J Plant Growth Regul (1984) 3:23–39

Journal of
Plant
Growth
Regulation

Phytohormones, *Rhizobium* Mutants, and Nodulation in Legumes. IV. Auxin Metabolites in Pea Root Nodules

Jane Badenoch-Jones, Roger E. Summons, Barry G. Rolfe, and David S. Letham

Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra A.C.T. 2601 Australia

Received August 10, 1983; accepted December 27, 1983

Abstract. High specific activity [^{3}H]indole-3-acetic acid (IAA) was applied directly to root nodules of intact pea plants. After 24 h, radioactivity was detected in all plant tissues. In nodule and root tissue, only 2–3% of ^{3}H remained as IAA, and analysis by thin layer chromatography suggested that indole-3-acetyl-L-aspartic acid (IAAsp) was a major metabolite. The occurrence of IAAsp in pea root and nodule tissue was confirmed unequivocally by gas chromatography-mass spectrometry (GC-MS). The following endogenous indole compounds were also unequivocally identified in pea root nodules by GC-MS: IAA, indole-3-pyruvic acid, indole-3-lactic acid, indole-3-propionic acid, indole-3-butyric acid, and indole-3-carboxylic acid. Evidence of the occurrence of indole-3-methanol was also obtained. With the exception of IAA and indole-3-propionic acid, these compounds have not previously been unequivocally identified in a higher plant tissue.

The level of auxin in an organ reflects the balance between the rates of its synthesis, degradation, conversion to conjugates, and transport into and out of the organ. We have previously examined the transport to the root nodule of [^{3}H]indole-3-acetic acid (IAA) applied to the apical bud of intact *Pisum sativum* plants (Badenoch-Jones et al. 1983). The metabolism within the nodule of the radioactive auxin received from the shoot was also investigated. Analysis by thin layer chromatography (TLC) suggested that the IAA conjugate indole-3-acetyl-L-aspartic acid (IAAsp) was a major metabolite. In the present com-

plementary study, [^{3}H]IAA was applied directly to pea root nodules *in situ* and its metabolism within the nodule and its transport out of the nodule were investigated. The results indicated that root nodules themselves have the ability to metabolize IAA to IAAsp. By gas chromatographic-mass spectrometric (GC-MS) analysis, IAAsp was identified unambiguously as a metabolite in extracts of combined root and nodule tissue, and a number of indole compounds were identified for the first time in nodule tissue.

Materials and Methods

Chemicals and Bacterial Strains

The chemicals and bacterial strains used have been described previously (Badenoch-Jones et al. 1982a, 1983). IAAsp and 4-chloro-indole-3-acetic acid were generous gifts from Drs. J. Cohen and R. Bandurski (Michigan State University, USA), and Dr. K. C. Engvild (Ris National Laboratory, Roskilde, Denmark), respectively.

Radiolabeling Experiments

1. *Plant culture.* For experiments in which [^{3}H]IAA was applied to root nodules, a method of plant culture was devised which allowed easy access to the root nodules of intact plants as well as aseptic growth conditions. Intact plants were used in order to maintain physiological conditions. Seeds of *Pisum sativum* L. cultivar Greenfeast were sterilized and germinated as described previously (Djordjevic et al. 1982).

Individual seedlings were aseptically transferred to an upright-placed large Petri dish (13.5 cm diameter) (Sterilin Ltd., Teddington, Middlesex, England) containing approximately 150 ml solid (1.5%, w/v, agar) modified Fåhraeus medium (Vincent 1970). A hole for the seedlings was cut in the agar in the Petri dish. The seedlings were etiolated by growing them in the dark. After approximately 6 d, the seedling shoots were pulled through holes that were made in the tops of the Petri dishes with a hot glass rod. Seedlings were etiolated for a further 3 d and then inoculated by streaking a fresh bacterial culture across the agar and the growing roots. The Petri dishes were sealed with Nescofilm strips (Nippon Shoji Kaishi Ltd., Osaka, Japan). Five small slits were made in the Nescofilm to allow gaseous exchange to take place. The Petri dishes were placed in black plastic bags to minimize illumination of the roots and were transferred to a growth cabinet (day: 16 h, 20°C, 100 μE $m^{-2}s^{-1}$; night: 8 h, 15°C).

2. *Application of radiolabel.* Pea plants (three per experiment) were grown in Petri dishes. Sixteen days after inoculation with strain ANU897, plants were transferred to a laminar flow hood for labeling. Using a microsyringe, either 5 μl (equivalent to 185 kBq and 30.2 ng IAA) or 10 μl of [^{3}H]IAA were distributed as evenly as possible over the surface of all accessible nodules lying on the agar. Care was taken not to contaminate the root tissue. Plants were returned to the growth cabinet for 24 h.

3. *Seedling dissection and extraction.* The following procedures were carried out in dim light. The whole seedlings were dissected into root, nodules, cotyledon, stem, and leaves. Nodules that did not receive radiolabel were discarded. Stems were further cut into segments of approximately 1 cm in length. Tissues from individual plants were pooled and were extracted in acetone (70%, v/v) as described previously (Badenoch-Jones et al. 1983). Extracts (pH 3.0) were partitioned with ethyl acetate using the procedures of Badenoch-Jones et al. (1982b), and then at pH 8.0 with dichloromethane.

4. *Thin layer chromatography (TLC).* TLC of ethyl acetate fractions was done as described by Badenoch-Jones et al. (1983) using the following TLC systems (solid support, solvent system [solvents are by volume]): 1, polyamide DC 6, benzene/ethyl acetate/acetic acid (14:5:1); 2, polyamide DC 6, butan-1-ol/14N ammonia/water (4:1:1; upper phase); 3, cellulose, isopropanol/14N ammonia/water (8:1:1); 4, silanized silica gel, benzene/ethyl acetate/acetic acid (14:5:1).

5. *Liquid scintillation counting.* Samples were eluted and counted using the procedures of Badenoch-Jones et al. (1983).

Experiments for the Identification of IAAsp by GC-MS

1. *Plant culture.* Thirty plants inoculated with strain ANU203 were grown in flasks using the procedures of Djordjevic et al. (1982). Thirteen d after inoculation, the plants were carefully transferred, under sterile conditions, to liquid modified Fåhraeus medium containing 0.17 mM IAA and returned to the growth cabinet for 24 h.

