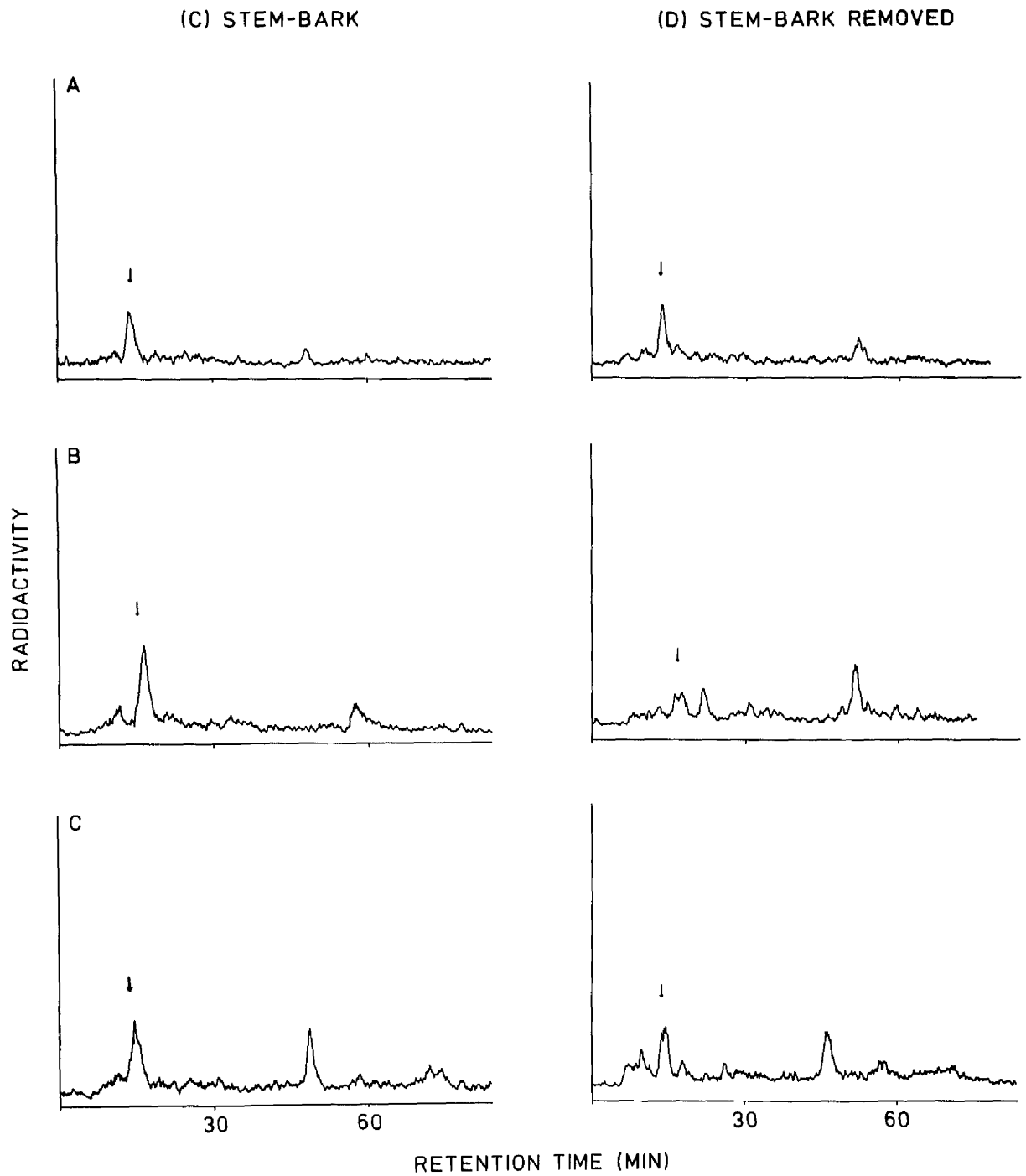
Column:- Partisil 10 or 20
Column temperature:- 30 ± 0.5°C
Stationary phase:- 40% 0.5 M formic acid or 40% 1M acetic acid
Mobile phase:- Gradient of 50-75% ethyl acetate in hexane in 40 minutes, extended when required to 100% ethyl acetate in hexane
Flor rate:- Mobile phase 4.6 ± 0.1 $ml.min^{-1}$.
scintillant 2.0 ± 0.1 $ml.min^{-1}$.

