Phytohormones, *Rhizobium* Mutants, and Nodulation in Legumes

V. CYTOKININ METABOLISM IN EFFECTIVE AND INEFFECTIVE PEA ROOT NODULES

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ABSTRACT

[1H]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 [1H] 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 [1H] 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 [1H] remained as zeatin riboside in root or nodule tissue at the end of the labeling period. Nodules metabolized [1H]zeatin riboside to the following cytokinins/cytokinin metabolites: zeatin, adenosine, adenine, the O-glucosides of zeatin and zeatin riboside, lupin 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 [1H] 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 [1H] [9R]Z 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)9RZ; and the alanine conjugate of Z, termed [9Ala]Z. The isoprenoid side chain can also be reduced to give (diH)Z, (diH)9RZ, dihydrozeatin nucleotides, and dihydro derivatives of the following: lupin acid, (OG)Z, (OG)9RZ, and (OG)9Ala Z, (diH OG)Z, (diH OG)9RZ, and (diH)9GZ, respectively. Data from the present study indicate that many of the above-mentioned metabolites are formed when [1H][9R]Z is administered to either effective or ineffective nodules.

MATERIALS AND METHODS

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

1 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)9RZ, 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-ribofuranosylhydridrozeatin; [9Ala]Z, lupin acid (1-β-[6-(4-hydroxy-3-methylbut-trans-2-enylamino)-purin-9-yl]alanine); (diH)[9Ala]Z, dihydrolupin acid; [7G]Z, 7-glucopyranosylzeatin; [9G]Z, 9-glucopyranosylzeatin; (OG)Z, (diH OG)Z, (OG)9RZ, and (diH OG)[9R]Z, O-glucosides; iP, N4(Δ2-isopentenyl)adenine; [9R] IP, 9-β-D-ribofuranosyl-iP.

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4 Supported by a Postdoctoral Fellowship from the Australian National University and by Public Health Service Grant GM28207 from the National Institutes of Health, and later by a Queen Elizabeth II Fellowship.

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weight tissue). By addition of the following compounds as carriers (normally 10
applied per plant). Tissue Dissection and Extraction. Plants were dissected into
free of nodules), and the tissues from individual plants were
grown in Petri dishes were labeled at 16 and 22 d after inoculation
strain ANU897, the above procedure was carried out except that
11
days 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 [3H][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

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 inocu-
lated with strain ANU203 were labeled later than those inocu-
lated with strain ANU897 to allow for the 6-d delay in nodulation
of the former strain compared with the latter. A solution of [3H]
[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 tech-
niques, 11 µl (0.5 MBq; 19.8 µg) of the [3H][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
[3H][9R]Z (equivalent to 0.61 KBq and 3.3 ng [9R]Z) were applied
per plant.

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

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, Eng-
land.

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RESULTS

[14]H/3RJZ Applied to Leaves. Distribution of 3H in the Plant. Of the total \(^{3}\text{H}\) 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, the 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 \(\text{NH}_4\text{OH}\) 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 \(\text{NH}_4\text{OH}\) 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 nodular tissue, acidic wash fraction, strain ANU203). The acidic wash could normally be readily distinguished from the \(\text{NH}_4\text{OH}\) eluate since little \(^{3}\text{H}\) in the latter chromatographed below dye A, whereas a considerable proportion of total \(^{3}\text{H}\) 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 \(^{3}\text{H}\) chromatographed above the location of dye C. TLC profiles of the \(\text{NH}_4\text{OH}\) 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 \(^{3}\text{H}\) chromatographing with \(9\text{RJZ}\) was evident, whereas for root tissue, the major peak of \(^{3}\text{H}\) 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 \(^{3}\text{H}\) 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 bio-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 [9RJZ/(diH)]9RJZ, and for some samples, [9Ala]Z and the \(\text{O}-\)glucosides. It should be noted that Z and (diH)Z are not separated from each other using this TLC system, nor are [9RZ and (diH)]9RZ. The \(^{3}\text{H}\) associated with Z and [9RJZ is thus designated as Z/(diH)Z and [9RJZ/(diH)]9RJZ, respectively. When sufficient \(^{3}\text{H}\)-labeled putative [9RJZ 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 \(^{3}\text{H}\) also co-chromatographed, as a discrete peak, with the authentic standard in the third TLC system mentioned.

Results of two-dimensional TLC of \(\text{NH}_4\text{OH}\) eluates confirmed
those obtained from one-dimensional analysis and indicated that [9R][dH][9R]Z was a major component of total 3H in leaf and stem tissue. However, there was less [9R][dH][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 [3H][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/[dH][9R]Z was similar (approximately 2.4). [9Ala]Z, [9G]Z, and the O-glucosides did not contribute appreciably to the 3H 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 [3H]Ado and [3H]Ado co-chromatographed with authentic marker. A considerable proportion of 3H 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.

