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Phytohormones, *Rhizobium* Mutants, and Nodulation in Legumes[1]

V. CYTOKININ METABOLISM IN EFFECTIVE AND INEFFECTIVE PEA ROOT NODULES

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ABSTRACT

[^{3}H]Zeatin riboside was supplied to intact pea (*Pisum sativum*) plants either onto the leaves or onto the root nodules. When applied directly to nodules, approximately 70% of recovered radioactivity remained in the nodules, approximately 15% was detected in the root system, and 15% was in the shoot. However, when supplied to the leaves, little ^{3}H was transported, with approximately 0.05% of recovered radioactivity being found in the root system and nodules. On a fresh weight basis, nodules accumulated more ^{3}H than the parent root. In both types of studies, metabolites with an intact zeatin moiety were detected in root nodules.

In all experiments, two-dimensional thin layer chromatography revealed that little ^{3}H remained as zeatin riboside in root or nodule tissue at the end of the labeling period. Nodules metabolized [^{3}H]zeatin riboside to the following cytokinins/cytokinin metabolites: zeatin, adenosine, adenine, the *O*-glucosides of zeatin and zeatin riboside, lupinic acid, nucleotides of adenine and zeatin, and the dihydro derivatives of many of these compounds.

Although a few small differences were observed, there were no major differences between root and nodule tissue in their metabolism of [^{3}H] zeatin riboside. Furthermore, any differences between effective and ineffective nodules were generally minor.

It has been proposed that cytokinins may be involved in the initiation and growth of root nodules. Circumstantial evidence for such a role for cytokinins comes from four types of studies. First, studies by Torrey and colleagues (18, 31) demonstrated that polyploid mitoses, characteristic of an early phase of nodule development, are initiated in the presence of both auxin and cytokinin, in mature root cells of cultured pea root segments. Second, application of exogenous cytokinins to roots can induce pseudonodules (22, 25), but their internal structure bears little resemblance to that of functional nodules. Third, there is evidence that at least some strains of *Rhizobium* in pure culture may produce cytokinins (see 8). Finally, cytokinin activity has been detected by bioassay in root nodules of *Pisum sativum* (28, 29, 32), *Phaseolus mungo* (13), and the non-legume, *Myrica gale* (23). It has been reported that root nodules of legumes (9, 21) and non-legumes (10) contain levels of cytokinins which are high relative to the parent root.

Syono *et al.* (28) found that the cytokinin content of *P. sativum* root nodules declined with age after 14 d, until at 35 d there was almost no extractable cytokinin. The identity, source, and fate of cytokinins in nodules and the contribution of nodules to the cytokinin economy of the whole plant are not known. Accordingly, we have studied the translocation and metabolism of [^{3}H][9R]Z[3] applied to leaves and directly to the root nodules of intact pea plants. Plants bearing either effective (nitrogen-fixing) or ineffective (unable to fix nitrogen) nodules were used in these experiments to ascertain whether the ability to fix nitrogen is associated with specific aspects of cytokinin metabolism. These studies complement a previous investigation of auxin metabolism in effective and ineffective pea root nodules (1). To the authors' knowledge, this is the first detailed examination of the metabolism of cytokinins in legume root nodules. The metabolism of Z in nodules and leaves of the non-legume *Alnus glutinosa* L. Gaertn. has been reported previously (11, 12).

When supplied exogenously to plant tissues, Z can be converted to a diversity of metabolites (see 7). These include: Ade and Ado, products of isoprenoid side chain cleavage; the riboside and nucleotides of Z; the *N*-glucosides of Z, [7G]Z, and [9G]Z; the *O*-glucosides of Z and [9R]Z, (OG)Z, and (OG)[9R]Z; and the alanine conjugate of Z, termed [9Ala]Z. The isoprenoid side chain can also be reduced to give (diH)Z, (diH)[9R]Z, dihydrozeatin nucleotides, and dihydro derivatives of the following: lupinic acid, (OG)Z, (OG)[9R]Z, and [9G]Z, termed (diH)[9Ala]Z, (diH OG)Z, (diH OG)[9R]Z, and (diH)[9G]Z, respectively. Data from the present study indicate that many of the above-mentioned metabolites are formed when [^{3}H][9R]Z is administered to either effective or ineffective nodules.

MATERIALS AND METHODS

Materials. [^{3}H][9R]Z (specific activity, 8.84 GBq mmol^{-1}) was prepared as described by Summons *et al.* (27). In one experiment

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[3] Abbreviations (which are those used in a recent review on cytokinins [17]): [9R]Z, zeatin riboside (9-β-D-ribofuranosylzeatin); Ado, adenosine; Ade, adenine; Z, zeatin (6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine); (diH)Z, dihydrozeatin (6-(4-hydroxy-3-methylbutylamino)purine); (diH)[9R]Z, dihydrozeatin riboside; (OG)Z, *O*-β-D-glucopyranosylzeatin; (diH OG)Z, *O*-β-D-glucopyranosyldihydrozeatin; (OG)[9R]Z, *O*-β-D-glucopyranosyl-9-β-D-ribofuranosylzeatin; (diH OG)[9R]Z, *O*-β-D-glucopyranosyl-9-β-D-ribofuranosyldihydrozeatin; [9Ala]Z, lupinic acid (L-β-[6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-purin-9-yl]alanine); (diH)[9Ala]Z, dihydrolupinic acid; [7G]Z, 7-glucopyranosylzeatin; [9G]Z, 9-glucopyranosylzeatin; (OG)Z, (diH OG)Z, (OG)[9R]Z, and (diH OG)[9R]Z, *O*-glucosides; iP, N^6-(Δ^2-isopentenyl)adenine; [9R]iP, 9-β-D-ribofuranosyl-iP.

(see below), [^{3}H][9R]Z of higher specific activity (65.1 GBq mmol^{-1}) was used. It was a generous gift from C. H. Hocart. [9Ala]Z, (diH)Z, and the cytokinin glucosides were synthesized by published methods (3, 5, 6, 14); (diH)[9R]Z was prepared from [9R]Z by hydrogenation at room temperature using a Pd catalyst (10% Pd on activated charcoal). Z and [9R]Z were purchased from Calbiochem-Behring Corp.

