

# Phytohormones, *Rhizobium* Mutants, and Nodulation in Legumes<sup>1</sup>

## III. AUXIN METABOLISM IN EFFECTIVE AND INEFFECTIVE PEA ROOT NODULES

Received for publication November 4, 1982 and in revised form June 10, 1983

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### ABSTRACT

High specific activity [<sup>3</sup>H]indole-3-acetic acid (IAA) was applied to the apical bud of intact pea (*Pisum sativum* L. cv Greenfeast) plants. Radioactivity was detected in all tissues after 24 hours. More radioactivity accumulated in the nodules than in the parent root on a fresh weight basis and more in effective (nitrogen-fixing) nodules than in ineffective nodules (which do not fix nitrogen).

For most samples, thin layer chromatography revealed major peaks of radioactivity at the R<sub>f</sub> values of IAA and indole-3-acetylaspatic acid (IAAsp) and further evidence of the identity of these compounds was obtained by chromatography in other systems. Disintegrations per minute due to IAA per unit fresh weight were significantly greater for root than for nodule tissue, but were not significantly different for effective and ineffective nodules. Radioactivity due to IAAsp, expressed both on a percentage basis and per unit fresh weight, was significantly greater for nodule than for root tissue and significantly greater for the effective nodules than for the ineffective nodules. When [<sup>3</sup>H]IAA was applied to effective nodules, IAAsp was the dominant metabolite in the nodule. The data suggest that metabolism of auxins may be important for the persistence of a functional root nodule.

A root nodule is a unique and highly organized structure developed as a result of the symbiotic relationship which normally occurs between leguminous plants and bacteria of the genus *Rhizobium*. The development and persistence of a functional root nodule clearly requires a high degree of regulation. It is generally believed that plant hormones are involved in triggering the initiation of root nodules, and that hormonal balance is an important factor in the control of nodule development, maintenance, and senescence. There is little fundamental evidence available, however, to support this belief.

There are few accurate data on the level of auxins in root nodules and none on the metabolism of auxins in intact root nodules. Several reports indicate that root nodules have greater auxin content than the parent roots in the legumes *Pisum sativum* (17), *Pisum arvense* (19), *Glycine max* (17), and *Lupinus luteus* (9, 16) and in the nonlegume, *Alnus glutinosa* (10). With the exception of the work of Dullaart (9, 10), who used spectro-

fluorimetry, these studies have relied on bioassay and need to be confirmed using an unambiguous quantitation method such as mass spectrometry. In the work presented here, we have examined in some detail the transport to the root nodule of radioactive auxin ([<sup>3</sup>H]IAA) applied to the apical bud and have investigated whether nodules differ from the parent root in their metabolism of IAA received from the shoot. Furthermore, by constructing a strain which forms ineffective (do not fix nitrogen) root nodules on pea plants, we were able to make a comparative study between plants inoculated with this strain and those inoculated with a strain that forms effective (nitrogen-fixing) nodules.

### MATERIALS AND METHODS

**Chemicals.** Radiochemical purity of the high specific activity [<sup>3</sup>H]IAA used ([5-<sup>3</sup>H]IAA from C.E.A., Gif-Sur-Yvette, France; 1073 Gbq mmol<sup>-1</sup>) was checked several times by TLC, using both systems 1 and 2 (see below), during the course of these experiments and was normally between 90 and 95%. When the percentage dropped below 85%, the radiolabeled IAA was purified by HPLC (see below). The sources of indole compounds were: IAAsp<sup>3</sup> (Drs. J. D. Cohen and R. S. Bandurski, Michigan State University, East Lansing), 4-Cl-IAA and Me-4-Cl-IAA (Dr. K. C. Engvild, Ris National Laboratory, Roskilde, Denmark), IAld, IAacAld, and IMetOH (Sigma), IAA (Calbiochem), ICA (Tokyo Kassi Co. Ltd., Tokyo, Japan), ILA (Aldrich Chemical Company), and IGA (Pfaltz and Bauer Inc.).

Solid supports used for TLC were silica gel 60 PF<sub>254</sub> and silanized silica gel 60 HF<sub>254</sub> (E. Merck, Darmstadt, Germany), polyamide DC 6 UV<sub>254</sub> (Macherey Nagel, Duren, Germany), Serva cellulose (Serva Feinbiochemica, Heidelberg, Germany), and D-5 silica gel (Camag, Muttentz, Switzerland). Woelm green fluorescent indicator (M. Woelm, Eschwege, Germany) was incorporated into the Camag silica gel and Serva cellulose (0.4% and 0.8% (w/w), respectively) prior to spreading the layers. Camag silica gel plates were washed prior to use by allowing methanol to flow to the top of the layer. Acetone, ethyl acetate, and dichloromethane were redistilled before use.