2. *Tissue extraction and sample purification.* The whole root system (i.e., roots plus nodules) of the plants was then extracted (see above) except that carrier IAA and IAAsp were not added. The samples (pH 3.0) were partitioned with ethyl acetate (see above) and the ethyl acetate fraction was subjected to TLC (system 2). Authentic IAAsp was parallel-chromatographed. The appropriate zone of polyamide was eluted with ethyl acetate. The sample was evaporated to dryness and further purified by higher performance liquid chromatography (HPLC). A μBondapak C_{18} column (300 mm × 7.9 mm) was used, with 20% ethanol (v/v) in 0.2 M acetic acid as solvent, at a flow rate of 3.5 ml min^{-1}. Equipment and the columns were supplied by Waters Associates, Milford, Massachusetts, USA, and details of the apparatus were as reported by Summons et al. (1979).

3. *Gas chromatography-mass spectrometry (GC-MS).* The dry sample was taken up in a small volume of methanol and transferred to a derivatization vial. It was evaporated under a stream of nitrogen and then thoroughly dried by azeotropic evaporation of dichloromethane. The trimethylsilyl (TMS) derivative was prepared by dissolving the sample in acetonitrile (15 μl) and *bis*(trimethylsilyl)trifluoroacetamide (15 μl) and then heating at 70°C for 10 min. An aliquot of the resulting solution was analyzed by GC-MS. Electron impact (EI) mass spectra were taken with a DuPont 21-491B instrument (ionization voltage 70 eV; source temperature 250°C; gas chromatographic column packing OV-101; GC temperature 160–200°C at 10°C min^{-1}) interfaced with a VG 2025

data system. Chemical ionization (CI) mass spectra were taken with a Finnigan 4500 instrument (ionization voltage 70 eV; source temperature 100°C) using a fused silica capillary column (SE-30) programmed from 70°C to 300°C at 10°C min^{-1} and Grob-type injection technique. The reagent gas was methane at 1 Torr.

Experiments for the Identification of Other Indole Compounds

1. *Plant culture.* Plants were grown in flasks (Djordjevic et al. 1982) and were inoculated with strain ANU897.

2. *Tissue extraction and sample purification.* Plants were harvested 30 d after inoculation. Root nodules were dissected clean of root tissue, frozen in liquid nitrogen, and stored at −198°C prior to use. Samples (approximately 1.3 g) were extracted with two solvents: acetone (70%, v/v) (see above) or methanol/water/formic acid (70:25:5, v/v/v). Frozen nodules were dropped into the latter solvent at −20°C (10 ml g^{-1} fresh weight tissue). After 24 h at −20°C, the tissue was ground in a mortar and the homogenate allowed to stand at −20°C for a further 24 h. The homogenate and mortar washings were then filtered through a Buchner funnel. The residue was resuspended in an equal volume of solvent, refiltered, and the filtrates pooled. The extracts, of either aqueous acetone or methanol, were evaporated to aqueous solution under reduced pressure at a temperature of less than 30°C. The concentrates were diluted with distilled water and their pH adjusted to 8.0. Samples were partitioned with dichloromethane (Badenoch-Jones et al. 1982b). Sample pH was then adjusted to 3.0, and samples were partitioned with dichloromethane followed by ethyl acetate (Badenoch-Jones et al. 1982b).

3. *GC-MS.* Samples were derivatized as described above. EI and CI mass spectra were taken with the Finnigan 4500 instrument, employing the conditions described above for it, except that on-column injection was used and the GC temperature program was as follows: 140°C for 1 min, 140–260°C at 20°C min^{-1}, 260–300°C at 8°C min^{-1}, 300°C for 1 min. Also, for the EI mass spectra, the source temperature was 150°C and no reagent gas was used.

Results

Radiolabeling Experiments

1. *The distribution of ^3H in the plant.* Results from the two experiments in which [^{3}H]IAA was applied to root nodules of pea plants are presented in Table 1. In both experiments radioactivity was detected in all plant tissues after the 24-h labeling period. As expected, the nodules remained the most highly labeled tissue. When experiment 1 (application of 10 µl [^{3}H]IAA), and experiment 2 (application of 5 µl [^{3}H]IAA) are compared, a greater proportion of the radiolabel was exported in experiment 1 (26% compared with 7%)[1] and a greater proportion of the transported radioactivity remained in the root system (88% compared with 62%). Very little radioactivity (approximately 0.2% of the ^{3}H

[1] Calculated as ([^{3}H recovered from root, cotyledon, stem and leaf] × 100)/total ^{3}H recovered from the plant.

Table 1. Radioactivity extracted from tissues of intact pea plants to which [^{3}H]IAA was applied to root nodules formed by strain ANU897, and the contribution of [^{3}H]IAA and [^{3}H]IAAsp to this radioactivity. For each experiment, tissues from three plants were extracted with acetone (70%, v/v), and extracts were purified by ethyl acetate partitioning (pH 3).

			Ethyl acetate fraction				
[^{3}H]IAA (μl/plant)	Tissue	Total dpm mg^{-1} f.w.t.[a] ($\times 10^{-2}$)	dpm mg^{-1} f.w.t.[a] ($\times 10^{-2}$) (A)	% ^{3}H due to IAA Mean ± S.E.M. (B)[b]	dpm as IAA mg^{-1} f.w.t.[a] ($\times 10^{-1}$) (C)[d]	% ^{3}H due to IAAsp (D)[c]	dpm as IAAsp mg^{-1} f.w.t.[a] ($\times 10^{-1}$) (E)[e]
10	Root	42.1	24.1	3.3 ± 0.83(4)	8.0	36	86.6
(expt.1)	Nodule	1706	938	2.2 ± 0.41(4)	206	52	4861
	Cotyledon	0.5	0.1	7.6 (2)	0.1	7	0.1
	Stem	5.1	1.4	13.1 ± 3.80(4)	1.9	3	0.5
	Leaf	5.0	1.0	7.7 ± 2.03(4)	0.8	2	0.2
5	Root	5.8	2.4	3.4 ± 0.82(3)	0.8	51	12.3
(expt.2)	Nodule	872	348	3.0 ± 1.00(3)	104	62	2143
	Cotyledon	0.1	0.1	9.6 (2)	0.1	14	0.2
	Stem	4.8	0.7	8.0 ± 2.22(3)	0.5	6	0.4
	Leaf	5.8	0.5	9.9 ± 2.65(3)	0.5	5	0.3

[a] f.w.t. = fresh weight of tissue

[b] Represents the percentage of dpm co-chromatographing with IAA in several (number in parentheses) TLC systems

[c] Represents the percentage of dpm co-chromatographing with IAAsp in TLC system 2