EXPERIMENT 1

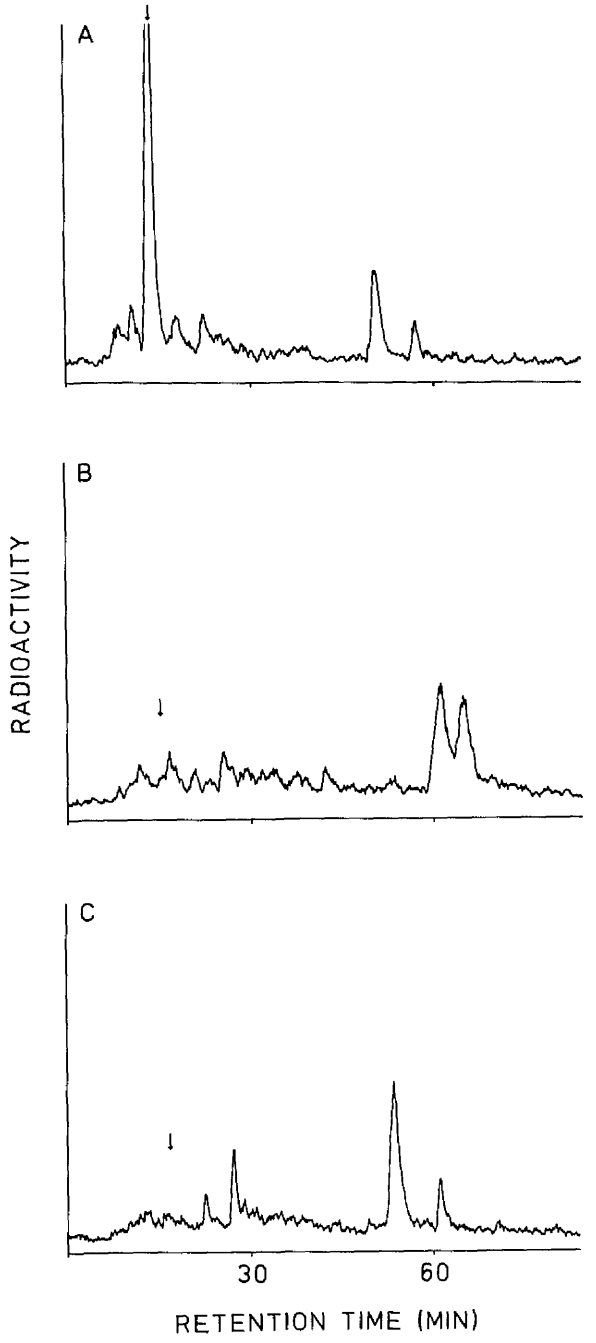


EXPERIMENT 2




(C) STEM-BARK
(D) STEM-BARK REMOVED
A
B
C
RADIOACTIVITY
30
60
30
60
RETENTION TIME (MIN)

(E) LEAVES



4. Discussion

The nature, metabolism and distribution within nodulated alder seedlings of radioactivity derived from labelled IAA, was investigated after application of the hormone to probable sites of auxin synthesis in the plant. These experiments were initial attempts to assess the validity of published speculation concerning possible roles for hormone movement in the integration of various activities of nodulated plants (see Introduction). Recovery of methanol soluble radioactivity in extracts of all plant parts, following application of 1-^{14}C-IAA both to the shoot apex and to the root nodules, demonstrated the movement of radiotracer within the nodulated plants, although most of the label remained in the fed organ, even after 72 hours (Table 12 a,b,c). Because of the possibility of decarboxylation of 1-^{14}C-IAA (Aåsheim and Iversen, 1971), introduced in a non-specific manner by micro-pipette into plant tissue, no attempt was made in these preliminary experiments, to establish critically the identity of the radioactive substances in the receiving tissues.