Silica Gel 60 PF_{254}, DF5 silica gel, and Serva cellulose for TLC were obtained from E. Merck (Darmstadt, Germany), Camag (Muttenz, Switzerland), and Serva Feinbiochemica (Heidelberg, Germany), respectively. Woelm green fluorescent indicator (M. Woelm, Eschwerge, Germany) was incorporated into the Serva cellulose at 0.8% (w/w) before spreading the layer. The following dyes were used as markers for TLC: dimarene brilliant blue K-BL (dye B) (Polysciences Inc., Warrington, PA), rhodamine B (dye C) (Hopkin and Williams Ltd., Chadwell Heath, Essex, England), toluidine blue (dye D) (George T. Gurr, Div. of Baird and Tatlock, Romford, England), bromocresol green D (dye F) (Aldrich Chemical Co., Milwaukee, WI), fast green FCF (dye E) and orange G (dye G) (Edward Gurr Ltd., London), and meldola blue (dye A) (Searle Diagnostic, High Wycombe, England), the latter being purified by TLC.

Alkaline phosphatase from *Escherichia coli* (Type III-S), almond β-glucosidase, Ado, Ade, iP, and [9R]iP were purchased from Sigma Chemical Co. Large Petri dishes (13.5-cm diameter) were purchased from Sterilin Ltd., Teddington, Middlesex, England.

Plant Culture. Pea plants were grown either in 250-ml flasks or in large Petri dishes (2, 4).

Bacterial Strains. Details of the strains of *R. leguminosarum* used in this study, strain ANU897 (Nod$^+$ Fix$^+$) and the ineffective strain ANU203 (Nod$^+$ Fix$^-$), have been reported previously (1).

Application of Radiolabel. In order to obtain representative results without carrying out a large number of time-consuming TLC analyses, we labeled a relatively large number of plants per experiment and pooled their tissues.

(a) Application to Leaves. Plants (five per experiment) were grown in flasks and were labeled 14 and 20 d after inoculation with strains ANU897 and ANU203, respectively. Plants inoculated with strain ANU203 were labeled later than those inoculated with strain ANU897 to allow for the 6-d delay in nodulation of the former strain compared with the latter. A solution of [^{3}H][9R]Z in 50% ethanol (45 μl, the equivalent of 2.04 MBq and 81 μg) was painted as evenly as possible over all leaves of a plant using a fine paint brush. Plants were harvested 8 d after labeling.

(b) Application to Root Nodules. The nodules of 15 plants grown in Petri dishes were labeled at 16 and 22 d after inoculation (strains ANU897 and ANU203, respectively). Using sterile techniques, 11 μl (0.5 MBq; 19.8 μg) of the [^{3}H][9R]Z solution used under (a) were applied as evenly as possible to all accessible nodules of a plant (in the order of 40 to 80). Care was taken not to contaminate root tissue with radiolabel. Plants were harvested 8 d after labeling. In one experiment with plants inoculated with strain ANU897, the above procedure was carried out except that 11 μl of a 50% ethanol solution of the higher specific activity [^{3}H][9R]Z (equivalent to 0.61 KBq and 3.3 ng [9R]Z) were applied per plant.

Tissue Dissection and Extraction. Plants were dissected into nodules, stem, leaves, and roots (the entire root system dissected free of nodules), and the tissues from individual plants were pooled. When [^{3}H][9R]Z was applied to the leaves, all nodules were collected, but when nodules were the site of [^{3}H][9R]Z application, only those to which [^{3}H][9R]Z was applied were collected. The tissues were weighed and dropped into ethanol/water/acetic acid (75:23:2, v/v/v) at −20°C (100 ml g^{-1} fresh weight tissue). By addition of the following compounds as carriers (normally 10 μg g^{-1} fresh weight of tissue), a recovery of 90% or more of the endogenous compound could be expected: Z, [9R]Z, (diH)Z, (diH)[9R]Z, [9G]Z, (OG)Z, (diH OG)Z, (OG)[9R]Z, (diH OG)[9R]Z, and [9Ala]Z. Enzymes were inactivated by leaving the samples at −20°C for 7 d, with occasional stirring. The tissues were then homogenized with a Waring blender and the homogenates were allowed to stand for 24 h at room temperature with occasional stirring. Samples were filtered through a Büchner funnel and the pellet was re-extracted for a further 24 h. The combined extracts were evaporated to dryness under reduced pressure at less than 40°C. The residues were dissolved in 50% ethanol (v/v) and an aliquot counted. The samples were then evaporated to dryness and the residues dissolved in water, adjusted to pH 3.0, and subjected to ion-exchange chromatography on cellulose phosphate (Whatman P1 (floc); Whatman Chemical Separation Ltd., England). The cellulose phosphate was used in the NH_4^+ form equilibrated to pH 3.0 by washing with 0.5 M acetic acid. Cytokinin nucleotides were washed from the column with 0.05 M acetic acid. The column was eluted with 0.5 M NH_4OH until the effluent was alkaline, followed by 2 column volumes. Recoveries of cytokinins from such columns were normally over 90%. The acidic wash and the NH_4OH eluate were evaporated to dryness and the residues were dissolved in 50% ethanol (v/v) for counting and analysis by TLC.

