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Phytohormones, *Rhizobium* Mutants, and Nodulation in Legumes. VI. Metabolism of Zeatin Riboside Applied Via the Tips of Nodulated Pea Roots

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Abstract. [^{3}H]zeatin riboside was supplied in physiological quantities to pea (*Pisum sativum* L. cv Greenfeast) plants by replacing the root tip with a small vial containing [^{3}H]zeatin riboside, to simulate the normal supply of cytokinin. Radioactivity was transported to the root nodules. Analysis by 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 (2, 5, or 8 d) and suggested that the following compounds were metabolites of [^{3}H]zeatin riboside: zeatin, adenosine, adenine, the O-glucosides of zeatin and zeatin riboside, nucleotides of adenine and zeatin, and the dihydro-derivatives of many of these compounds.

The O-glucosides (and in particular, O-β-D-glucopyranosyl-9-β-D-ribofuranosylzeatin) appeared to be more prominent metabolites in the effective nodules formed by strain ANU897 than in the ineffective nodules produced by strain ANU203. However, no other appreciable differences were detected between effective and ineffective nodules in their metabolism of zeatin riboside. There were few marked differences between root and nodule tissue; however, in some experiments, the nodules contained a higher proportion of O-glucoside metabolites, and generally root tissue contained a greater proportion of zeatin and/or dihydro-

Abbreviations: TLC, thin layer chromatography; Ados, adenosine; Ade, adenine; Z, zeatin (6-[4-hydroxy-3-methylbut-*trans*-2-enylamino]purine); [9R]Z, zeatin riboside (9-β-D-ribofuranosylzeatin); (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.

zeatin, zeatin riboside and/or dihydrozeatin riboside, adenine and the nucleotides of zeatin and adenine, as metabolites.

While much work has been done on the metabolism of radiolabeled cytokinins in a variety of tissues (see Entsch et al. 1980), there have been few studies on root nodules (Henson and Wheeler 1977, Badenoch-Jones et al. 1983a), even though cytokinins have been implicated in the development and maintenance of root nodules (see Badenoch-Jones et al. 1983a). In a previous study (Badenoch-Jones et al. 1983a), we examined the metabolism by root nodules of ^{3}H-labeled zeatin riboside ([^{3}H][9R]Z) applied directly to the nodules or received by the nodules from the shoot. The results indicated that little [^{3}H][9R]Z was transported from the leaf laminae. In contrast, the root tip is generally believed to be a site of cytokinin biosynthesis and to supply cytokinin to the shoot (see Skene 1975). In the current study, we have attempted to simulate the normal situation by replacing the root tip with a vial containing [^{3}H][9R]Z. Our previous work suggested that the metabolism of [^{3}H][9R]Z by root nodules depends on the amount of [9R]Z supplied. Care was therefore taken in the present study to render the concentration of [^{3}H][9R]Z, supplied via the root tip, within the physiological range.

Materials and Methods

Materials, Plant Culture, and Bacterial Strains

The materials used have been described previously (Badenoch-Jones et al. 1983a). Plants were grown in large (13.5 cm diameter) upright-placed Petri dishes as described by Badenoch-Jones et al. (1983b). The plant stem was pulled through a hole made in the top of the Petri dish. Plant root growth took place largely over the surface of the agar, and seedlings were inoculated by streaking a fresh bacterial culture across the agar and growing roots. Details of the bacterial strains were given by Badenoch-Jones et al. (1983c).

Application of Radiolabel

Plants were normally labeled 16 d after inoculation with strain ANU897 or 22 d after inoculation with strain ANU203 (to allow for the 6-d delay in nodulation of the latter strain compared with the former). Using sterile techniques, accessible root tips were excised approximately 1–2 mm from the tip. Root tips were replaced with a small vial containing 1.1 ml solid (0.9%, w/v, agar) modified Fåhraeus medium (Vincent 1970) and [^{3}H][9R]Z (50 μM, specific activity 8.84 GBq mmol^{-1}). Approximately seven plants were selected from a total of twelve plants grown for the experiment, and two vials were used per plant. The vials were inserted into holes cut in the agar. In order to hold them in a fixed position, molten Fåhraeus agar (1.5%, w/v) was pipetted in any space between the vials and the agar in the dish. When this agar had solidified, the Petri dishes were returned to the growth cabinet in an upright position. In some experiments, the roots were excised at a greater distance behind the tip so that the vial was in closer proximity to the nodules (within

10 mm of the nodules nearest the root tip). Nodules and roots were harvested 2, 5, or 8 d after labeling.