[d] C = A × B/100

[e] E = A × D/100

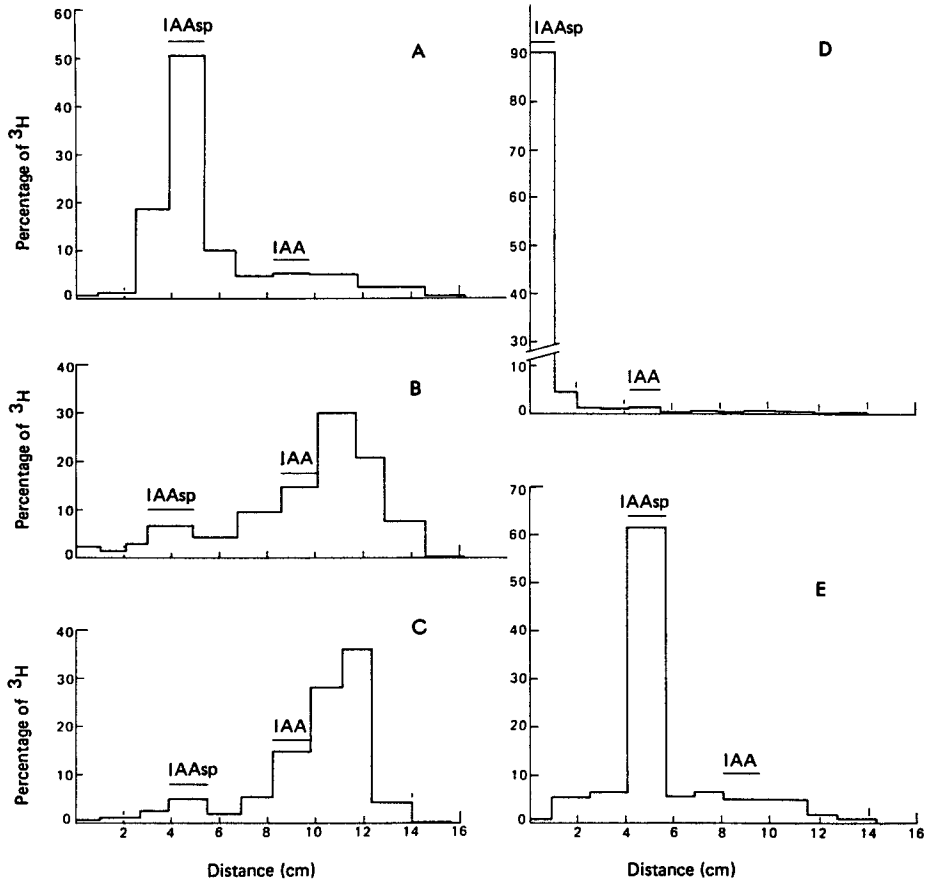


Fig. 1. Distribution of radioactivity of ethyl acetate fractions of tissue extracts on polyamide DC 6 thin layer chromatograms. Solvent: butan-1-ol/14N ammonia/water (4:1:1, v/v/v, upper phase) (i.e., TLC system 2) for A, B, C, and E; benzene/ethyl acetate/acetic acid (14:5:1, v/v/v) (i.e., TLC system 1) for D. Radioactivity is expressed as the percentage of the total radioactivity recovered from the chromatogram. The positions of co-chromatographed marker compounds are indicated. Chromatograms are of samples from experiment 2. A = root, B = stem, C = leaf, D and E = nodule.

transported out of the nodules) accumulated in the cotyledon. Leaf tissue accounted for a greater percentage of transported radioactivity (8% and 23%, in experiments 1 and 2, respectively) than did stem tissue (4% and 15% for experiments 1 and 2, respectively).

The proportion of total radioactivity that partitioned into the ethyl acetate fraction was similar for root and nodule, lower for stem, and lowest for leaf tissue. Less than 1% of sample ^3H partitioned into the dichloromethane phase at pH 8.

2. *Chromatographic characterization and identity of metabolites.* The ethyl acetate fractions were subjected to TLC to identify the metabolites present (see Table 1). The TLC profiles of root and nodule tissue were similar, with one major peak of ^3H co-chromatographing with IAAsp in all systems (Fig. 1).

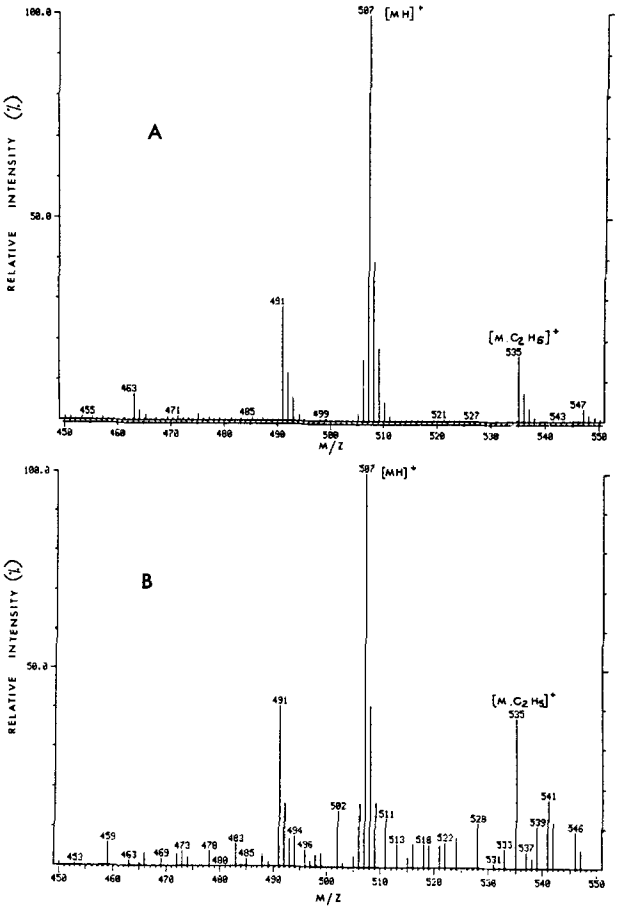


Fig. 2. Chemical ionization mass spectrum of the TMS-IAAsp peak, A = authentic IAAsp, B = putative IAAsp from plant extract. MH^+ denotes the protonated molecular ion.

For stem and leaf extracts there was a more even chromatographic distribution of ^3H, with no major peaks of ^3H at the Rfs of IAA and IAAsp (Fig. 1).

For each sample, a similar percentage of ^3H in the extract chromatographed with IAA in every TLC system. The mean values (±S.E.M.) are given in Table 1. Of the TLC systems used, system 2 gives the best estimate of percentage of ^3H due to IAAsp (see Badenoch-Jones et al. 1983). The percentages of sample ^3H co-chromatographing with IAAsp in TLC system 2 are therefore given in Table 1.