Following the demonstration of movement of methanol soluble radioactivity, derived from 1-^{14}C-IAA, within alder seedlings, further experiments with 5-^{3}H-IAA were performed and more detailed analysis conducted of the radioactivity extracted from the various plant parts. IAA, labelled on the indole ring, was used to avoid enzymic fixation of decarboxylated $^{14}CO_2$ into other compounds. It was confirmed that most of the methanol soluble radioactivity was associated with the fed organ and that only low levels of radioactivity were present in the unfed nodules. The low proportion of methanol soluble radioactivity (0.1 - 5.5%, Table 13) which reached the roots following feeding of the stem apex, compared to that which moved into the shoot following nodule feeding (1.4 - 43.9%, Tables 18, 25) suggests the existence of a slower transport pathway from the stem apex, which might be similar to that reported previously by several authors (see Introduction).

These results demonstrate clearly the widespread movement of radioactivity within the plant, which occurred following micro-injection of radiotracer. However, all experiments designed to study plant processes by following the fate of applied radiotracer, are subject to a variety of experimental artefacts peculiar to the application

technique used. For example, Wheeler et al. (1978) using nodulated root segments (not intact plants, where nodule activity may be less impaired), showed that the insertion of micropipettes into nodule lobes of Alnus glutinosa reduced nitrogenase activity, although activity often recovered after 35-40 minutes. The effect noted was on nitrogenase activity, a sensitive indicator of nodule disturbance, but it is probable that other nodule processes might also be affected to some degree for example, transport processes into and out of the nodules.

Apart from damage and wounding effects, additional artefacts undoubtedly arise in 'fed' tissues by the introduction of radiotracer into sites within the tissue, to which the compound of interest does not normally have free access. For example, in this project in nodule-fed plants, when IAA was applied to nodule lobes by micropipette, it was possible that the micropipette might have penetrated the stele, effectively injecting radiotracer directly into the conducting tissues and therefore bypassing the barriers which normally control movement of IAA within the nodule. This possibility was checked by employing two methods of nodule feeding; firstly by micropipette inserted into the intact nodule lobe, and secondly via the exposed vascular strand of a nodule cluster. Direct access of nodule lobe-fed tracer to the conducting tissues could produce similar patterns of movement and metabolism of the radiotracer with both methods of application, whereas different patterns would support the possibility of injection of at least some radiotracer into the nodule cortex rather than directly into the stele. Injection into the cortex, would increase the possibility of at least some radiotracer following similar transport pathways within the nodule, to those of endogenous IAA.

The greater accumulation in the shoot (5-44% of total plant methanol soluble radioactivity, Table 25), of radioactivity derived from 5-^{3}H-IAA applied via the vascular strand, compared to that when label was applied via the nodule lobe (1-35% of total plant methanol soluble radioactivity, Table 18), supports a route for micro-injection of the intact nodule other than directly into the stele. Eventual transfer to the stele of label applied via the nodule lobe, followed by export from the nodule in the xylem, would presumably delay the movement