Tissue Dissection and Extraction

The tissue collected consisted of (1) all nodules on the piece of root the tip of which was replaced by a vial containing [^{3}H][9R]Z, and (2) the portion of root between the top of the vial and the nodule farthest from the vial on the same piece of root. Tissues collected from the seven individual plants were pooled. Tissues were extracted as described previously (Badenoch-Jones et al. 1983a), except that they were homogenized with a hand grinder and were centrifuged (8,800 × *g*, 30 min, 5°C) rather than filtered. Chromatography on cellulose phosphate was done as described by Badenoch-Jones et al. (1983a).

Thin Layer Chromatography (TLC)

Both analytical and preparative TLC were carried out using the procedures of Badenoch-Jones et al. (1983a). The solid supports and solvents used were as follows (solvents are by volume): for one-dimensional TLC of acidic washes and NH_4OH eluates from cellulose phosphate columns—Merck silica-gel 60 PF_{254}, butan-1-ol/acetic acid/water (12:3:5) (solvent A); for two-dimensional TLC of cellulose phosphate NH_4OH eluates—Merck silica-gel, solvent A, followed by butan-1-ol/14N ammonia/water (6:1:2, upper phase) (solvent B); for TLC of putative [^{3}H]Ados and [^{3}H]Ade—cellulose, solvent B; for TLC of putative [^{3}H][9R]Z and (diH)[9R]Z and putative [^{3}H]Z and (diH)Z—Camag silica-gel, methyl acetate/ethanol/acetic acid/2,2-dimethoxypropane/formic acid (120:13:7:1.4:0.35); for TLC of hydrolyzed putative [^{3}H]O-glucosides and [^{3}H]nucleotides—Merck silica-gel, solvent B. Dye markers were used to locate metabolites as reported previously (Badenoch-Jones et al. 1983a).

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. (1978) and Letham et al. (1975), respectively. Samples were extracted with butan-1-ol (Badenoch-Jones et al. 1983a) prior to rechromatography.

Liquid Scintillation Spectrometry

Samples were counted by the procedures of Badenoch-Jones et al. (1983c).

Results

Cellulose Phosphate Chromatography and One-dimensional TLC

When plants were labeled for 8 d, the proportion of radioactivity in the acidic wash and NH_4OH eluate from cellulose phosphate columns was similar for all samples, averaging 33% and 67% for the wash and eluate, respectively, for

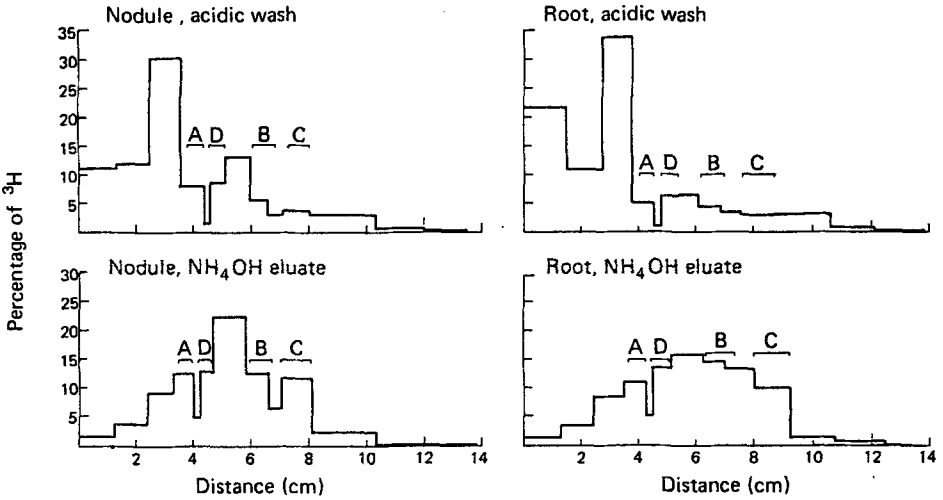

Fig. 1. One-dimensional TLC profiles (Merck silica-gel, with butan-1-ol/acetic acid/water (12:3:5, v/v/v) is solvent) of extracts of root and nodule tissue from pea plants labeled for 8 d with [^{3}H][9R]Z via the root tip. Plants were labeled 16 d after inoculation with strain ANU203. Extracts were applied to cellulose phosphate columns, and the acidic washes and NH_4OH eluates were analyzed by TLC. The positions of dyes A, B, C, and D are indicated.