**Bacterial Strains.** The *Rhizobium leguminosarum* strains used were strain ANU897 (Nod<sup>+</sup> Fix<sup>+</sup>) and strain ANU203 (Nod<sup>+</sup> Fix<sup>-</sup>). Strain ANU897 is a derivative of strain 300 and has the following genetic markers: phe-1, tryp-123, and str-37 (15). Strain ANU897 was kindly provided by Dr. J. E. Beringer, Rothamsted Experimental Station, Hertfordshire, England. Strain ANU203

<sup>1</sup> Part II of this series is J. Badenoch-Jones, R. E. Summons, B. Entsch, B. G. Rolfe, C. W. Parker, and D. S. Letham 1982 Biomed Mass Spectrom 9: 429–437. Part I is Reference 1.

<sup>2</sup> Supported by a Postdoctoral Fellowship from the Australian National University and by Public Health Service Grant GM28027 from the National Institutes of Health.

<sup>3</sup> Abbreviations: IAAsp, indole-3-acetylaspatic acid; 4-Cl-IAA, 4-chloroindole-3-acetic acid; Me-4-Cl-IAA, monomethyl-4-chloroindole-3-acetic acid; IAacAld, indole-3-acetaldehyde; IAld, indole 3-aldehyde; ICA, indole-3-carboxylic acid; IGA, indole-3-glycolic acid; ILA, indole-3-lactic acid; IMet, indole-3-methanol.

was constructed for use in the present study by transferring into strain 202 (kindly provided by Dr. J. M. Vincent, University of Sydney, Australia) the transmissible pea nodulation plasmid (pJB5JI). Strain 202 is a derivative of the naturally occurring *R. trifolii* coryn strain (6), for which the symbiosis does not continue long enough to achieve much nitrogen fixation (25). This mutant is classed as a nodule persistence (Nop) mutant under the scheme of Vincent (25). Strain ANU203 was ineffective as indicated by the stunted growth, yellow leaves, and lack of acetylene reduction of plants inoculated with this strain.

**Plant Culture.** Intact plants were used in all the experiments in order to maintain physiological conditions and to avoid any effect of wounding such as might occur in experiments where IAA is applied to decapitated stems.

Seeds of *Pisum sativum* L. cultivar Greenfeast were grown as described previously (8), except when nodules were labeled directly, when plants were grown in large upright-placed Petri dishes (13.5 cm diameter) containing 150 ml of sterile solid (1.5% (w/v) agar) modified Fahraeus medium (24). The plant stem was pulled through a hole made in the top of the Petri dish and the dish was sealed with Nescofilm (20).

**Application of Radiolabel.** Plants (5/experiment) were grown in flasks and had radiolabel applied to the apical bud either at 16 or 24 d after inoculation with strain ANU897, or at 22 and 30 d after inoculation with strain ANU203. Plants inoculated with strain ANU203 were labeled later than those inoculated with strain ANU897 because of the delayed appearance of nodules (approximately 6 d) on the former plants. Using a microsyringe, 5  $\mu$ l of [ $^3$ H]IAA (equivalent to 185 kBq and 30.2 ng IAA; ethanol solution) were slowly and carefully applied to the apical bud and the two youngest expanding leaves surrounding the bud (referred to for simplicity as the apical bud). Plants were harvested 24 h after the application of the radiolabel. Experiments, each using five plants, were done on two separate occasions. When root nodules were directly labeled, [ $^3$ H]IAA (5  $\mu$ l) was applied using aseptic techniques, to accessible root nodules lying on the surface of the agar. Three plants inoculated with strain ANU897 were labeled 16 d after inoculation and were harvested 24 h after labeling.

**Seedling Dissection and Extraction.** Entire plants were dissected into roots, nodules, cotyledon, stem, apical bud, and leaves. Tissues from the individual plants were pooled. Stems were further cut into segments of approximately 1 cm in length.

The extraction procedure was based on that developed by Bandurski and Schulze (2) to minimize hydrolysis of IAA complexes. Manipulations were carried out in dim light. Unlabeled carrier IAA and IAAsp (each at 10  $\mu$ g g $^{-1}$  fresh weight of tissue) were added to the sample. After complete evaporation of the acetone, the concentrate was diluted to an equal volume with distilled water and counted. The sample (pH 3.0) was partitioned with ethyl acetate using the procedures of Badenoch-Jones *et al.* (1). It was then adjusted to pH 8.0 and partitioned with dichloromethane (1). The residues were dissolved in 50% ethanol (v/v) and aliquots were counted and analyzed by TLC. Analyses were carried out within 15 h of sample preparation.

Control experiments, using both root and nodule tissue, were done in duplicate to estimate recovery of added [ $^3$ H]IAA during sample extraction and purification by the procedures routinely employed, and to check that these procedures did not cause appreciable breakdown of the [ $^3$ H]IAA. Before extraction with ethyl acetate, recovery of radiolabel averaged 92% for both tissues, while after extraction, mean recovery was 84% for both tissues. Radiochemical purity of the [ $^3$ H]IAA, as evidenced by TLC, was reduced by less than 5%. In one experiment, samples of root and leaf were extracted for shorter periods (three extractions of 2.5 h), with stirring. This extraction procedure was found to yield results almost identical to those obtained by the method

routinely used. Data presented in "Results" are not corrected for recovery, but it is clear that the techniques employed ensured the recovery of a large and consistent proportion of radioactivity. It should be noted, however, that any IAA derivatives insoluble in 70% (v/v) acetone would not be recovered from samples.