of radiotracer to the shoot compared to those plants fed directly via the vascular tissue. This might account for the greater accumulation, within a 24 hour period, of radioactivity in the shoot of vascular strand-fed plants compared to those fed via a nodule lobe. A further difference resulting from the two methods of feeding, emerged in later experiments when the bark and wood of the stem were analysed separately. While plants fed by either route showed little difference in the distribution of methanol soluble radioactivity between bark and wood extracts, in vascular strand-fed plants, the proportion of radioactivity in the acidic ether fraction (containing IAA and radioactive derivatives) of the wood was much greater (7-30 times, Table 28) than that of the bark. Further comparison of the specific radioactivities of the acidic ether fraction in vascular strand-fed plants, confirmed that the concentration of label in the wood was higher (5-20 times, Table 29) than that in the bark. These results could reflect a greater translocation of an unchanged or closely related form of 5-^{3}H-IAA in the xylem transpiration stream of vascular strand-fed plants compared to plants fed via the intact nodule. In the latter group of plants there would be increased opportunity for metabolism of IAA in the nodule before it reached the conducting tissues. However, the similar patterns of distribution of radioactivity, shown by HPLC of the acidic ether extracts of bark and wood from vascular strand-fed plants (Figure 15), suggested in both tissues qualitatively similar acidic ether soluble products of metabolism of 5-^{3}H-IAA derived radioactivity (the nature of the radioactivity in the aqueous fraction was not investigated) even though their rates of production were apparently different.

The fate of that portion of the original 5-^{3}H-IAA which remained as IAA following movement to various plant parts from the site of application, was of primary interest in this project. An indication of the extensive metabolism of the radiotracer, is provided by the initial solvent partitioning of radioactivity in methanolic extracts of the plant parts. A major portion of the radioactivity in most plant parts, remained in the aqueous fraction both in apical bud fed plants (21-64%, although in some of the leaves, 100% Table 15) and in the nodule lobe and vascular strand-fed plants (42-79% Tables 20,27).

This radioactivity cannot be attributed to exchange of ^{3}H with ^{2}H from water, since aliquots were dried before counting but isotope exchange with other water soluble compounds, as well as metabolism or conjugation of IAA to produce water soluble compounds, is a possibility. A preliminary examination of the aqueous fraction using dialysis, suggested that a proportion (20%) might be associated with higher molecular weight compounds. The recovery of water soluble IAA derivatives or IAA conjugates as a result of feeding labelled IAA to intact plants has been previously reported (Eschrich 1968; Morris et al., 1969; Bourbouloux and Bonnemain, 1974). Of particular interest, is the incorporation of a substantial proportion (45%) of the applied radioactivity into five amino acid conjugates (three of the five formed were identified by mass spectrometry and the remaining two by TLC and hydrolysis) after incubation of 2-^{14}C-IAA with Parthenocissus tricuspidata crown gall tissue (Feung et al., 1976). Both root nodule and crown gall tumours arise following infection by bacteria, so it is not inconceivable that there may be similarities in IAA metabolism in the two tissues. The partition coefficients of several of the amino acid conjugates identified by Feung, were such that they were present in both ethereal and aqueous fractions, so their identification would be necessary before assessment of their relative importance as metabolites in a particular tissue, could be made. In the present investigation, the precise nature of the radioactivity in the aqueous fraction of plant part extracts, including the nodules, awaits further elucidation.

Further study of the fate of 5-^{3}H-IAA, centred on a more detailed examination of the relative proportions and nature of the label extracted from the separated plant parts, which partitioned into ether from the aqueous phase at pH3. Generally, the proportion of total radioactivity recovered in this fraction was low (apical bud feeding experiments, 24-26% of radioactivity, excluding that remaining in the residue, Table 14; nodule and vascular strand feeding experiments, 0.3 - 22%, of total radioactivity, Appendix Tables 2a,b, 3), indicating, as mentioned above, that substantial metabolism or inactivation of IAA occurred in, or en route to, the various plant parts. Since the radioactivity detected in the acidic ether fraction of the various plant