Identification of IAAsp by GC-MS

When the purified root and nodule extract was derivatized and analyzed by GC-MS, a peak was present which had a GC retention time similar to that of authentic TMS-IAAsp. It also exhibited a CI mass spectrum that was identical in the molecular ion region to that of authentic TMS-IAAsp. The CI mass spectrum (Fig. 2) showed a protonated molecular ion at *m/z* 507, together with $M^+C_2H_5$ adduct ion at *m/z* 535 and M − 15 ion at *m/z* 491. TMS-IAAsp also exhibits a characteristic EI mass spectrum (Fig. 3) with a molecular ion at *m/z*

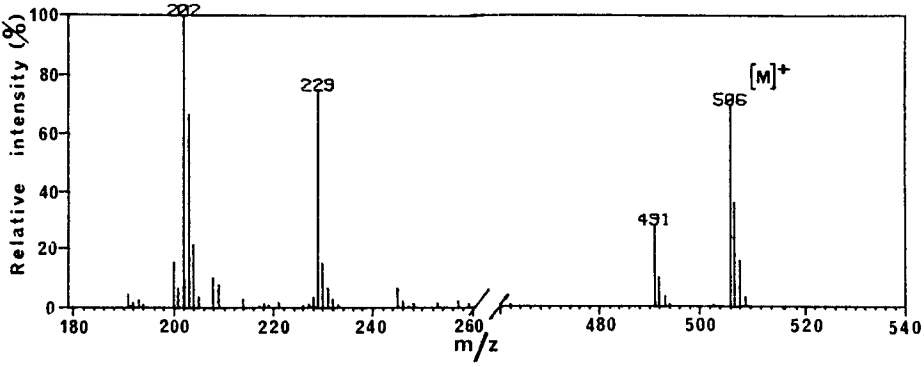


Fig. 3. Electron impact mass spectrum of the TMS-IAAsp peak for authentic standard. In the region deleted from the spectrum there were no ions of relative intensity greater than 3.5%.

506, methyl loss at *m/z* 491, and the commonly encountered indole-containing fragment ions at *m/z* 202 and *m/z* 229.

Identification of Other Indole Compounds

The total ion currents for the solvent-partitioned fractions of nodule extract were complex (Fig. 4). However, from the ethyl acetate fraction of the sample extracted with aqueous methanol/formic acid, it was possible to identify the following indole compounds directly by GC-MS: IAA; indole-3-pyruvic acid (IPyA); indole-3-lactic acid (ILA); indole-3-propionic acid (IPrA); indole-3-butyric acid (IBA); indole-3-carboxylic acid (ICA); and indole-3-methanol (IMet). These indole compounds identified in the nodule sample gave GC peaks having the same retention times as those observed for authentic standards. The first four compounds were identified by both CI mass spectra (Fig. 5a–d) and EI mass spectra (Fig. 5h–k). IBA and ICA were identified by CI mass spectra (Fig. 5e–f). Although several of the spectra showed peaks due to impurities, both the EI and CI spectra were in accord with those of the respective authentic compounds (compare Badenoch-Jones et al. 1982a for EI spectra and Table 2 for CI spectra). Mass spectral data (Fig. 5g) also indicated the presence of IMet. The ability to detect IMet in the ethyl acetate acid (pH 3) fraction presumably reflects incomplete partitioning of this compound into the neutral (pH 8) dichloromethane fraction. For IMet, the relative intensities of the ions at *m/z* 276 and 292 in the CI mass spectrum for the nodule sample differed from those given in Table 2 for authentic IMet. For this compound, however, some variability was observed for these parameters over a number of GC-MS analyses of authentic standard.

The mass spectral data, coupled with the fact that the compounds eluted at the correct retention time during GC, provide unambiguous identification. The EI spectra exhibited characteristic fragmentations and molecular ions of weak or moderate intensity, while the CI spectra showed intense protonated molecular ions (MH^+), usually the base peak, and associated weaker $MH - CH_3$ ions and $M{\cdot}C_2H_5$ adduct ions.

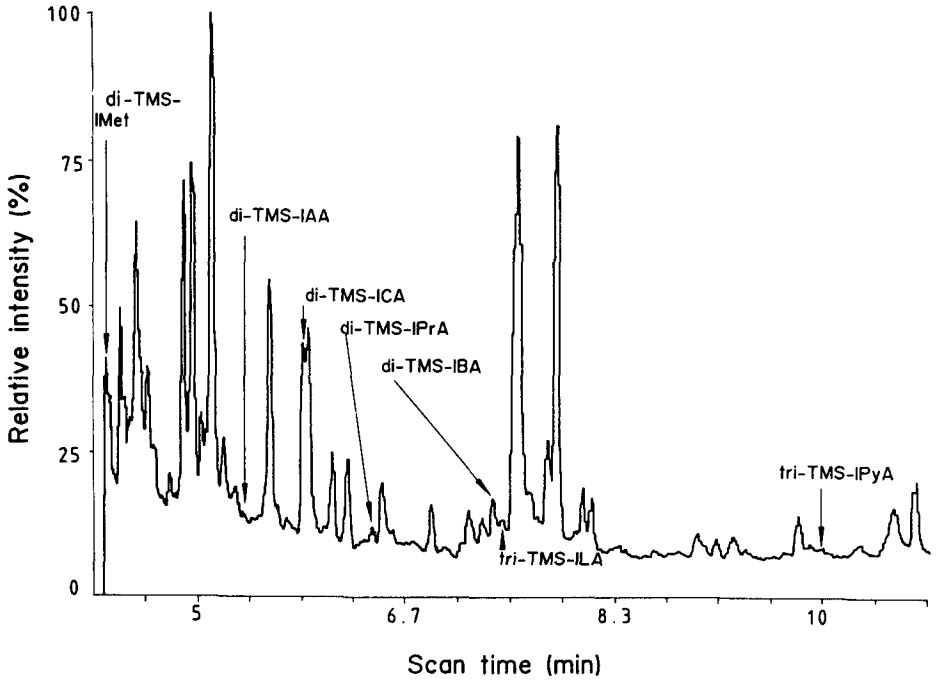
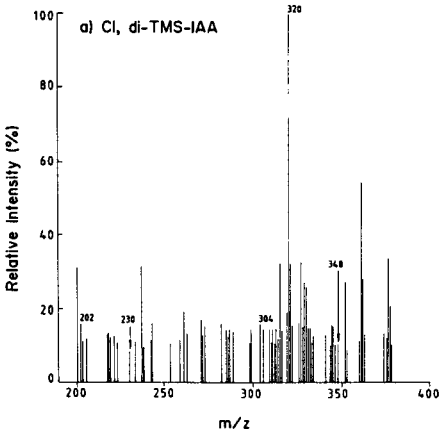


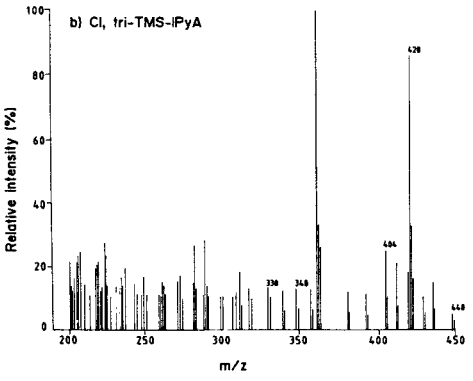
Fig. 4. Profile of the total ion current taken during GC-MS (CI) of the ethyl acetate fraction (pH 3) of nodule tissue extracted in methanol/water/formic acid (70:25:5, v/v/v).