**Chromatography.** *Thin Layer Chromatography.* Because some metabolites and conjugates of IAA have TLC mobilities similar to IAA itself, all samples were analyzed by two or more of the TLC systems which are outlined in Table I. Due to instability of IAA and some of its metabolites, it was considered preferable to analyze samples by chromatography in several one-dimensional systems rather than by two-dimensional chromatography.

Spotting and developing of TLC plates (20  $\times$  20 cm, 0.25-mm thick layer) were carried out in dim light and darkness, respectively. Unlabeled marker compounds were co- or parallel-chromatographed with samples. Following chromatography, marker compounds were visualized under UV light at 254 nm. Chromatogram zones for the determination of radioactivity by liquid scintillation counting were eluted with 0.5 ml water in counting vials at 20°C for approximately 18 h.

*High Performance Liquid Chromatography.* HPLC was carried out using a  $\mu$ Bondapak C $_{18}$  column (300  $\times$  3.9 mm) (Waters Associates) and 30% (v/v) ethanol in 0.2 N acetic acid as solvent at a flow rate of 1.5 ml min $^{-1}$ . Details of the HPLC apparatus have been reported previously (22).

**Liquid Scintillation Counting.** The following scintillation fluid (10 ml/1 ml of sample) was used: 6 g PPO, 1 L Triton X-100, and 2 L toluene. Samples were counted twice in an LKB 1215 Rackbeta II liquid scintillation spectrometer (Wallac Oy, Turku, Finland) and cpm were corrected for background, and for quenching using the external standard ratio method. In order to count leaf extracts accurately, it was necessary to construct a quench curve using unlabeled leaf extract as the quenching agent.

**Statistics.** Mean values are presented with SEM and significance levels were calculated using Student's *t* test.

## RESULTS

**Distribution of  $^3$ H in the Plant.** When [ $^3$ H]IAA was applied to the apical bud, radioactivity was detected in all tissues. In most experiments, tissues could be listed in the following order with respect to accumulation of radioactivity per unit fresh weight: bud  $\gg$  stem > nodule > leaf > root > cotyledon (Table II). Accumulation of radiolabel per unit fresh weight appeared to decline as the plants aged. The decline was of similar magnitude for tissues of plants inoculated with either *Rhizobium* strain and there was little indication of a change with age in the relative accumulation of radioactivity between the different tissues.

In every experiment, the amount of radioactivity per unit fresh weight of nodule tissue was greater than that for root tissue, especially in the case of plants inoculated with strain ANU897. Averaging over the two ages, for these plants, the mean difference in accumulation of radioactivity between root and nodule tissue on a fresh weight basis was significant ( $P < 0.05$ ); the mean value for total radioactivity per unit fresh weight was significantly ( $P < 0.001$ ) higher for nodules formed by strain ANU897 than for those formed by strain ANU203, as was the mean value for the ratio, total dpm mg $^{-1}$  fresh weight nodule/total dpm mg $^{-1}$  fresh weight root ( $5.4 \pm 0.32$  and  $2.0 \pm 0.06$ , respectively). A ratio of 1.8 was found for plants inoculated with strain ANU203 and harvested 8 d after labeling instead of the usual 24 h.

On a per organ basis, accumulation of radioactivity was generally in the following sequence for plants inoculated with strain ANU897: bud  $\gg$  stem > root > nodule > leaf > cotyledon, while the following sequence applied to plants inoculated with strain ANU203: bud  $\gg$  stem > root > leaf > nodule > cotyledon (Table II). Nodule tissue accounted for approximately 4 and 13% of exported radioactivity for plants inoculated with strain

Table I.  $R_f$  Values for a Number of Authentic Indole Compounds in the TLC Systems Used in the Present Study

TLC System Number	Solid Support	Solvent System <sup>a</sup>	$R_f$ Values									
			IAA	IAAsp	IACald	IAlD	ICA	IGA	ILA	IMet	4-Cl-IAA	Me-4-Cl-IAA
1.	Polyamide	A	0.31	0	0	0 and 0.46	0.35	0 and 0.04	0.09	0.40	0.24	0.74
2.	Polyamide	B	0.60	0.34	0	0.70	0.52	0.50	0.56	0.67	0.60	0.80
3.	Cellulose	C	0.38	0.11	0.43	0.87	0.30	0.29	0.39 and 0.93	0.84	0.38	0.86
4.	Silanized silica gel	A	0.62	0.26	0.64	0.44	0.59	0.27	0.36	0.71	0.61	0.62
5.	Merck silica gel	D	0.77	0.06	0.56	0.71	0.72	0.13	0.16	0.72	0.77	0.82
6.	Camag silica gel	A	0.58	0.05	0.64	0.29	0.54	0.05	0.09	0.73	0.47	0.57

<sup>a</sup> Solvent systems (solvents are by volume): A, benzene:ethyl acetate:acetic acid (14:5:1); B, butan-1-ol:14 N ammonia:water (4:1:1; upper phase); C, isopropanol:14 N ammonia:water (8:1:1); D, ethylmethyl ketone:ethyl acetate:ethanol:water (3:5:1:1).