parts, is indicative only of the presence of the labelled indole ring and extensive breakdown of the ring is unlikely to occur, the nature of the radioactivity was analysed further both by TLC and preparative HPLC. The higher resolution of HPLC showed that the proportion of radioactivity which co-eluted with the IAA marker was usually a very much smaller quantity (Figures 12,15) than that indicated by TLC analysis (Figures 11, 14). For example, in experiment 4 of the nodule feeding experiments, TLC of the wood extract (from plant B), showed one main peak which accounted for 34% of the radioactivity eluted from the plate (Table 23), whereas on HPLC analysis, the label which co-chromatographed with the IAA marker, represented only 6% of the eluted radioactivity (Table 24). Similarly, in experiment 2 of the vascular strand feeding experiments, TLC of the wood (plant B) showed two main peaks, the first of which corresponded to the IAA marker and represented 35% of the radioactivity eluted from the plate (Table 30), whereas on HPLC analysis, only 4% of the eluted radioactivity corresponded with the IAA marker (Table 31). The higher resolution of acidic ether soluble 5-^{3}H-IAA derivatives by HPLC, provides much more information than TLC concerning the metabolism of IAA and should afford a more definitive measurement of the amount of unchanged IAA in the receiving tissue.

Evidence for movement of radioactive IAA from the apical bud of nodulated alder seedlings was based on co-chromatography with IAA by TLC. In subsequent experiments with nodule lobe and vascular strand-fed plants, HPLC of acidic ether extracts, as mentioned above, showed that a large proportion of the radioactivity which had co-chromatographed with IAA by TLC, could be resolved to reveal other compounds. Evidence from TLC alone for movement of IAA within alder seedlings, is obviously therefore inadequate and can at best only reinforce the possibility of translocation of IAA from the apex to other plant parts.

As was shown for endogenous IAA analyses in Part I, Sections 2.3 and 2.4, analysis of the IAA region of acidic ether soluble extracts by preparative HPLC rather than TLC, still does not completely separate IAA from contaminants, since further purification of the IAA region of preparative HPLC eluates from endogenous extracts, successively removed U.V. absorbing components (Figures 4,5,8). Even after

additional chromatography by preparative HPLC therefore, employed with extracts from nodule lobe and vascular strand-fed plants, definite conclusions regarding the identity of the radioactivity co-eluting with IAA cannot be drawn. Nevertheless, in these studies, the additional information provided by the use of two chromatographic systems for analysis of radioactive metabolites derived from 5-^{3}H-IAA, does provide somewhat stronger evidence for movement of an IAA-like compound between the root nodules and other parts of alder seedlings. In many published accounts of movement of radioactive IAA in other species, either TLC alone of ethanol soluble extracts, or TLC following solvent partitioning, has been used for assessment of the nature of the radioactivity. These include studies of movement of IAA from the shoot apices of dwarf pea (Morris et al., 1969) and Vicia faba (Bourbouloux and Bonnemain, 1974). Although conclusions concerning the movement of IAA based on TLC may be suspect for the reasons discussed above, studies noting accumulation of radioactivity in the lateral root primordia of plants fed radioactive IAA via the shoot apex are of interest (Morris et al., 1969; Rowntree and Morris, 1979), since they suggest a way in which movement of IAA from the shoot could influence root development by regulating, for example, initiation of lateral root primordia. Although the precise location of radiotracer in the alder root system was not determined in the present study, the occurrence of such movement would have important implications for nodule development since in non-legumes, nodules may be viewed anatomically as modified lateral roots (Callaham and Torrey, 1977).

There is a paucity of published information concerning movement of IAA from roots to shoot in intact plants. However, a potential for movement to all plant parts of the cytokinin, zeatin, following application by micropipette of ^{14}C-zeatin to alder nodules, has been demonstrated (Henson and Wheeler, 1977d). Zeatin and a number of metabolites were detected by a variety of chromatographic procedures including ion-exchange, paper, TLC and Sephadex LH-20; the occurrence of more polar metabolites such as glucosides was particularly notable. In the present experiments, acidic ether extracts of roots generally contained the highest proportion of radioactivity associated with IAA compared to other plant parts. For example, HPLC analysis of root

extracts from nodule lobe and vascular strand-fed plants showed that 4-69% (Table 24) and 16-62% (Table 31) of radioactivity respectively, eluted at the IAA marker position, suggesting the least metabolism of all the extracts in the acidic ether soluble fraction. Most metabolism of applied IAA was shown generally by the leaves from both nodule lobe and vascular strand feeding experiments where HPLC showed usually, least radioactivity associated with IAA (1-12%, Table 24 and 2-17%, Table 31, respectively).