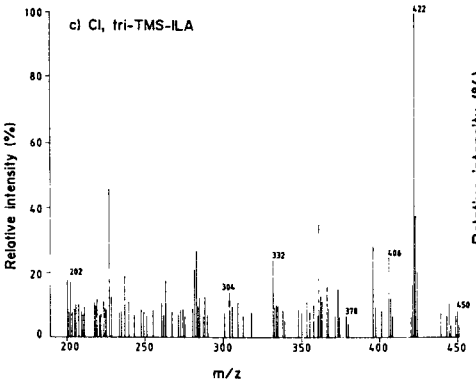
A search was also made for the following indole compounds in the nodule extracts: indole-3-aldehyde; tryptophol (2-[indol-3-yl]ethanol); N-acetyl-L-tryptophan; indole-3-acetaldehyde; indole-2-carboxylic acid; indole-3-acrylic acid; indole-3-acetone; indole-3-glyoxylic acid; indole-3-acetonitrile; indole-3-glycolic acid; and 4-chloro-indole-3-acetic acid. Although none of these compounds was detected, the possibility that they may be present, particularly at very low levels, cannot be eliminated. When the extracting solvents alone were processed in a manner identical to that of the nodule extracts, no indole compounds were detected.

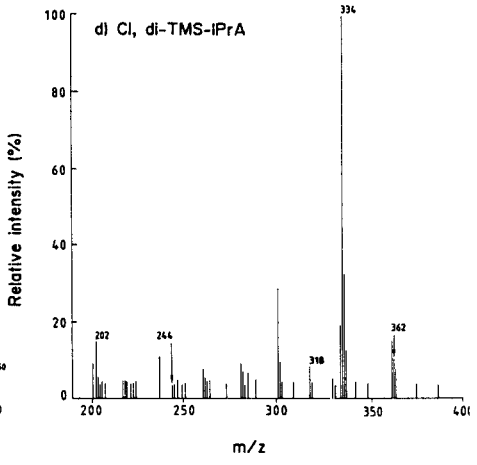
Discussion

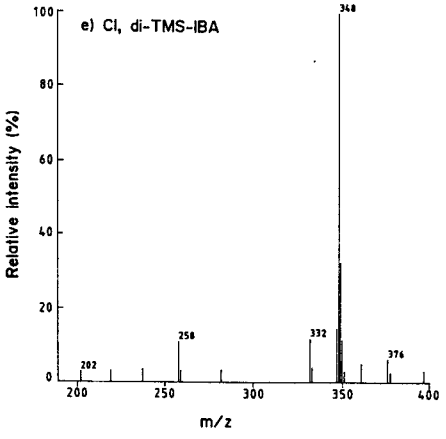
Data from the present experiments indicate that when [^{3}H]IAA is applied to root nodules, radioactivity is exported to all parts of the plant, and it is probable that endogenous auxin in root nodules is likewise transported, although the possibility that exogenous auxin enters a transport system not entered by endogenous IAA cannot be discounted. Root nodules have been shown to import IAA from other parts of the plant (Badenoch-Jones et al. 1983), and the export of IAA or its metabolites from the nodules presumably occurs concurrently with the transport of IAA into the nodules. Insufficient data are available to determine whether net movement was positive or negative. A considerable proportion of the auxin transported out of the nodule remained