TLC. Solvents used were as follows (proportions are by volume): 1, butan-1-ol/acetic acid/water (12:3:5); 2, butan-1-ol/14 N ammonia/water (6:1:2, upper phase); 3, methyl acetate/ethanol/acetic acid/2,2-dimethoxypropane/formic acid (120:13:7:1.4:0.35); 4, methylethyl ketone/acetic acid/water (16:1:4). Layers, 0.25 or 1.0 mm thick, were used for analytical and preparative TLC, respectively. Merck silica gel was used for TLC of plant extracts (acidic wash and NH_4OH eluate from cellulose phosphate columns). Solvent 1 was used for one-dimensional TLC. NH_4OH eluates were also developed in two dimensions: in solvent 1 and then, after thorough drying, in solvent 2. When separation of the four *O*-glucosides was desired, plates were run twice in solvent 2. After one-dimensional chromatography, location of the labeled cytokinins and their metabolites was determined with respect to the dyes A, B, C, and D which were added to the sample before chromatography (see Fig. 1 for location of authentic standards with respect to the dyes). After two-dimensional TLC, UV light (254 nm) was normally used to visualize unlabeled marker compounds which were co-chromatographed with the sample (see Fig. 1). In some experiments, the *O*-glucoside complex and [9Ala]Z were located after chromatography in the second dimension by reference to the dyes E, F, and G which were applied to the plate after TLC in the first dimension (see Fig. 1). The entire two-dimensional TLC layers were cut into zones for counting. Each plate was divided into appropriate horizontal lanes, of approximately equal height, according to the location of the marker compounds. Each horizontal lane containing a marker was divided into a number of zones of equal width to the marker spot. Zones were eluted with 0.5 ml (or 1.0 ml for larger zones) of water in scintillation vials for approximately 18 h at 20°C, before the addition of scintillation fluid. Normally, a sample was analyzed only once by two-dimensional TLC, but some samples were assayed in duplicate and the results found to be reproducible.

Chromatogram zones cut from preparative plates were packed into columns and eluted exhaustively with 50% methanol (v/v), or for Z-containing zones with methanol/water/acetic acid (50:48:2, v/v/v). Ado and Ade zone eluates were directy rechromatographed on Serva cellulose using solvent 2. Z and [9R]Z eluates were purified on a cellulose phosphate column and then rechromatographed either on Camag silica gel using solvent 3, or by HPLC (see below), for separation of Z and [9R]Z from the corresponding dihydro compounds. The Camag silica gel plates were heated to 100°C for 1 h and then allowed to cool before

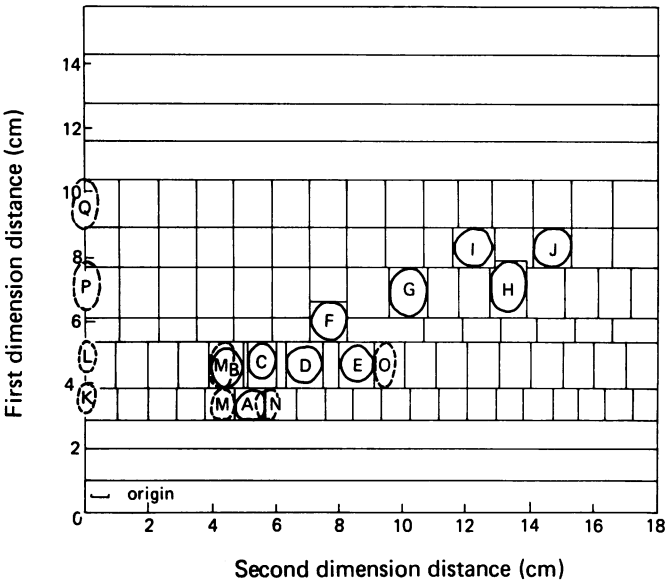

FIG. 1. Positions of authentic cytokinins/cytokinin metabolites and dyes after two-dimensional TLC on Merck silica gel using butan-1-ol/acetic acid/water (12:3:5, v/v/v) as solvent in the first dimension and butan-1-ol/14 N ammonia/water (6:1:2, v/v/v; upper phase) as solvent in the second dimension. A, [9Ala]Z; B, (OG)[9R]Z; C, (diH OG)[9R]Z; D, (OG)Z; E, (diH OG)Z; F, [9G]Z; G, Ado; H, Ade; I, [9R]Z; J, Z; K, dye A; L, dye D; M, dye G; N, dye E; O, dye F; P, dye B; Q, dye C. After one-dimensional TLC, [9Ala]Z chromatographed with and just below dye A, the *O*-glucosides chromatographed with dye D and between dyes A and D, Ado and Ade chromatographed with and just below dye B, and Z and [9R]Z chromatographed largely between dyes B and C, but running just into the top of dye B. Nucleotides chromatographed below dye A and iP and [9R]iP chromatographed above dye C.

spotting the sample. [9Ala]Z eluates were purified on a cellulose phosphate column and then rechromatographed on Serva cellulose using solvent 4, followed by solvent 2, for separation of [9Ala]Z from its dihydro derivative. The cellulose [9Ala]Z eluate was then subjected to HPLC (see below). All of the above compounds were located by co-chromatography with unlabeled authentic standards. The eluates containing *O*-glucosides were treated with β-glucosidase (see below), repurified by extraction at pH 8 into butan-1-ol (3 times, equal volume), and then rechromatographed on Merck silica gel in one dimension using solvent 2, with the appropriate unlabeled *O*-glucoside and its hydrolysis product as markers.

Nucleotide-containing fractions (cellulose phosphate acidic wash), after hydrolysis with alkaline phosphatase (see below), were purified by butanol extraction as described above and chromatographed on Merck silica gel using solvent 2 with unlabeled Ado and [9R]Z as markers.

High Performance Liquid Chromatography. HPLC was carried out on a Zorbax C8 column (9.4 × 250 mm) (DuPont Co.) using methanol in 0.2 M acetic acid as solvent, at a flow rate of 5.0 ml min^{-1}. The proportion of methanol (v/v) was as follows: for chromatography of [9Ala]Z, 20%; for separation of [9R]Z and (diH)[9R]Z, 30%; and for separation of Z and (diH)Z, 45%. Details of the HPLC apparatus have been reported previously (26).

Enzyme Incubations. Dephosphorylation of nucleotides with alkaline phosphatase and hydrolysis of the *O*-glucosides with β-glucosidase were done as previously described by Parker *et al.* (20) and Letham *et al.* (15), respectively.

Liquid Scintillation Spectrometry. Samples were counted by the procedures of Badenoch-Jones *et al.* (1).