In the nodule lobe and vascular strand feeding experiments, separate analysis of the bark and stem provided some information concerning the pathways for transport of radiotracer within the plant. The occurrence of IAA-like radioactivity both in the bark (phloem and/or cambium) and wood (xylem) of alder stems is in accordance with published reports, based on a variety of analytical techniques, for the presence of IAA in the phloem and xylem of several different species (Hoad et al., 1971; Hall and Baker, 1972; Hall and Medlow, 1974; Sheldrake, 1973; Allen et al., 1979).

In nodule fed plants, the presence of IAA-like radioactivity in bark extracts suggests either transfer from the xylem, which would be the usual export route from the nodule, or re-export from other plant parts, after they had initially received radioactivity derived from labelled IAA, via the xylem. Xylem to phloem transfer has been shown in the stem of other woody species such as willow (Hoad et al., 1971) and sycamore (Zamski and Wareing, 1974). Re-export of nodule-derived fixed nitrogen compounds has been demonstrated in legumes, from the shoot to other plant parts (Oghoghorie and Pate, 1972; Pate and Flinn, 1973). As well as providing an explanation for the presence of IAA-like radioactivity in the phloem, such recycling of nodule products might account for the accumulation of only very low levels of radioactivity by the unfed nodules in all experiments. All nitrogen fixed by the nodule leaves via the xylem and transpiration stream, so that distal regions of the root and the growing apices of nodules only acquire fixed nitrogen after it has been cycled through the shoot and returned to the root in the translocation stream. In the case of radiotracer fed to a nodule, this cycling process could result in only a low level of movement of radioactivity to the unfed nodules compared to other plant parts which receive radiotracer earlier in the recycling process.

These studies on the mobility of radioactively labelled IAA, have shown that small amounts of hormone-derived label, co-chromatographing with IAA on preparative HPLC, can move from fed nodules of alder seedlings to the shoot of the host plant. In addition, movement of radioactivity from the apical bud of alder seedlings to the roots and nodules has been shown, a proportion of the acidic ether soluble radioactivity co-chromatographing with IAA by TLC; the potential errors of this analytical method have, however, been discussed.

The significance of nodule hormone movement suggested by the mobility of applied radioactive hormone is unknown. However, in a previously published report, in which movement of ^{14}C-zeatin from root nodules to all parts of alder seedlings including the shoot was demonstrated (Henson and Wheeler, 1977d), subsequent comparison of endogenous cytokinin levels in nodulated and non-nodulated plants suggested that the contribution of nodule cytokinins to the complement of the host plant was negligible (Wheeler and Henson, 1978). Therefore, should further studies conclusively prove the mobility of radioactive IAA derived from applied label, between the shoot and nodulated root system of alder seedlings, the relevance to endogenous movement must be questioned in more detail.

The presence of large amounts of IAA may reflect the accumulation of immobile auxin derivatives rather than metabolism, whereas low levels may indicate a high rate of turnover and a low level of immobilisation. Due consideration would be required, therefore, in further work, to be given to the physiological significance in receiving tissues of the movement of even very small amounts of endogenous IAA.

Table 1

Application of 5-^{3}H-IAA to apical buds. The distribution of radioactivity between the acidic ether and aqueous fractions of the separated plant parts.

Plant part	Fractionated radioactivity (d.p.m. x 10^{-4})					
	Aqueous			pH3		
	A	B	C	A	B	C
Fed apical bud	650	290	500	150	130	120
Stem	77	36	47	100	140	100
Top leaves (2)	7.9	3.5	2.1	1.7	0.8	0.3
Lower leaves	0.2	0.0	0.5	0.0	0.0	0.0
Roots	1.7	1.2	0.6	3.2	3.6	1.9
Nodules	0.3	0.03	0.1	0.2	0.1	0.07

A P P E N D I X

Table 2 a

Application of 5-^{3}H-IAA to root nodules. The distribution of radioactivity between the aqueous, basic ether, acidic ether and residual fractions of the separated plant parts.