Table II. Radioactivity Extracted from Tissues of Intact Pea Plants to Which [<sup>3</sup>H]IAA Was Applied to the Apical Bud, and the Contribution of [<sup>3</sup>H]IAA and [<sup>3</sup>H]IAAsp to This Radioactivity

Tissues were extracted with acetone (70% [v/v]) and extracts were purified by ethyl acetate partitioning (pH 3). Results are means of two experiments (five plants per experiment).

Time of Labeling (d.a.i.) <sup>a</sup>	Strain of <i>Rhizobium</i>	Tissue	Total dpm mg <sup>-1</sup> f.w.t. <sup>b</sup> (A)	Transported dpm in whole tissue (B) <sup>c</sup>	Ethyl Acetate Fraction				
					dpm mg <sup>-1</sup> f.w.t. (C)	dpm due to IAA, mean ± SEM (D) <sup>d</sup>	dpm as IAA mg <sup>-1</sup> f.w.t. (E) <sup>e</sup>	dpm due to IAAsp (F) <sup>f</sup>	dpm as IAAsp mg <sup>-1</sup> f.w.t. (G) <sup>g</sup>
			×10 <sup>-2</sup>	%	×10 <sup>-2</sup>	%	×10 <sup>-1</sup>	%	×10 <sup>-1</sup>
16	ANU897	Root	3.5	30	2.5	42 ± 1.47 (5)	10.3	20	5.0
		Nodule	17.7	15	11.4	0.9 ± 0.21 (5)	1.0	47	53.6
		Cot. <sup>h</sup>	1.0	0.6	0.5				
		Stem	14.6	35	9.1	15.1 ± 0.55 (3)	13.7	45	41.1
		Leaf	4.4	19	1.3	14.4 ± 1.13 (3)	1.9	27	3.6
		Bud	3347		1854	22.7 ± 0.94 (3)	4208	35	6451
24	ANU897	Root	1.6	16	1.0	48.6 ± 0.83 (4)	4.7	21	2.0
		Nodule	8.7	11	6.4	1.7 ± 0.15 (4)	1.1	55	35.4
		Cot.	0.4	0.3	0.2				
		Stem	11.1	60	6.5	12.1 (2)	7.8	46	30.0
		Leaf	1.7	13	0.5	16.8 (2)	0.8	18	0.8
		Bud	3379		1652	21.4 (2)	3536	35	5849
22	ANU203	Root	2.0	15	0.9	45.7 ± 1.23 (5)	4.2	14	1.3
		Nodule	3.8	3	1.8	13.8 ± 0.36 (5)	2.4	38	6.7
		Cot.	0.9	0.7	0.5				
		Stem	10.3	56	6.0	11.8 ± 0.56 (4)	7.1	39	23.3
		Leaf	6.4	25	2.6	42.1 ± 0.93 (4)	10.9	15	4.0
		Bud	3920		2006	25.8 ± 3.56 (4)	5175	22	4373
30	ANU203	Root	0.9	17	0.6	39.5 ± 0.70 (4)	2.4	16	0.9
		Nodule	1.5	4	1.6	6.2 ± 0.36 (4)	1.0	36	5.7
		Cot.	0.1	0.1	0.2				
		Stem	12.0	74	6.4	9.1 ± 0.81 (3)	5.8	42	26.6
		Leaf	0.9	5	0.6	15.6 ± 0.93 (3)	0.9	10	0.6
		Bud	3252		1671	18.4 ± 0.94 (3)	3075	23	3760

<sup>a</sup> d.a.i., days after inoculation.

<sup>b</sup> f.w.t., fresh weight tissue.

<sup>c</sup> Calculated as  $[A \times \text{fresh weight tissue (data not shown)} \times 100] / \Sigma B$  for root, nodule, cotyledon, stem, and leaf.

<sup>d</sup> Represents the percentage of dpm co-chromatographing with IAA in several (number in parentheses) TLC systems.

<sup>e</sup>  $E = C \times D/100$ .

<sup>f</sup> Represents the percentage of dpm co-chromatographing with IAAsp in TLC system 2.

<sup>g</sup>  $G = C \times F/100$ .

<sup>h</sup> Cot., cotyledon.

ANU203 and ANU897, respectively. An average of 88% of applied radiolabel remained at the site of application (the apical bud).