root samples and 39% and 61% for the wash and eluate, respectively, for nodule samples. Similarly, when roots were labeled above the root tip, the corresponding percentages were 35% and 65% for root samples and 36% and 64% for nodule samples. For the shorter labeling periods (2 or 5 d), the proportion of ^{3}H in the cellulose phosphate NH_4OH eluate averaged 53% for the root samples and 86% for the nodule samples. Typical examples, for both root and nodule tissue, of one-dimensional TLC profiles of the acidic wash and NH_4OH eluate from cellulose phosphate columns are presented in Fig. 1. The profiles emphasize the complexity of the metabolism; [9R]Z, if present at all, would be centered between dyes B and C. As observed previously for samples from plants in which the nodules or leaves were the site of [^{3}H][9R]Z application (Badenoch-Jones et al. 1983a), the acidic wash could be readily distinguished from the NH_4OH eluate by the percentage of ^{3}H that chromatographed between the origin and dye A. In chromatograms of the former fractions, a prominent peak of ^{3}H occurred in the zone just below dye A, which would contain the nucleotides of Z and Ade. Also, as observed previously, very little ^{3}H in any sample chromatographed above the location of dye C (the position of N^6-(Δ^2-isopentenyl)adenine and 9-β-D-ribofuranosyl-N^6-(Δ^2-isopentenyl)adenine). In most samples of NH_4OH eluate, there were no particularly prominent peaks of radioactivity, but a high proportion of the ^{3}H chromatographed with and between dyes B and D, the known location of the O-glucosides, adenine, and adenosine. TLC profiles for nodule and root samples from the same experiment were usually quite similar.

Two-dimensional TLC

Data from two-dimensional TLC analyses are given in Table 1. Using this TLC system, Z and [9R]Z were not separated from their corresponding dihydro-derivatives. The radioactivity associated with the Z and [9R]Z markers is thus designated as Z/(diH)Z and [9R]Z/(diH)[9R]Z, respectively. It should also be borne in mind that a result from such analysis for the percentage of sample ^{3}H co-chromatographing with an authentic standard represents only the maximum value for the percentage of sample ^{3}H actually due to that compound. The results in parentheses in Table 1 represent the original data corrected by the proportion of radioactivity of the putative ^{3}H-labeled metabolite that co-chromatographed with authentic standard in a third TLC system.

Discrete peaks of radioactivity were detected on the two-dimensional chromatograms at the positions of most of the marker compounds, with the exception of [9G]Z, and for some samples, [9Ala]Z and the O-glucosides. The following points evident from Table 1 merit comment:

(1) In all experiments very little radioactivity remained as the cytokinin supplied to the plant ([9R]Z) in root tissue, and generally even less in nodule tissue. The percentage of total ^{3}H remaining as [9R]Z/(diH)[9R]Z appeared to be less after the longer labeling period (8 d) than after the shorter labeling periods (2 or 5 d). When sufficient ^{3}H was available for rechromatography of the putative [^{3}H][9R]Z/(diH)[9R]Z and [^{3}H]Z/(diH)Z by TLC on Camag silica-gel, to achieve separation of the dihydro-derivative from the corresponding unsaturated compound, radioactivity co-chromatographing with [9R]Z and (diH)[9R]Z, or with Z and (diH)Z, was detected as discrete peaks and accounted for a large proportion (60–90%) of total ^{3}H. Furthermore, for most samples more ^{3}H co-chromatographed with the dihydro-derivative than with the unsaturated compound (see results in parentheses, Table 1).

(2) A relatively large proportion of the total radioactivity was not detected in known cytokinins and cytokinin metabolites, in the order of 70% after the 8-d labeling period, and slightly less, approximately 60%, after the shorter labeling periods.

(3) [9G]Z was apparently not a metabolite formed after the application of [^{3}H][9R]Z, and [9Ala]Z was a very minor metabolite, if it formed at all. In most instances, Ados and Ade were the major metabolites formed. While the O-glucosides generally appeared to be fairly minor metabolites of [^{3}H][9R]Z, reasonable quantities of [^{3}H]O-glucosides, especially (OG)[9R]Z, seemed to be present in some samples, most notably in nodules formed by strain ANU897. When the O-glucosides from these samples were eluted from preparative TLC plates, hydrolyzed with β-glucosidase, butanol-extracted, and rechromatographed, 63% to 100% (mean 89%) of dpm co-chromatographed with the appropriate aglycone.