Plant part	Fractionated radioactivity (d.p.m. x 10^{-4})											
	Aqueous			pH8			pH3			Residue		
	A	B	C	A	B	C	A	B	C	A	B	C
Experiment 1												
Fed nodule	370	700	480	16	42	34	43	57	79	13	25	2.4
Roots	280	70	240	44	4.9	26	51	14	41	14	6.7	40
Leaves	52	20	43	0.6	1.5	0.8	1.1	2.4	2.6	47	31	49
Stem	30	9.4	15	3.6	0.7	0.6	6.1	1.7	1.1	2.6	12	16
Nodules	160	6.0	3.1	120	0.3	0.6	12	0.7	0.2	4.2	9.9	2.4
Apical bud	3.6	0.2	1.0	0.04	0.0	0.0	0.05	0.0	0.02	5.2	4.8	1.1
Experiment 2												
Fed nodule	2500	2000	2600				16	8.6	9.8	1700	1500	1600
Roots	23	59	21				1.4	0.4	0.9	9.8	44	15
Leaves	29	130	26				0.6	2.6	1.7	57	270	41
Stem	34	47	9.5				1.4	1.3	0.2	28	44	8.7
Nodules	0.9	16	1.2				0.2	0.6	0.1	1.8	39	1.1

(i) - Solvent partitioning at pH8 only performed in experiment 1.

Table 2b

Plant part	Fractionated radioactivity (d.p.m. x 10^{-4})								
	Aqueous			pH3			Residue		
	A	B	C	A	B	C	A	B	C
Experiment 3									
Fed nodule	1500	1900	1400	420	430	300	1300	1300	1200
Roots	17	50	260	92	100	260	13	38	240
Leaves	66	210	390	7.2	40	130	83	170	280
Stem	19	69	180	8.6	26	480	18	62	180
Nodules	1.3	5.2	5.4	0.2	1.0	1.2	3.8	7.7	6.3
Experiment 4									
Fed nodule	1500	1500	1600	170	780	380	840	1000	1000
Roots	930	420	130	270	190	57	260	180	74
Leaves	1100	630	500	110	82	39	300	220	12
Stem - bark	170	150	53	32	31	6.8	42	42	8.6
- wood	220	140	57	38	33	8.5	54	50	14
Nodules	89	110	4.0	30	7.7	0.8	130	92	8.9

Table 3

Application of 5-^{3}H-IAA to root nodule vascular strands. The distribution of radioactivity between the aqueous, basic ether, acidic ether and residual fractions of the separated plant parts.

Plant part	Fractionated radioactivity (d.p.m. x 10^{-4})											
	Aqueous			pH8			pH3			Residue		
	A	B	C	A	B	C	A	B	C	A	B	C
Experiment 1												
Fed vascular strand	740	92	760	64	20	82	68	12	120	6.3	140	150
Roots	140	340	110	9.0	20	7.6	36	92	47	120	310	120
Leaves	100	640	20	6.3	24	2.3	37	8.4	2.5	85	94	32
Stem	44	140	7.9	5.3	18	1.5	5.7	20	1.2	68	110	7.1
Nodules	38	14	3.1	0.2	0.5	0.1	0.4	1.5	0.5	5.1	10	1.2
Experiment 2												
Fed vascular strand	820	880	1300				400	200	110	780	760	1100
Roots	940	970	280				10	30	27	500	530	280
Leaves	340	1200	800				67	170	160	120	330	170
Stem - bark	53	230	230				6.6	27	28	8.7	38	27
- wood	59	280	240				180	260	210	21	110	74
Nodules	12	24	110				0.9	1.8	12	8.9	14	77

(1) - Solvent partitioning at pH8 performed only in experiment 1.

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