RESULTS

[^{3}H][9R]Z Applied to Leaves. *Distribution of ^{3}H in the Plant.* Of the total ^{3}H recovered from the whole plant, only 3 to 4% had been exported from the leaves, and of this 98% remained in the stem. Thus, for plants inoculated with strain ANU203, root tissue accounted for only 1.1% of exported radioactivity and 0.03% of total radioactivity recovered from the plant and the corresponding values for nodule tissue were 0.5 and 0.02%, respectively. For plants inoculated with strain ANU897, root and nodule tissue each accounted for 0.9% of radioactivity exported and 0.03% of total radioactivity recovered from the plant. For plants inoculated with strain ANU897, dpm mg^{-1} fresh weight of tissue was 69.0 for nodules and 7.5 for roots, giving a nodule/root ratio of 9.2; the corresponding values for plants inoculated with strain ANU203 were 38.1 and 6.7, giving a nodule/root ratio of 5.7. The percentage of total dpm in the cellulose phosphate NH_4OH eluate was very similar for the same tissues from plants inoculated with either strain, and also differed little between the tissues, averaging 84% for nodule, 74% for root, 76% for stem, and 83% for leaf tissue.

TLC Profiles. Initially, the acidic wash and NH_4OH eluates from cellulose phosphate columns were chromatographed in one dimension. TLC profiles of the acidic wash fractions for the various tissues of plants inoculated with strain ANU897 were very similar to those for the same tissues of plants inoculated with strain ANU203 (see Fig. 2 for TLC profile of nodule tissue, acidic wash fraction, strain ANU203). The acidic wash could normally be readily distinguished from the NH_4OH eluate since little ^{3}H in the latter chromatographed below dye A, whereas a considerable proportion of total ^{3}H in the acidic wash chromatographed well below dye A, in an R_F region known to contain nucleotides of Ade and Z. This trend was particularly pronounced with leaf, stem, and root extracts. In all samples, very little ^{3}H chromatographed above the location of dye C. TLC profiles of the NH_4OH eluates for stem, leaf, and root tissues of plants inoculated with strain ANU897 were very similar to those for the same tissues of plants inoculated with strain ANU203. In Figure 2, therefore, only the profiles for plants inoculated with one of the strains (ANU203) are given. In the leaf and stem profiles, a major peak of ^{3}H chromatographing with Z/[9R]Z was evident, whereas for root tissue, the major peak of ^{3}H was at the position of Ado/Ade. Some differences were evident in the TLC profiles for nodule tissue formed by the different strains (Fig. 2); for nodules formed by strain ANU897, a greater proportion of ^{3}H chromatographed with Ado/Ade than for nodules formed by strain ANU203.

Data from two-dimensional TLC analyses (see "Materials and Methods") are given in Table I. The identification of radioactive compounds based on co-chromatography with authentic standards in only two different solvent systems is clearly equivocal. Hence, the results of these, and other two-dimensional analyses carried out during this study may best be considered upper limits. Nevertheless, for most samples, discrete peaks of radioactivity were detected on the chromatograms at the positions of Ade, Ado, Z/(diH)Z, and [9R]Z/(diH)[9R]Z, and for some samples, [9Ala]Z and the *O*-glucosides. It should be noted that Z and (diH)Z are not separated from each other using this TLC system, nor are [9R]Z and (diH)[9R]Z. The ^{3}H associated with Z and [9R]Z is thus designated as Z/(diH)Z and [9R]Z/(diH)[9R]Z, respectively. When sufficient ^{3}H-labeled putative [9R]Z and [9R]Z metabolites were available, preparative TLC was carried out and further chromatography performed in order to more rigorously establish the identity of these compounds (see data in brackets in Table I). In each case, when a bracketed value is given, this ^{3}H also co-chromatographed, as a discrete peak, with the authentic standard in the third TLC system mentioned.