In an overall assessment, the ethyl acetate fractions derived from leaf, apical bud, stem, root, and nodule tissue contained 40 ± 5.7, 52 ± 5.0, 58 ± 1.5, 61 ± 4.4, and 72 ± 7.6%, respectively,

of the total <sup>3</sup>H extracted from that tissue.

The dichloromethane fractions usually contained less than 2% of the radioactivity in the corresponding ethyl acetate fractions, and hence only the latter were characterized chromatographically as outlined in the next section.

#### Chromatographic Characterization and Identity of Metabolites

in Ethyl Acetate Fractions. Results for several tissues chromatographed by TLC systems 1, 2, and 3 are shown in Figures 1, 2, and 3, respectively. For most samples, there were major peaks of radioactivity at the  $R_f$  values of IAA and IAAsp. Exceptions to this generalization were nodules formed by strain ANU897 for which there was little or no  $^3\text{H}$  at the  $R_f$  of IAA. In root tissue, the peak at the  $R_f$  of IAA was usually the major peak, but for other tissues, the peak at the  $R_f$  of IAAsp was usually dominant. Whereas IAAsp remained at the origin, together with several other indole compounds, in TLC system 1, in system 2 this compound moved a considerable distance from the origin and none of the indole compounds tested co-chromatographed with it (Table I). Extracts of all tissues when subjected to TLC (system 1) exhibited a minor peak of  $^3\text{H}$  immediately after the origin, where IGA and ILA chromatographed, and in TLC system 3, many samples had minor peaks of  $^3\text{H}$  in the zones before and after IAAsp, but none of the marker compounds chromatographed in these zones.

Data for the percentages of sample dpm (ethyl acetate fraction) that co-chromatographed with authentic IAA marker are given in Table II, except for the cotyledon, for which the mean value over all experiments was  $20 \pm 3.7\%$ . In almost all cases, the results obtained for the same sample using several different TLC systems corresponded closely (see SEM in Table II). This is despite the fact that in many of the TLC systems used, IAA did not separate from one or more potential IAA metabolites (Table I).

In order to confirm that radioactivity co-chromatographing with IAA in system 1 was in fact  $^3\text{H}$ IAA, the ethyl acetate

fraction of a root extract was chromatographed by TLC and the zone at the  $R_f$  of IAA was eluted with ethyl acetate. The eluate was evaporated to dryness, and then subjected to HPLC. For a  $^3\text{H}$ IAA standard, 94.6 and 95.5% (duplicates) of the radioactivity which co-chromatographed with IAA during TLC co-eluted with IAA on HPLC. For the root extract, the corresponding value was 92.2%. For both standard and sample, the remaining 5 to 8% of radioactivity chromatographed largely in the fraction eluting immediately before IAA.

Averaging over all experiments, the root had the highest percentage of radioactivity (ethyl acetate fraction) as IAA ( $43 \pm 1.1\%$ ) of all the plant tissues, whereas nodule tissue had the lowest ( $6 \pm 1.2\%$ ). Values for leaf and bud tissue were similar ( $23 \pm 2.6$  and  $23 \pm 2.1\%$ , respectively), and higher than the value for stem tissue ( $12 \pm 0.8\%$ ). Nodules formed by strain ANU897 had a significantly ( $P < 0.001$ ) lower percentage of radioactivity present as IAA ( $1.3 \pm 0.2\%$ ) than nodules formed by strain ANU203 ( $10 \pm 2.0\%$ ) (Table II).

Radioactivity as IAA  $\text{mg}^{-1}$  fresh weight of tissue was significantly ( $P < 0.05$ ) greater for root tissue than for nodule tissue; the mean ratio, root (dpm as IAA)/nodule (dpm as IAA) was  $4.6 \pm 1.91$ . Nodules from plants inoculated with either strain ANU897 or strain ANU203 did not differ significantly ( $P > 0.05$ ) in their content of  $^3\text{H}$ IAA per mg fresh weight (Table II).

Data for the percentage of radioactivity in the ethyl acetate fraction co-chromatographing with IAAsp in TLC system 2 are shown in Table II, except for the cotyledon, for which the mean value over all experiments was  $16 \pm 3.9\%$ . When TLC systems 1, 2, and 3 were compared, it was found that, for almost all samples, values from system 1 were highest and values from system 2 were lowest (data not shown). Inasmuch as IAAsp does not move from the origin in TLC system 1, it is not surprising that values for the percentage of radioactivity co-chromatographing with IAAsp overestimate the percentage of total  $^3\text{H}$  due to IAAsp in the samples. It is suggested that the values for system 2 are closest to the true values for percentage of sample radioactivity due to IAAsp. Results from the following experiment provide evidence to support this suggestion. Ethyl acetate fractions derived from two bud extracts were chromatographed, together with marker IAAsp, by TLC system 2 in the first dimension. Of the  $^3\text{H}$  in the IAAsp zone, 88% co-chromatographed with this marker when the TLC plate was developed in the second dimension (solvent:butan-1-ol:acetic acid:water, 12:3:5, v/v/v). The occurrence of IAAsp in root and nodule tissues of pea plants was also confirmed by mass spectrometry. An extract of roots plus nodules (plants inoculated with strain ANU203) was purified by solvent partitioning, TLC (system 2), and HPLC ( $\mu$ Bondapak  $C_{18}$  column ( $300 \times 7.9$  mm); 20% (v/v) ethanol in 0.2 N acetic acid as solvent). GC-MS of the resulting fraction detected a compound with the same retention time and mass spectrum as authentic IAAsp.