[3H][9R]Z Applied to Root Nodules. Distribution of 3H in the Plant. Whereas only a very small proportion of [3H][9R]Z was translocated from the leaf, a considerable quantity of [3H][9R]Z was exported from the root nodules (44 and 23% for plants inoculated with strains ANU897 and ANU203, respectively).
Table 1. Contribution of Various Cytokinins and Cytokinin Metabolites to Radioactivity Extracted from Plant Tissues after Application of [3H] [9R]Z to Leaves (A) or Nodules (B) of Intact Plants

<table>
<thead>
<tr>
<th>Strain of Rhizobium</th>
<th>Tissue</th>
<th>Total dpm mg⁻¹ Fresh Wt. Tissue</th>
<th>Ammonia Eluates from Cellulose Phosphate Columns</th>
<th>Acidic Washes from Cellulose Phosphate Columns</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. ANU897</td>
<td>Leaf</td>
<td>51,947</td>
<td>0.1</td>
<td>1.2 (0.2, 0.4, 0.4, 0.2)</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>2,749</td>
<td>0.3</td>
<td>2.5 (1.5, 0.3, 0.5, 0.2)</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>7</td>
<td>3.4</td>
<td>1.6 (1.3, 1.3, 0.5, 0.2)</td>
</tr>
<tr>
<td></td>
<td>Nodule</td>
<td>69</td>
<td>1.1</td>
<td>0.3 (1.0, 0.6, 0.6)</td>
</tr>
<tr>
<td>ANU203</td>
<td>Leaf</td>
<td>47,398</td>
<td>0.1</td>
<td>1.4 (0.3, 0.4, 0.4, 0.2)</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>1,974</td>
<td>0.1</td>
<td>3.2 (2.1, 0.8, 0.5, 0.4)</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>7</td>
<td>0.2</td>
<td>2.3 (1.2, 0.1, 0.5, 0.5)</td>
</tr>
<tr>
<td></td>
<td>Nodule</td>
<td>38</td>
<td>3.5</td>
<td>6.5 (2.9, 1.4, 0.2, 0.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. ANU897</td>
<td>Leaf</td>
<td>448</td>
<td>3.8</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>246</td>
<td>3.1</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>87</td>
<td>3.2</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Nodule</td>
<td>9,157</td>
<td>19.3</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU203</td>
<td>Leaf</td>
<td>337</td>
<td>4.7</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>177</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>186</td>
<td>4.1</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Nodule</td>
<td>10,268</td>
<td>13.2</td>
<td>5.3</td>
</tr>
</tbody>
</table>

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

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

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

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

For details of further chromatography, see Results.

The percentage of transported 3H 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 NH₄OH 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 NH₄OH 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 3H chromatographing with [9Ala] Z in the latter. The profiles for stem and leaf tissue were quite different from those obtained when 3H[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 3H 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 3H co-chromatographing 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 3H in all samples. Little radioactivity remained in [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 3H 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 3H 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)[9R] Z as metabolites, as previously indicated by TLC on Camag silica gel, was therefore confirmed by HPLC on Zorbax C8. A large proportion (averag-
that effective nodules 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 [3H][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 column was attributable to nucleotides of Ado and to a lesser extent to nucleotides of Z (Table 1).

**High Specific Activity [3H][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 [3H][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 [3H][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 NH₄OH eluate, which contained 97% of the total ³H in the nodule extract, revealed a major ³H peak chromatographing with the O-glucosides (dye D). Two-dimensional analysis revealed that, of the total ³H 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 [3H][OG][9R]Z with β-glucosidase, only 2% of ³H chromatographed with (OG)[9R]Z, whereas 62% of ³H 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 [3H][9R]Z was applied to leaves, the ratio of nodule ³H to root ³H 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 ³H in the cellulose phosphate NH₄OH 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 ³H was collected in the NH₄OH eluate after chromatography on cellulose phosphate. Thus, no more than 1 to 2% of ³H 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 ³H in the cellulose phosphate NH₄OH 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 ³H might lead one to believe, since, once formed, these compounds may rapidly be further metabolized to a large number of other compounds.

When [3H][9R]Z was applied to the leaf, a relatively large proportion of total ³H in the leaf (approximately 35%) and in the stem (approximately 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 [3H][9R]Z was applied
to root nodules, more extensive metabolism of [9R]Z was observed in stem and leaf tissue than when [3H][9R]Z was applied to the leaf surface.

In most samples, a considerable proportion of 3H 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 3H in the acidic wash, it could be calculated that the nucleotides of Ade and Z contributed quite substantially (averaging approximately 7.5%) to total 3H 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 quantities of [9R]Z relative to endogenous concentrations. Although the amounts of cytokinin/cytokinin metabolites received by the root system from [3H][9R]Z applied to the leaves, and by the shoot, from [3H][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 [3H][9R]Z were applied directly to root nodules with the experiment carried out under identical conditions except that larger amounts of lower specific activity [3H][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 3H to the root system when [3H][9R]Z was applied to the leaves compared with the transport of 3H observed when [3H][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 [3H][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 [3H][9R]Z. The only notable difference between the two nodule types was the greater proportion of 3H which chromatographed with Ado during TLC in two dimensions for the effective rather than the ineffective nodules following the application of [3H][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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