Results of two-dimensional TLC of NH_4OH eluates confirmed

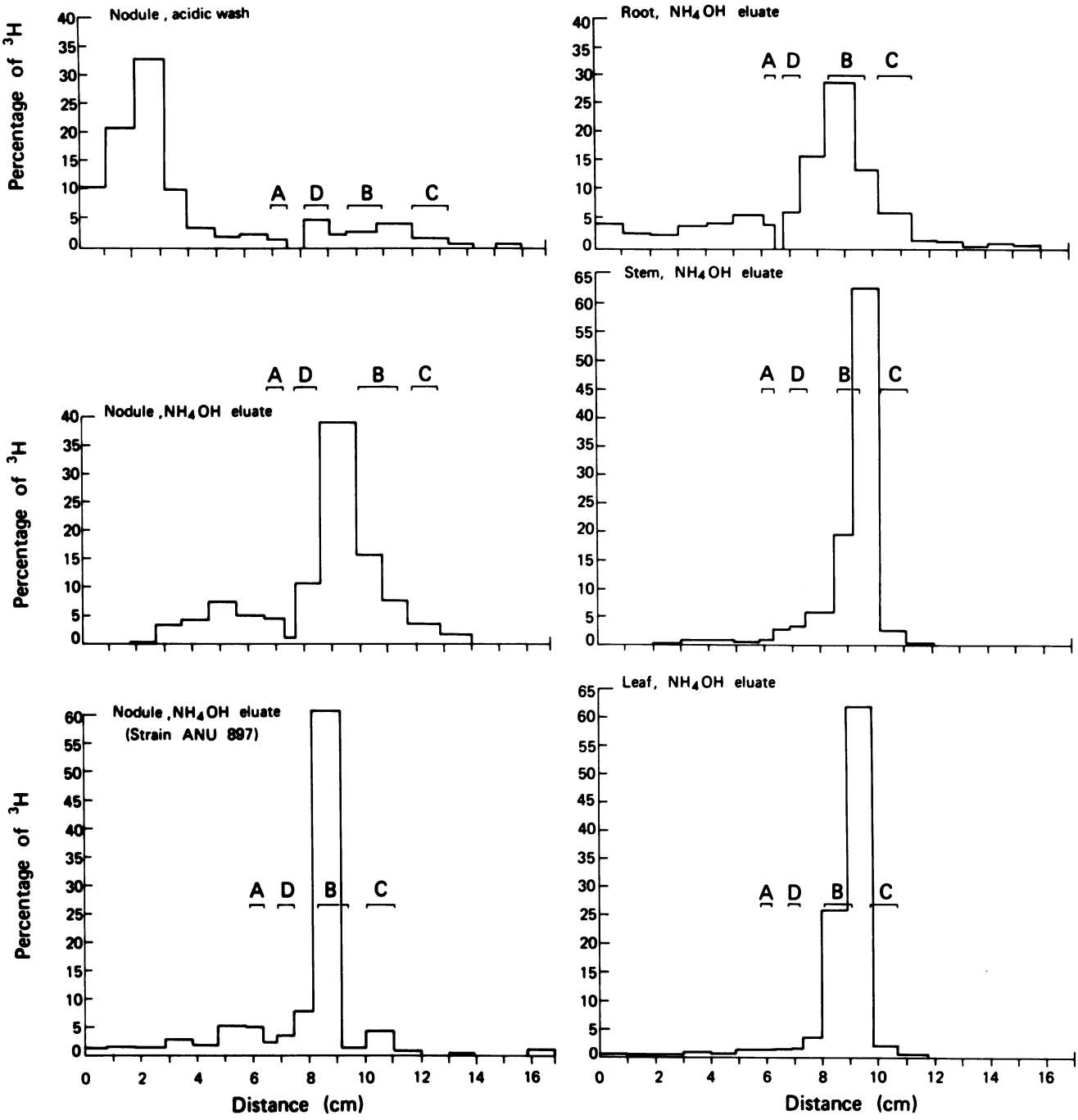

FIG. 2. One-dimensional TLC profiles (Merck silica gel, with butan-1-ol/acetic acid/water (12:3:5, v/v/v) as solvent) of extracts of various tissues from pea plants (inoculated with strain ANU203, unless otherwise indicated) and labeled for 8 d with [^{3}H][9R]Z via the leaves. Each extract was applied to a cellulose phosphate column and the acidic wash and NH_4OH eluate were analyzed by TLC. The positions of dyes A, B, C, and D are indicated.

those obtained from one-dimensional analysis and indicated that [9R]Z/(diH)[9R]Z was a major component of total ^{3}H in leaf and stem tissue. However, there was less [9R]Z/(diH)[9R]Z in stem than in leaf tissue, and considerably less in root, and even less in nodule tissue. For all samples, 80 to 90% of the putative [^{3}H][9R]Z/(diH)[9R]Z co-chromatographed with these markers in a third TLC system (Camag silica gel; solvent 3) and for all samples, the ratio of [9R]Z:(diH)[9R]Z was similar (approximately 2.4). [9Ala]Z, [9G]Z, and the *O*-glucosides did not contribute appreciably to the ^{3}H in any tissue, with the possible exception of the *O*-glucosides in nodules formed by strain ANU203. Ado appeared to be a major metabolite in all tissues, whereas Ade appeared to be a metabolite in root and nodule tissue. When rechromatographed on cellulose, a large proportion of the putative [^{3}H]Ade and [^{3}H]Ado co-chromatographed with authentic marker. A considerable proportion of ^{3}H in the cellulose phosphate acidic wash of all extracts was attributable to nucleotides of Z and Ade (see Table I), but the proportion was lower for nodule tissue than for the other tissues.

[^{3}H][9R]Z Applied to Root Nodules. *Distribution of ^{3}H in the Plant.* Whereas only a very small proportion of [^{3}H][9R]Z was translocated from the leaf, a considerable quantity of [^{3}H][9R]Z was exported from the root nodules (44 and 23% for plants inoculated with strains ANU897 and ANU203, respectively).

Table I. *Contribution of Various Cytokinins and Cytokinin Metabolites to Radioactivity Extracted from Plant Tissues after Application of [^{3}H][9R]Z to Leaves (A) or Nodules (B) of Intact Plants*

Ammonia eluates from the cellulose phosphate columns were analyzed by two-dimensional TLC. The proportion of dpm in the total extract that appeared in the ammonia eluate was similar for all tissues (see text). The radioactivity co-chromatographing with each marker compound is expressed as a percentage of total dpm eluted from the plate. Acidic washes from cellulose phosphate columns were hydrolyzed with alkaline phosphatase, butanol-extracted, and analyzed by TLC (Merck silica gel, solvent 2). The radioactivity co-chromatographing with Ado and [9R]Z is expressed as a percentage of total dpm in the acidic wash.