Averaging over all experiments, root tissue had a significantly ( $P < 0.001$ ) lower percentage ( $18 \pm 0.7\%$ ) of sample  $^3\text{H}$  (ethyl acetate fraction) co-chromatographing with IAAsp (TLC system 2) than nodule tissue ( $44 \pm 3.7\%$ ) and a significantly ( $P < 0.001$ ) lower  $^3\text{H}$ IAAsp content on a fresh weight basis than nodule tissue. However, nodules from plants inoculated with strain ANU203 had a significantly ( $P < 0.05$ ) lower percentage of sample radioactivity (ethyl acetate fraction) co-chromatographing with IAAsp ( $37 \pm 4.1\%$ ) than nodules from plants inoculated with strain ANU897 ( $51 \pm 3.6\%$ ) and a significantly ( $P < 0.01$ ) lower  $^3\text{H}$ IAAsp content (Table II). When root nodules were labeled directly, IAAsp was the major metabolite detected in the nodules, accounting for approximately 60% of  $^3\text{H}$  in the ethyl acetate fraction.

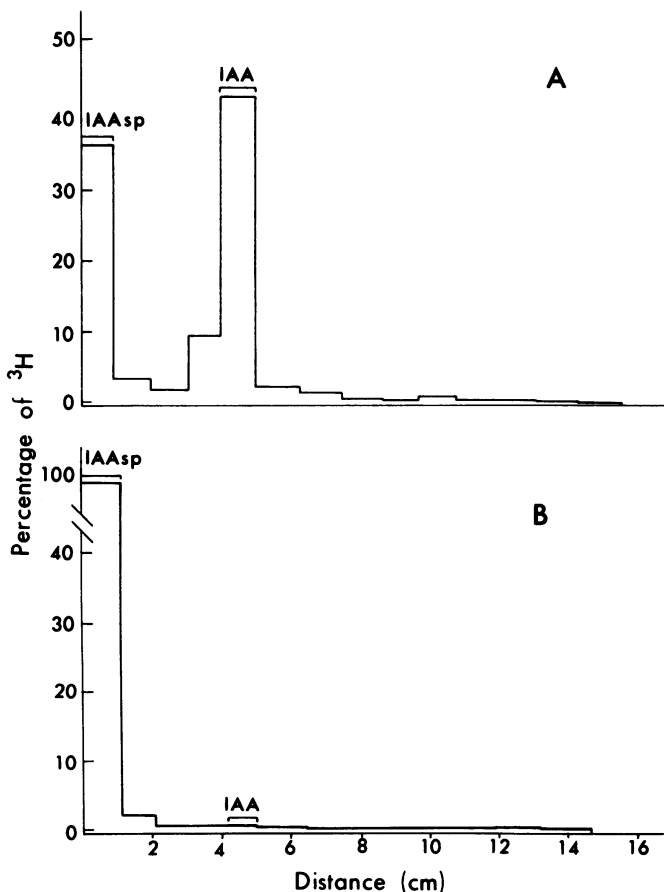


FIG. 1. Typical distribution of radioactivity of ethyl acetate fractions of extracts of root tissue (A) and nodule tissue, strain ANU897 (B) using TLC system 1 (Table I). Radioactivity is expressed as the percentage of the total radioactivity recovered from the chromatogram. The positions of co-chromatographed marker compounds are indicated.