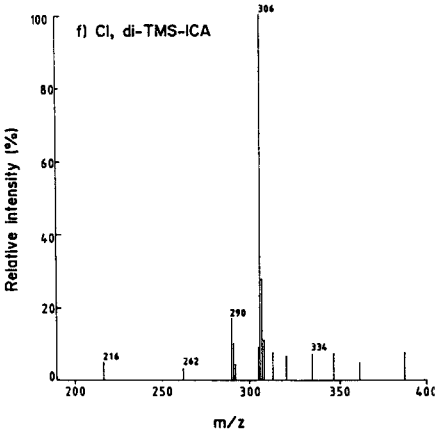

a) CI, di-TMS-IAA
Relative intensity (%)
m/z
320
348
202
230
304

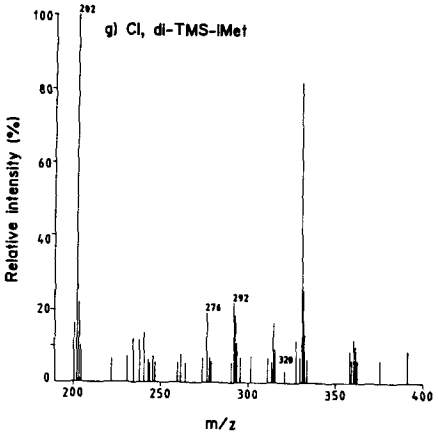

b) CI, tri-TMS-IPyA
Relative intensity (%)
m/z
420
404
330
348
448

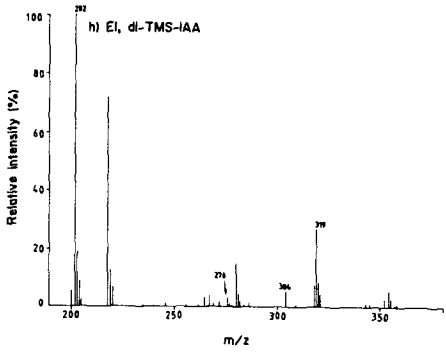

c) CI, tri-TMS-ILA
Relative intensity (%)
m/z
422
332
406
202
304
378
450

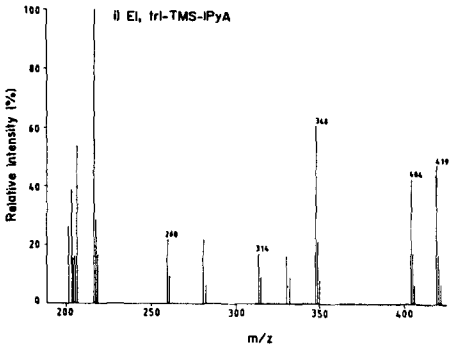

d) CI, di-TMS-IPrA
Relative intensity (%)
m/z
334
202
244
318
362

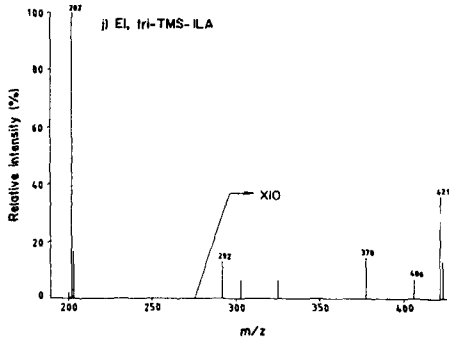

e) CI, di-TMS-IBA
Relative intensity (%)
m/z
348
258
332
202
376


f) CI, di-TMS-ICA
Relative intensity (%)
m/z
306
290
216
242
334









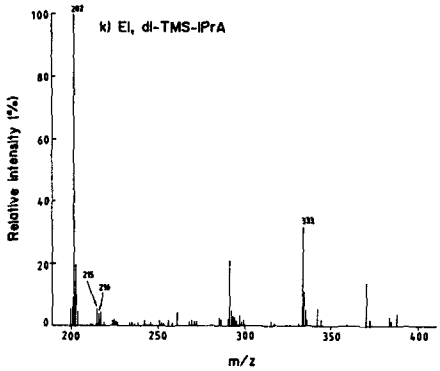


Fig. 5. a–g: Chemical ionization mass spectra of the peaks shown in Fig. 4: (a) di-TMS-IAA (b) tri-TMS-IPyA; (c) tri-TMS-ILA; (d) di-TMS-IPrA; (e) di-TMS-IBA; (f) di-TMS-ICA; (g) di-TMS-IMet. h–k: Electron impact mass spectra of corresponding peaks taken during GC-MS analysis in the EI mode: (h) di-TMS-IAA; (i) tri-TMS-IPyA; (j) tri-TMS-ILA; (k) di-TMS-IPrA.

Table 2. Characteristic features of the chemical ionization mass spectra of TMS derivatives of authentic indole standards in order of their elution from OV-101. Relative intensities as a percentage are given in parentheses. T denotes a TMS moiety.

Compound		Base peak	$[MH]^+$	Other diagnostic ions					
Indole-3-methanol	Di-T	202	292 (98)	276(99)	320(16)				
Indole-3-acetonitrile	Mono-T	202	229 (25)	213 (1)	257 (8)				
Indole-3-aldehyde	Mono-T	218	218(100)	246(32)					
Indole-2-carboxylic acid	Di-T	306	306(100)	216(37)	290(72)	334 (7)			
Tryptophol	Di-T	306	306(100)	216(18)	290(59)	334 (9)	202 (1)		
Indole-3-acetic acid	Di-T	320	320(100)	230 (1)	304(15)	348(17)	202 (3)		
Indole-3-aldehyde	Di-T	290	290(100)	318 (3)					
Indole-3-acetaldehyde	Di-T	304	304(100)	288(20)	332(14)				
Indole-3-carboxylic acid	Di-T	306	306(100)	216 (2)	290(22)	334(18)	262 (1)		
Indole-3-acetone	Di-T	318	318(100)	302(11)	346(11)				
Indole-3-propionic acid	Di-T	334	334(100)	244(1)	318 (8)	362(13)	202 (4)		
4-Chloro-indole-3-acetic acid	Di-T	354	$354(Cl^{35},100)$	$264(Cl^{35},3)$	$338(Cl^{35},12)$	$382(Cl^{35},6)$	$236(Cl^{35},21)$		
			$356(Cl^{37}, 43)$	$266(Cl^{37},1)$	$340(Cl^{37}, 5)$	$384(Cl^{37},3)$	$238(Cl^{37}, 8)$		
Indole-3-butyric acid	Di-T	348	348(100)	258 (5)	332(19)	376(17)			
Indole-3-lactic acid	Tri-T	422	422(100)	332(30)	406(70)	450 (9)	202(10)	304(6)	378(7)
Indole-3-acrylic acid	Di-T	332	332(100)	242 (5)	316(21)	360(20)			
N-acetyl-L-tryptophan	Di-T	391	391(100)	301 (2)	375(12)	419(18)	202 (2)		
Indole-3-pyruvic acid	Tri-T	420	420(100)	330 (7)	404(50)	448(13)	348 (1)		

in the root system. This is not unexpected since, although both acropetal (i.e., towards the root tip) and basipetal movement of IAA takes place in roots, basipetal movement has usually been found to extend only over a limited distance (see Batra et al. 1975). However, radioactivity was also detected in the stem and leaves, implying acropetal movement of auxin in the stem. Indeed, McCready (1968) and Tsurumi and Wada (1980) reported both acropetal and basipetal movement of [^{14}C]IAA after its application to petiole segments of *Phaseolus vulgaris* and to the cotyledon of seedlings of *Vicia faba*, respectively, and Bourbouloux and Bonnemain (1979) reported that some of the radioactive auxin translocated to the roots from a young leaf of *Vicia faba* was returned to the shoot. Very little of the radioactive auxin transported from the nodules accumulated in the cotyledon, despite its proximity to the root system. The lack of accumulation of radioactivity in the cotyledon was also observed when [^{3}H]IAA was applied to the apical bud (Badenoch-Jones et al. 1983) and was attributed to the senescing state of the cotyledon (see Elkinawy 1982).

Since the results of the current experiments suggest that root nodules can export auxin to other parts of the plant, possibly in appreciable quantities, they raise the question of whether root nodules synthesize IAA. This question is currently under investigation.

The mean percentages of radioactivity as IAA and IAAsp in ethyl acetate fractions of nodule extracts in the current experiments (2.6% and 57%, respectively) were very similar to the mean values of 1.3% and 51%, respectively, obtained when plants inoculated with the same strain were labeled at the apical bud (Badenoch-Jones et al. 1983). Although feeding parts of a whole plant does not give good evidence of sites of synthesis, two results from the present study, namely: (1) the large proportion of radioactivity as IAAsp in nodule tissue and the little remaining as IAA, and (2) the relatively large proportion of radioactivity remaining in the nodule tissue, when taken together, suggest that nodules themselves have the capacity to form IAAsp. The mean percentages of radioactivity as IAA and IAAsp in ethyl acetate fractions of root extracts in the current experiments (3.4% and 44%) were very similar to the values obtained for nodule tissue. In contrast, they were markedly different to the respective mean values obtained when plants inoculated with the same strain were labeled at the apical bud (45% and 21%). From these data it might be speculated that, when plants were labeled at the apical bud, a considerable amount of the radioactive auxin reaching the root system was IAA itself, whereas nodules probably export labeled auxin as a metabolite of IAA (presumably IAAsp) rather than as IAA itself.