Strain of *Rhizobium*	Tissue	Total dpm mg^{-1} Fresh Wt. Tissue	Ammonia Eluates from Cellulose Phosphate Columns							Acidic Washes from Cellulose Phosphate Columns	
			[9A1a]Z	*O*-Glucoside complex[a]	[9G]Z	Ado[b]	Ade[b]	[9R]Z/(diH)-[9R]Z[c]	Z/(diH)Z[c]	Ado	[9R]Z
A. ANU897	Leaf	51,947	0.1	1.2 (0.2, 0.4, 0.4, 0.2)	0.1	7.6 (4.2)	0.3	63.9 (40.6, 13.6)	0.8	27.2	19.3
	Stem	2,749	0.3	2.5 (1.5, 0.3, 0.5, 0.2)	0.2	6.4	0.8	53.1 (33.8, 11.0)	1.0	15.3	32.2
	Root	7	0	3.4 (1.6, 1.3, 0.5, 0)	0.7	7.2 (5.5)	2.7 (2.2)	8.6 (4.9, 2.2)	1.0	16.5	20.8
	Nodule	69	1.1	1.3 (0.1, 0, 0.6, 0.6)	0	49.8	2.0 (1.7)	3.0	0	9.6	5.6
ANU203	Leaf	47,398	0.1	1.4 (0.3, 0.4, 0.5, 0.2)	0.2	7.7 (5.1)	0.4	58.1 (34.0, 12.8)	0.8	50.1	21.3
	Stem	1,974	0.1	3.8 (2.1, 0.8, 0.5, 0.4)	0.3	7.8 (5.3)	1.0	41.5 (25.1, 8.0)	0.3	19.8	36.7
	Root	7	0.4	2.3 (1.2, 0.1, 0.5, 0.5)	1.0	10.9 (3.9)	9.4 (5.6)	8.8 (5.0, 2.0)	1.3	44.3	6.4
	Nodule	38	1.5	6.5 (2.9, 1.4, 0.2, 2.0)	0.8	17.3	3.0 (1.7)	2.9 (1.7, 0.7)	0	11.3	13.6
			[d]	[d]							
B. ANU897	Leaf	448	5.8	11.9	2.0	7.1 (4.1)	3.0 (2.0)	4.3	1.4		
	Stem	248	1.5	9.8	2.6	12.3 (8.0)	7.7 (5.6)	4.4 (0.6, 2.2)	1.4 (0.5, 0.4)	25.4	15.6
	Root	487	4.0	8.6	1.6	4.5 (3.0)	3.7 (3.4)	2.6 (0.4, 1.9)	1.5 (0.4, 0.5)	31.7	5.8
	Nodule	9,572	19.3[e]	6.1	2.4	3.7 (3.0)	2.3 (1.7)	3.4 (0.9, 0.3)	1.3 (0.7, 0.5)	20.2	3.6
			[d]	[d]							
ANU203	Leaf	337	4.7	7.6	3.1	10.1 (5.9)	5.0 (3.8)	5.9	2.4		
	Stem	177	5.6	5.6	2.4	11.6 (8.6)	8.7 (6.7)	4.9 (1.7, 1.9)	1.3 (0.5, 0.4)	41.3	8.8
	Root	186	4.1	8.5	1.7	5.6 (4.3)	4.3 (3.2)	2.4 (0.7, 0.6)	1.6 (0.6, 0.8)	32.9	3.4
	Nodule	10,268	13.2[e]	5.3	1.5	4.4 (2.6)	1.9 (1.1)	2.0 (0.7, 0.4)	1.7 (0.8, 0.6)	23.9	5.0

[a] Values for the individual *O*-glucosides are given in parentheses in the following order: (OG)[9R]Z, (diH OG)[9R]Z, (OG)Z, (diH OG)Z.

[b] The values in parentheses denote the data multiplied by the fraction of ^{3}H co-chromatographing with marker compound in a third TLC system (cellulose, solvent 2).

[c] The values in parentheses denote the data multiplied by the fraction of ^{3}H co-chromatographing with the unsaturated compound and its dihydro derivative, respectively (Camag silica gel and solvent 3, experiment A; HPLC, experiment B).

[d] Dyes, rather than authentic standards, were used as markers (see "Materials and Methods").

[e] For details of further chromatography, see Results.

The percentage of transported ^{3}H that was detected in the different tissues was similar for plants inoculated with either strain; whereas 59 and 49% of transported radioactivity remained in the root system for plants inoculated with strains ANU897 and ANU203, respectively, a considerable proportion was also detected in the stem (14 and 16%, respectively, and even more in the leaves, 27 and 35%, respectively). The percentage of total dpm in the NH_4OH eluate of the cellulose phosphate column was similar for the same tissues from plants inoculated with the different strains, being approximately 98% for leaf, 90% for stem, and 63% for root and nodule tissue.

TLC Profiles. One-dimensional TLC profiles of the cellulose phosphate NH_4OH eluates of the various tissues of plants inoculated with strain ANU897 were very similar to those for the corresponding tissues of plants inoculated with strain ANU203. In Figure 3, therefore, only the profiles for tissues from plants inoculated with one of the strains (ANU897) are given. The profiles for root and nodule tissue were distinguishable by the relatively greater proportion of ^{3}H chromatographing with [9Ala]Z in the latter. The profiles for stem and leaf tissue were quite different from those obtained when [^{3}H][9R]Z was applied to the leaf (compare Fig. 2). Satisfactory chromatography of the acidic wash was only achieved for root and nodule samples, but again, similar profiles were obtained for the corresponding tissues of plants inoculated with the two strains (data not shown). A greater proportion of ^{3}H chromatographed in the nucleotide region for root than nodule tissue. Results from two-dimensional TLC (Table I) further elucidated those obtained from one-dimensional analysis and revealed a relatively large proportion of ^{3}H co-chromotographing with [9Ala]Z in nodule extracts, and with Ado, and to a lesser extent, Ade, in leaf and stem extracts. The *O*-glucosides appeared to make a small, but notable, contribution to total ^{3}H in all samples. Little radioactivity remained as [9R]Z in any tissue. HPLC on Zorbax C8 enabled separation of [9R]Z and Z from their corresponding dihydro derivatives; between 10 and 75% of the ^{3}H which co-chromatographed with [9R]Z or Z during two-dimensional TLC was the dihydro derivative (Table I). For all samples, there were discrete peaks of ^{3}H that co-eluted with Z and (diH)Z or with [9R]Z and (diH)[9R]Z. Very similar data were obtained when selected samples were chromatographed on both Camag silica gel and HPLC. The occurrence of (diH)[9R]Z and (diH)Z as metabolites, as previously indicated by TLC on Camag silica gel, was therefore confirmed by HPLC on Zorbax C8. A large proportion (averag-