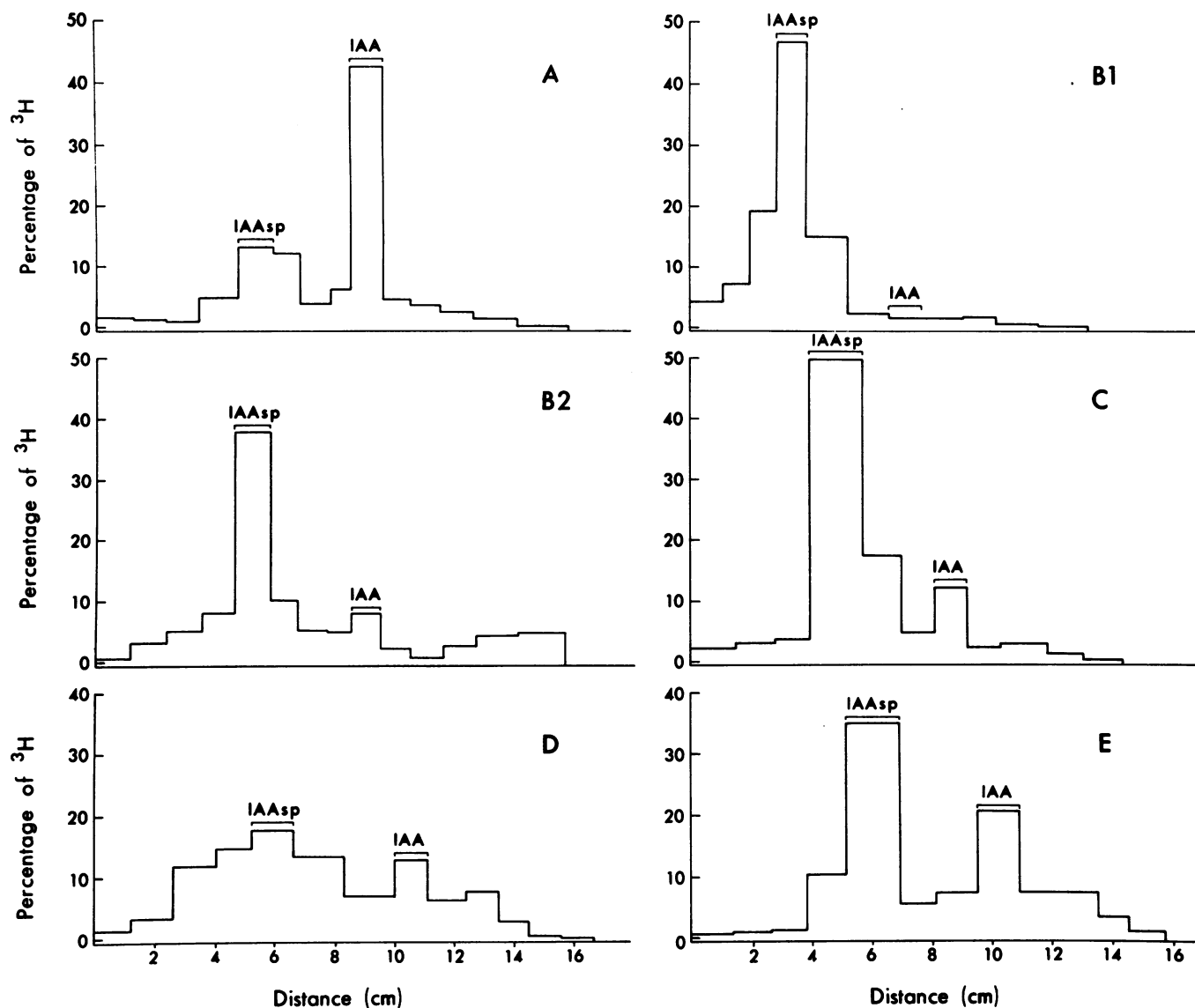


FIG. 2. Typical distribution of radioactivity of ethyl acetate fractions of tissue extracts of root (A), nodule, strain ANU897 (B1), nodule, strain ANU203 (B2), stem (C), leaf (D), and apical bud (E) using TLC system 2. Radioactivity is expressed as percentage of the total radioactivity recovered from the chromatogram. The positions of co-chromatographed marker compounds are indicated.

### DISCUSSION

The young expanding leaves are considered one of the most important sites of IAA synthesis in the plant (26). Radioactivity applied as [ $^3\text{H}$ ]IAA to the apical bud and young leaves of nodulated pea plants (calculated to represent less than 5 times the tissue pool of total IAA [compare Bandurski and Schulze, Ref. 3]) moved within 24 h to all tissues of the plant, and it is probable that endogenous auxin is similarly transported.

Although there are many reports that auxin applied to the shoot can be transported to the root (4), the present study is the first to examine in particular, transport of auxin to, and metabolism of auxin by, the root nodule in relation to the whole plant.

Very little radioactivity accumulated in the cotyledon, as might be expected in a tissue that is senescing and in which the concentration of IAA has declined to a low level (11). In contrast, considering their distance from the apical bud, the root system and root nodules represent a considerable sink for IAA applied to the apical bud.

Chromatographic studies indicated that in most tissues some radioactivity remained as IAA and a considerable proportion was present as the conjugate IAAsp. Similar results have often,

but not always, been reported in legumes. After application of labeled IAA to the apical bud of intact pea (18) and *Vicia faba* plants (5), radioactivity was detected as IAA and IAAsp in various tissues. However, substantial quantities of a labeled compound were also found in root tissue and tentatively identified as IAld (18). Tsurumi and Wada (23), who applied [ $^{14}\text{C}$ ]IAA to the cotyledon of *V. faba* plants, found that approximately 35% of radioactivity in root tissue was as IAA, but none of the remaining activity co-chromatographed with IAAsp.