IAAsp was tentatively identified by TLC as a metabolite of [^{3}H]IAA in tissues of pea plants in the present study and a previous study (Badenoch-Jones et al. 1983). The occurrence of IAAsp following the application of IAA to various tissues of pea plants has also been indicated by other workers on the basis of hydrolysis products as well as chromatographic behavior and color reaction (Andreae and Good 1955, Andreae and van Ysselstein 1956, 1960, Winter and Thimann 1966). We have now unequivocally established the identity of this metabolite in combined root and nodule tissue of peas by GC-MS. Rigorous characterization of IAAsp by GC-MS has also recently been presented for seeds of *Glycine max* (Cohen 1982) and for shoots of *Pinus sylvestris*

L. (Anderssen and Sandberg 1982). These workers examined IAAsp by GC-MS as the IAAsp-*bis*-methyl ester, in contrast to our analysis of the TMS derivative of IAAsp.

The presence of IAA in pea root nodules was also unequivocally established by GC-MS in the present study. Wang et al. (1982) and Badenoch-Jones et al. (1982b) unambiguously identified IAA in culture supernatants of *Rhizobium* strains, but the identification of IAA in root nodules has, until now, been only tentative, being based largely on chromatographic properties, bioassay, and chromogenic reaction (Thimann 1936, Link and Eggers 1940, Pate 1958, Silver et al. 1966, Kretlovich et al. 1972) or on spectrofluorimetry (Dullaart 1967, 1968, 1970).

The present study also describes the first successful isolation and unequivocal identification of IPyA from a higher plant tissue. IPyA is an unstable compound (Stowe and Thimann 1954, Vlitos and Meudt 1954, Bentley et al. 1956, Jepson 1958, Kaper and Veldstra 1958, Schwarz and Bitancourt 1960, Kaper et al. 1963a, b, Atsumi et al. 1976) and has therefore proved very difficult to isolate from plant tissues. The unstable nature of IPyA has, in fact, resulted in there being some controversy concerning the presence of IPyA in plant tissues. Several workers have claimed to have demonstrated its presence or its synthesis in various plant tissues (see Sembdner et al. 1980) based largely on Rf and color reaction. However, others (Srivastava 1964, Stowe et al. 1968) were unable to confirm these claims. Furthermore, IPyA breaks down to a considerable extent when chromatographed with the basic solvent systems used in many studies. Until now, perhaps the most convincing evidence for the occurrence of IPyA in plant tissues was the isolation of radiolabeled IPyA (stabilized as the dinitrophenylhydrazine derivative) from tissues that had been incubated with radiolabeled tryptophan (Moore and Shaner 1968, Gibson et al. 1972).

By mass spectrometry, four other indole compounds were unequivocally identified in nodule extracts in the current experiments, namely ILA, IPrA, IBA, and ICA. Evidence for the occurrence of IMet was also obtained. IPrA has previously been unequivocally identified by GC-MS in hypocotyls of squash seedlings (Segal and Wightman 1982). The other compounds, as well as IPrA, have previously been tentatively identified, based on chromatographic behavior, color reaction, biological activity, and spectral characteristics, in plant tissues: ILA in tomato shoots (Schneider et al. 1972); IPrA in tobacco stems and leaves (Bayer 1969, Wightman 1977); IBA in tobacco stems (Bayer 1969) and potato tubers (Blommaert 1954); and ICA in a number of plant systems such as wheat and pea (see Sembdner et al. 1980). IMet is a reduction product of indole-3-aldehyde (IAld) (Magnus et al. 1971) and IAld appears to occur naturally in some plants (see Sembdner et al. 1980). There have been very few studies of the naturally occurring indole compounds in root nodules. Dullaart (1967) identified IAA and ICA in root nodules of *Lupinus luteus* by spectrofluorimetry, and these identifications have been confirmed with pea root nodules in the present study. The physiological significance of the presence of these indole compounds remains to be elucidated, but it is of interest that some, particularly IBA and IPrA, are active growth substances (Wightman 1962).

Acknowledgments. The excellent technical assistance of J. Wicks and P. Kell is gratefully acknowledged. J.B-J. was supported by a Queen Elizabeth II Fellowship.

References

Anderssen B, Sandberg G (1982) Identification of endogenous N-(3-indoleacetyl)aspartic acid in Scots pine (*Pinus sylvestris* L.) by combined gas chromatography-mass spectrometry, using high performance liquid chromatography for quantification. J Chromatog 238:151–156

Andreae WA, Good NE (1955) The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol 30:380–382

Andreae WA, van Ysselstein MWH (1956) Studies on 3-indoleacetic acid metabolism. III. The uptake of 3-indoleacetic acid by pea epicotyls and its conversion to 3-indoleacetyl aspartic acid. Plant Physiol 31:235–240

Andreae WA, van Ysselstein MWH (1960) Studies on 3-indoleacetic acid metabolism. VI. 3-indoleacetic acid uptake and metabolism by pea roots and epicotyls. Plant Physiol 35:225–232

Atsumi S, Kuraishi S, Hayashi T (1976) An improvement of auxin extraction procedure and its application to cultured plant cells. Planta 129:245–247

Badenoch-Jones J, Summons RE, Entsch B, Rolfe BG, Parker CW, Letham DS (1982a) Mass spectrometric identification of indole compounds produced by *Rhizobium* strains. Biomed Mass Spectrom 9:429–437

Badenoch-Jones J, Summons RE, Djordjevic MA, Shine J, Letham DS, Rolfe BG (1982b) Mass spectrometric quantification of indole-3-acetic acid in *Rhizobium* culture supernatants: Relation to root hair curling and nodule initiation. Appl Environ Microbiol 44:275–280

Badenoch-Jones J, Rolfe BG, Letham DS (1983) Phytohormones, *Rhizobium* mutants and nodulation in legumes. III. Auxin metabolism in effective and ineffective pea root nodules. Plant Physiol 73:347–352

Batra MW, Edwards KL, Scott TK (1975) Auxin transport in roots: Its characteristics and relation to growth. In: Torrey JG, Clarkson DT (eds) The development and function of roots. Third Cabot Symposium. Academic Press, London, pp 299–325

Bayer MH (1969) Gas chromatographic analysis of acidic indole auxins in *Nicotiana*. Plant Physiol 44:267–271

Bentley JA, Farrar KR, Housley S, Smith GF, Taylor WC (1956) Some chemical and physiological properties of 3-indolylpyruvic acid. Biochem J 64:44–49

Blommaert KLJ (1964) Growth and inhibiting substances in relation to the rest period of the potato tuber. Nature 174:970–972