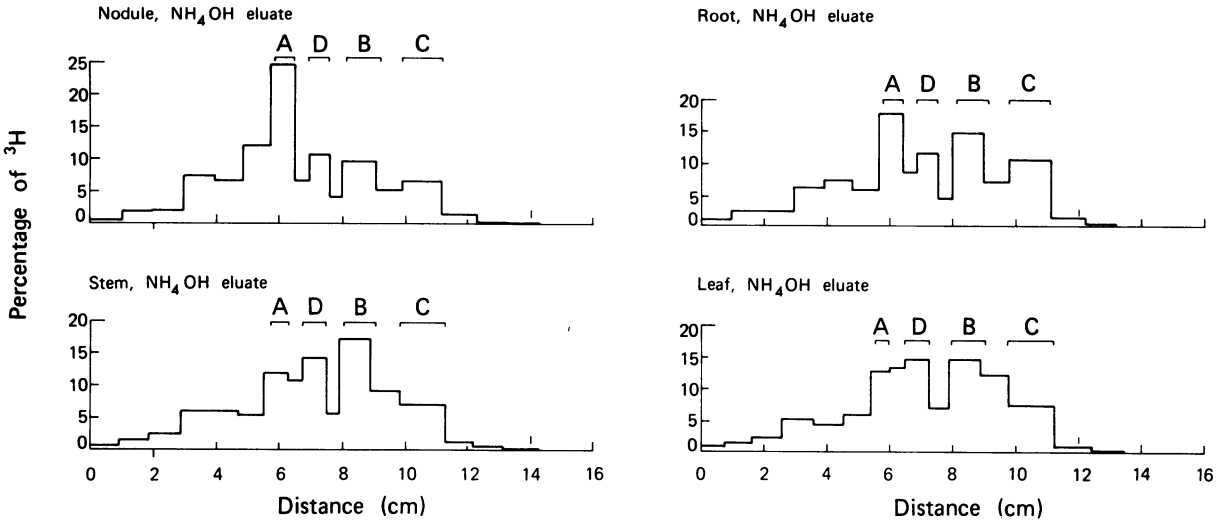

FIG. 3. One-dimensional TLC profiles (Merck silica gel, with butan-1-ol/acetic acid/water (12:3:5, v/v/v) as solvent) of cellulose phosphate NH_4OH eluates of extracts of various tissues from pea plants inoculated with strain ANU897 and labeled for 8 d with [^{3}H][9R]Z via the root nodules. The positions of dyes A, B, C, and D are indicated.

ing more than 70%) of radioactivity that co-chromatographed with Ado or Ade in two dimensions on silica gel, also co-chromatographed with the appropriate marker in a third system (cellulose, solvent 2). A considerable proportion (41% for the effective nodules and 49% for the ineffective nodules) of radioactivity that co-chromatographed with [9Ala]Z in two dimensions on silica gel also co-chromatographed with authentic standard in a third system (cellulose; solvent 4, followed by solvent 2), whereas 14 and 13%, for the two respective nodule types, co-chromatographed with (diH)[9Ala]Z. The occurrence of [9Ala]Z as a metabolite was supported by HPLC on a Zorbax C8 column; for the two respective nodule types, 73 and 71% of the putative [^{3}H][9Ala]Z eluted from the cellulose layer co-eluted with authentic [9Ala]Z when subjected to HPLC.

For all tissues (except leaf samples for which satisfactory chromatography was not achieved), an appreciable percentage of radioactivity in the acidic wash from cellulose phosphate columns was attributable to nucleotides of Ade and to a lesser extent to nucleotides of Z (Table I).

High Specific Activity [^{3}H][9R]Z Applied to Root Nodules in Small Quantities. In order to have sufficient radioactivity, especially in leaf and stem samples, so that samples could be counted accurately without encountering problems of excessive quenching and chemiluminescence, and could be analyzed by TLC without encountering problems of poor chromatography due to sample overloading, it was necessary to apply relatively large quantities of [^{3}H][9R]Z to the root nodules. Even then, for some samples (see above) satisfactory chromatography was not achieved. An experiment (using plants inoculated with strain ANU897) was carried out to determine whether metabolism of [9R]Z varied with the amount of [9R]Z applied. For this experiment, the highest specific activity [^{3}H][9R]Z available was used and the amount applied to the nodules was reduced to 3.3 ng (compared with 19.8 μg used previously). Radioactivity was detected in root and stem tissue. Only the nodule sample contained sufficient radioactivity for satisfactory TLC analysis. One-dimensional TLC of the NH_4OH eluate, which contained 97% of the total ^{3}H in the nodule extract, revealed a major ^{3}H peak chromatographing with the *O*-glucosides (dye D). Two-dimensional analysis revealed that, of the total ^{3}H recovered from the layer, 66% co-chromatographed with the *O*-glucosides (60% with (OG)[9R]Z, 5% with (diH OG)[9R]Z, 1% with (OG)Z, 0.5% with (diH OG)Z), 2% with [9Ala]Z, 0.8% with [9G]Z, 4% with Ado, 0.4% with Ade, 0.5% with [9R]Z/(diH)[9R]Z, and 0.2% with Z/(diH)Z. After hydrolysis of putative [^{3}H](OG)[9R]Z with β-glucosidase, only 2% of ^{3}H chromatographed with (OG)[9R]Z, while 62% of ^{3}H was found to co-chromatograph with [9R]Z. Hence, *O*-glucoside formation becomes more dominant as the amount of [9R]Z applied to nodules is reduced.

DISCUSSION

Zeatin riboside appeared to be metabolized to a considerable extent when applied directly to root nodules, be they either effective or ineffective, and when received by the nodules from the shoot. When [^{3}H][9R]Z was applied to leaves, the ratio of nodule ^{3}H to root ^{3}H per unit weight was considerably greater than 1, indicating that nodules accumulate more cytokinin than the parent root.

In all experiments, less than 4% of ^{3}H in the cellulose phosphate NH_4OH eluate from nodule tissue co-chromatographed bidimensionally with [9R]Z and in the order of 30% of this radioactivity was (diH)[9R]Z. Furthermore, normally only approximately 75% of total ^{3}H was collected in the NH_4OH eluate after chromatography on cellulose phosphate. Thus, no more than 1 to 2% of ^{3}H in nodule tissue was due to [9R]Z, irrespective of the route by which [9R]Z was supplied to the nodules. The spectrum of metabolites in nodule tissue included products of alanine conjugation, and of side chain cleavage, reduction, and glucosylation. However, in both root and nodule tissue, a considerable portion of radioactivity was not detected in known cytokinin metabolites. Averaging over all experiments, only approximately 20% of ^{3}H in the cellulose phosphate NH_4OH eluates from these tissues co-chromatographed with the authentic markers. This may indicate that side chain cleavage to Ado and Ade was more extensive than the values for their contribution to total ^{3}H might lead one to believe, since, once formed, these compounds may rapidly be further metabolized to a large number of other compounds.