Data from the current investigation suggested that IAAsp was the major compound in root nodules *in vivo* resulting from the metabolism of auxin applied to the apical bud, and accounted for up to 40% of the  $^3\text{H}$ . Furthermore, results from the experiment in which root nodules were labeled directly indicate that nodule tissue itself has the capacity to form IAAsp. IAAsp appears to occur naturally in several species (3) and the possible functions of the naturally occurring conjugation of IAA have been recently reviewed by Cohen and Bandurski (7) and include a mechanism for maintaining hormonal homeostasis that would enable nodules to accumulate IAA (in conjugate form) without causing major changes in their steady state concentration of free

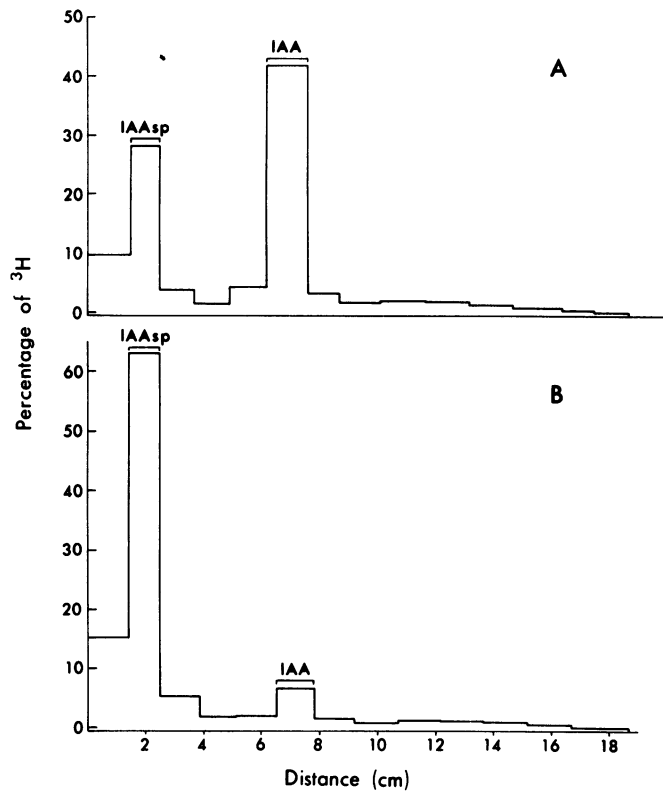


FIG. 3. Typical distribution of radioactivity of ethyl acetate fractions of extracts of root tissue (A) and nodule tissue, strain ANU203 (B) using TLC system 3. Radioactivity is expressed as percentage of the total radioactivity recovered from the chromatogram. The positions of co-chromatographed marker compounds are indicated.

#### IAA.

Labeled IAA was metabolized to some extent prior to reaching the root system, definitely at the site of its application, and probably also in the stem. Other workers have shown that IAA is also metabolized during transport from the seed to the shoot (13). The transported form of IAA cannot be determined from the present experiments, although when auxin is applied to the apical bud, it is believed to move in a system that is specific for auxins and does not involve transport of IAAsp (12).

Whereas IAAsp was identified as a major metabolite of IAA in the present study, unidentified minor metabolites were also detected by TLC as indicated by the presence of radioactivity at positions where none of the marker indole compounds chromatographed. Indole compounds that have been identified in plants and are likely to be derived from IAA include IAld, ICA, IMet, ILA, IGA, and IAld (21). Other IAA derivatives which have been identified in legumes, at least in immature seeds of the tribe *Vicieae*, are 4-Cl-IAA and its methyl ester (14). In the plant tissues examined in the present study, these metabolites, if present at all, made a minor contribution to metabolite radioactivity, since there was little radioactivity at the  $R_f$  values of these compounds. Furthermore, all TLC systems gave very similar results for the percentage of  $^3\text{H}$  due to IAA in most samples even though in some of these systems ICA, ILA, IAld, 4-Cl-IAA, or Me-4-Cl-IAA co-chromatographed with IAA.

Our data indicate that when [ $^3\text{H}$ ]IAA is applied to the apical bud of pea plants, the root nodules, be they either effective or ineffective in fixing nitrogen, accumulate more radioactivity on a fresh weight basis, and have a greater proportion of this radioactivity as IAAsp, than corresponding root tissue. It is of particular interest that functional nodules which fix nitrogen

have a greater capacity to accumulate auxin transported from the shoot and to metabolize it to IAAsp than nodules which cannot fix nitrogen. These results provide evidence that the root nodule, which has already been noted to be a morphologically unique and highly organized structure formed following infection of legume roots by *Rhizobium*, is also markedly different from the root tissue from which it is derived in its auxin relationships with the whole plant. They also suggest that the metabolism of auxins received from the shoot apex may be an important factor in the persistence of a functional root nodule.

*Acknowledgments*—The authors wish to thank J. McIver and C. M. Bates for excellent assistance and Professor R. S. Bandurski for advice and encouragement which he gave generously during a visit to this laboratory.

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