Bourbouloux A, Bonnemain J-L (1979) The different components of the movement and the areas of retention of labelled molecules after the application of [^{3}H]indolyl-acetic acid to the apical bud of *Vicia faba*. Physiol Plant 47:260–268

Cohen JD (1982) Identification and quantitative analysis of indole-3-acetyl-L-aspartate from seeds of *Glycine max* L. Plant Physiol 70:749–753

Djordjevic MA, Zurkowski W, Rolfe BG (1982) Plasmids and stability of symbiotic properties of *Rhizobium trifolii*. J Bacteriol 151:560–568

Dullaart J (1967) Quantitative estimation of indoleacetic acid and indolecarboxylic acid in nodules and roots of *Lupinus luteus* L. Acta Bot Neerl 16:222–230

Dullaart J (1968) The indole acetic acid content of root nodules and roots of *Cycas circinalis* L. with regard to other root nodule systems. Acta Bot Neerl 17:496–498

Dullaart J (1970) The auxin content of root nodules and roots of *Alnus glutinosa* (L.) Vill. J Exp Bot 21:975–984

Elkinawy M (1982) Physiological significance of indoleacetic acid and factors determining its level in cotyledons of *Lupinus albus* during germination and growth. Physiol Plant 54:302–308

Gibson RA, Schneider EA, Wightman F (1972) Biosynthesis and metabolism of indol-3yl-acetic acid. II. *In vivo* experiments with ^{14}C-labelled precursors of IAA in tomato and barley shoots. J Exp Bot 23:381–399

Jepson JB (1958) Indolylacetamide: A chromatographic artifact from the natural indoles, indolylacetylglucosiduronic acid and indolylpyruvic acid. Biochem J 69:22P

Kaper JM, Veldstra H (1958) On the metabolism of tryptophan by *Agrobacterium tumefaciens*. Biochim Biophys Acta 30:401–420

Kaper JM, Gebhard O, van den Berg C, Veldstra H (1963a) Studies on indolepyruvic acid. I. Synthesis and paper chromatography. Arch Biochem Biophys 103:469–474

Kaper JM, Gebhard O, van den Berg C, Veldstra H (1963b) Studies on indolepyruvic acid. II. Ultraviolet spectrophotometry. Arch Biochem Biophys 103:475–487

Kretovich VL, Alekseeva II, Tsivina NZ (1972) Content of β-indolylacetic in root nodules and roots of Lupine. Sov Plant Physiol 19:421–424

Link JKK, Eggers V (1940) Avena coleoptile assay of ether extracts of nodules and roots of bean, soybean and pea. Bot Gaz 101:650–657

Magnus V, Iskrić S, Kveder S (1971) Indole-3-methanol: A metabolite of indole-3-acetic acid in pea seedlings. Planta 97:116–125

McCready CC (1968) The polarity of auxin movement in segments excised from petioles of *Phaseolus vulgaris*. In: Wightman F, Setterfield G (eds) Biochemistry and physiology of plant growth substances. The Runge Press, Ltd., Ottawa, Canada, pp 1005–1023

Moore TC and Shaner CA (1968) Synthesis of indoleacetic acid from tryptophan via indolepyruvic acid in cell-free extracts of pea seedlings. Arch Biochem Biophys 127:613–621

Pate JS (1958) Studies of the growth substances of legume nodules using paper chromatography. Aust J Biol Sci 11:516–528

Schneider EA, Gibson RA, Wightman F (1972) Biosynthesis and metabolism of 3-indoleacetic acid. I. The native indoles of barley and tomato shoots. J Exp Bot 23:152–170

Schwarz K, Bitancourt AA (1960) Further evidence of tautomerism in chromatograms of indolyl-3-pyruvic acid. Biochem J 75:182–187

Segal LM, Wightman F (1982) Gas chromatographic and GC-MS evidence for the occurrence of 3-indolylpropionic acid and 3-indolylacetic acid in seedlings of *Cucurbita pepo*. Physiol Plant 56:367–370

Sembdner G, Gross D, Liebisch H-W, Schneider G (1980) Biosynthesis and metabolism of plant hormones. In: MacMillan J (ed) Encyclopedia of plant physiology. New Series, Vol. 9. Springer-Verlag, Berlin Heidelberg New York, pp 281–444

Silver WS, Bendana FE, Powell RD (1966) Root nodule symbiosis. II. The relation of auxin to root geotropism in roots and root nodules of non-legumes. Physiol Plant 19:207–218

Srivastava BIS (1964) Investigations on the occurrence and biosynthesis of indole-pyruvic acid in plant tissues and bacteria. Plant Physiol 39:781–785

Stowe B, Thimann KV (1954) The paper chromatography of indole compounds and some indole-containing auxins of plant tissues. Arch Biochem Biophys 51:499–516

Stowe B, Vendrell M, Epstein E (1968) Separation and identification of indoles in maize and woad. In: Wightman F, Setterfield G (eds) Biochemistry and physiology of plant growth substances. The Runge Press, Ltd., Ottawa, Canada, pp 173–182

Summons RE, Duke CC, Eichholzer JV, Entsch B, Letham DS, MacLeod JK, Parker CW (1979) Mass spectrometric analysis of cytokinins in plant tissues. II. Quantitation of cytokinins in *Zea mays* kernels using deuterium labelled standards. Biomed Mass Spectrom 6:407–413

Thimann KV (1936) On the physiology of the formation of nodules on legume roots. Proc Natl Acad Sci 22:511–514

Tsurumi S, Wada S (1980) Transport of shoot- and cotyledon-applied indole-3-acetic acid to *Vicia faba* root. Plant Cell Physiol 21:803–816

Vincent JM (1970) A manual for the practical study of root-nodule bacteria. IBM Handbook No. 15. Blackwell Scientific Publications, Oxford, p 145

Vlitos AJ, Meudt W (1954) The role of auxin in plant flowering. III. Free indole acids in short-day plants grown under photoinductive and nonphotoinductive daylengths. Contrib Boyce Thompson Inst 17:413–417

Wang TL, Wood EA, Brewin NJ (1982) Growth regulators, *Rhizobium* and nodulation in peas: Indole-3-acetic acid from the culture medium of nodulating and non-nodulating strains of *R. leguminosarum*. Planta 155:345–349

Wightman F (1962) Metabolism and biosynthesis of 3-indoleacetic acid and related indole compounds in plants. Can J Bot 40:689–718

Wightman F (1977) Gas chromatographic identification and quantitative estimation of natural auxins in developing plant organs. In: Pilet PE (ed) Plant growth regulation. Springer-Verlag, Berlin Heidelberg New York, pp 77–90

Winter A, Thimann KV (1966) Bound indoleacetic acid in *Avena* coleoptiles. Plant Physiol 41: 335–342