When [^{3}H][9R]Z was applied to the leaf, a relatively large proportion of total ^{3}H in the leaf (approximately 35%) and in the stem (aproximately 22%) remained as [9R]Z and reduction to (diH)[9R]Z was the dominant form of metabolism. Thus, the leaf and stem appeared to metabolize [9R]Z to a much lesser extent than root and nodule tissue, although it is possible that some of the applied [9R]Z was not taken up by the leaf, but remained on its surface. However, when [^{3}H][9R]Z was applied

to root nodules, more extensive metabolism of [9R]Z was observed in stem and leaf tissue than when [^{3}H][9R]Z was applied to the leaf surface.

In most samples, a considerable proportion of ^{3}H in the alkaline phosphatase-treated acidic wash from cellulose phosphate columns co-chromatographed with Ado and [9R]Z. After taking into account the proportion of total extract ^{3}H in the acidic wash, it could be calculated that the nucleotides of Ade and Z contributed quite substantially (averaging approximately 7.5%) to total ^{3}H in the extract. The occurrence of cytokinin nucleotides in the plant tissues from our experiments was expected since the cytokinin 5′-phosphates appear to be the principal metabolites initially formed when cytokinin bases are supplied to many plant tissues, although they usually decrease quite rapidly after the early period of metabolism (17). Evidence is emerging that cytokinin nucleotides may play an important role in cytokinin metabolism in many plant tissues (see 17).

There have been relatively few studies of the metabolism of naturally occurring cytokinins in intact plants. We chose to use intact plants in order to maintain physiological conditions and to examine cytokinin metabolism in the nodule in relation to that in the whole plant. However, in order to label tissues sufficiently, it was necessary in some experiments to apply large quantitities of [9R]Z relative to endogenous concentrations. Although the amounts of cytokinin/cytokinin metabolites received by the root system from [^{3}H][9R]Z applied to the leaves, and by the shoot, from [^{3}H][9R]Z applied to the nodules, were within the physiological range, there remains some uncertainty as to whether the metabolism observed is indicative of that of endogenous cytokinins. A comparison of the experiment in which small amounts of the higher specific activity [^{3}H][9R]Z were applied directly to root nodules with the experiment carried out under identical conditions except that larger amounts of lower specific activity [^{3}H][9R]Z were applied, suggests that the metabolism of [9R]Z depends on the amount supplied to the nodules. At low concentrations, cytokinin appears to be conserved (stabilized) as *O*-glucosides; at high and perhaps unphysiological concentrations, side chain cleavage to yield Ado and Ade appears to predominate. This may be induced by the applied cytokinin itself (30).

Although it would appear that movement of cytokinin from shoot to root is possible via the phloem, we detected very little movement of ^{3}H to the root system when [^{3}H][9R]Z was applied to the leaves compared with the transport of ^{3}H observed when [^{3}H][9R]Z was applied to the nodules. Our results are therefore in accord with the generally accepted view that the root supplies cytokinin to the shoot (see 24) and that cytokinin applied to leaf laminae is essentially immobile (16).

Evidence from the present study and from the literature indicates that nodules may accumulate cytokinins in excess of the quantities in the parent root. If nodules functioned in storing cytokinins, one might expect them to metabolize a large proportion of the cytokinin that they received to storage forms of cytokinins. It has been proposed that the *O*-glucosides represent low activity storage forms of cytokinins, although their physiological role has not yet been clearly elucidated. Results from the present work show that, given the appropriate conditions, root nodules have the capacity to convert a considerable proportion of applied [9R]Z to *O*-glucosides. The high glucosylating capacity of nodule tissue has also been observed by Henson and Wheeler (11). However, under the experimental conditions in the current work, there were no real indications of greater metabolism of [9R]Z to *O*-glucosides in nodules compared with roots. The most notable difference between root and nodule tissue was the occurrence of [9Ala]Z as a prominent metabolite of [9R]Z in the latter, but not in the former, following the application of [^{3}H][9R]Z directly to the nodules. The metabolic significance of this difference remains uncertain. While [9Ala]Z is a stable compound and has only weak cytokinin activity in most bioassays, in some tissues for example, soybean callus, it gradually releases Z and may therefore be regarded as a possible slow release storage form of cytokinin (19).

Under the experimental conditions we employed, no major differences were found between effective and ineffective nodules in the number and nature of metabolites formed from [^{3}H][9R]Z. The only notable difference between the two nodule types was the greater proportion of ^{3}H which chromatographed with Ado during TLC in two dimensions for the effective rather than the ineffective nodules following the application of [^{3}H][9R]Z to the leaf. Nitrogen fixation by the ineffective nodules at the ages examined would have been negligible; thus, at least for nodules formed by strain ANU203, their defect in nitrogen fixation does not appear to be associated with a major alteration in their ability to metabolize cytokinins. It remains possible, however, that the quantitative levels of cytokinins may be very different in the nodules formed by the two different strains of *Rhizobium*. It is of interest that Henson and Wheeler (11) also found little difference in the metabolism of zeatin between nodules from nondormant and dormant plants of *Alnus glutinosa* L. Gaertn., where the latter would again, in contrast to the former, be inactive in fixing nitrogen.

Cytokinins or cytokinin metabolites move from nodule to root and then to the shoot. Further work is required, however, to fully elucidate the role of nodules in the cytokinin economy of the whole plant. While it would be of particular interest to know if nodules contribute to the cytokinin pool of the whole plant by synthesis of free cytokinins, this is a difficult question to address because of the low concentrations of cytokinins in plant tissues, including root nodules, and the central role of the most likely precursor (adenine) in cellular metabolism.

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