(4) In most experiments, root tissue contained a higher proportion of radioactivity as zeatin and/or dihydrozeatin, and as adenine, than did nodule tissue.

To identify the nucleotide metabolites formed, the acidic wash from the cellulose phosphate column was treated with alkaline phosphatase, extracted with butanol, and then subjected to TLC. For all samples, appreciable proportions of ^{3}H in the butanol extract co-chromatographed with Ados and [9R]Z,

Table 1. The contribution of various cytokinins and cytokinin metabolites to radioactivity extracted from root and nodule tissue after application of [^{3}H][9R]Z via the root tip. Ammonia eluates from cellulose phosphate columns were analyzed by two-dimensional TLC. The radioactivity co-chromatographing with each marker compound is expressed as a percentage of total dpm eluted from the plate.

Strain of *Rhizobium*	Time of labeling (dai)[b]	Labeling period (days)	Tissue	Position of vial containing [^{3}H][9R]Z	Marker compound						
					[9A1a]Z	O-Glucosides[d]	[9G]Z	Ados[f]	Ade[f]	[9R]Z/(diH)[9R]Z[g]	Z/(diH)Z[g]
			Root	A[c]	2.0	10.4 (1.5,2.1,1.4,5.4)	1.1	4.0	3.5	3.7	3.3
				B	1.3	6.7 (1.4,1.4,2.0,1.9)	0.9	10.4	4.9	4.1	3.3
ANU897[a]	16	8	Nodule	A	2.1	17.0 (10.3,1.4,1.0,4.3)	0.7	5.2	3.2	2.5	1.5
				B	0.5	32.2 (26.5,1.6,3.0,1.1)	0.6	4.3	4.0	1.5	3.4
			Root[h]		1.9	15.0 (1.9,2.0,8.5,2.6)	1.0	5.5	3.4	3.8	1.9
ANU203[a]	16	8	Root	A	1.6	8.6 (2.1,1.7,1.6,3.2)	0.9	6.0 (4.4)	4.2	3.4	2.1
				B	1.8	12.6 (2.2,2.5,2.4,5.5)	0.5	4.0	2.6	2.1	2.2
			Nodule	A	1.6	5.5 (1.5,1.6,1.4,1.0)	0.6	4.1	1.9	3.0	1.3 (0.3,0.6)
				B	2.1	6.9 (0.8,1.0,1.1,4.0)	0.4	3.5	1.6	3.1	1.0
ANU203	22	8	Root	A	1.2	6.0 (1.7,1.6,1.0,1.7)	0.7	10.1 (7.2)	7.5 (6.3)	4.5	5.6
			Nodule		2.2	6.9 (2.2,1.2,1.0,2.5)	0.4	6.6 (3.9)	2.6	3.3	1.1
ANU897	16	2	Root	A	2.3[e]	3.1[e]	0.6	9.3 (7.8)	8.1 (7.8)	8.4 (2.1,3.2)	7.3 (1.6,3.7)
			Nodule		2.5	3.1	1.6	9.4 (6.4)	3.6 (3.1)	8.0 (3.4,2.4)	2.4
ANU203	22	2	Root	A	2.4[e]	4.2[e]	1.1	9.1 (7.8)	8.6 (8.3)	12.2 (3.1,4.2)	4.9 (1.7,2.7)
			Nodule		3.2	2.7	0	11.9 (10.7)	5.3 (5.3)	4.5 (1.1,2.1)	1.6
ANU203	22	5	Root	A	3.6[e]	4.8[e]	1.1	11.0 (10.5)	12.1 (11.7)	7.9 (1.7,2.9)	5.6 (1.6,2.8)
			Nodule		5.6	13.3	1.8	15.9 (14.3)	4.1 (3.6)	4.3 (1.1,1.5)	0.9

[a] Data are means of two experiments carried out on separate occasions.

[b] dai = days after inoculation.

[c] A = at root tip; B = within 10 mm of root nodules nearest the root tip.

[d] The values in parentheses denote the individual O-glucosides in the following order: (OG) [9R]Z; (diH OG) [9R]Z; (OG)Z; (diH OG)Z.

[e] Dyes, rather than authentic standards, were used as markers (see Badenoch-Jones et al. 1983a).

[f] 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 B).

[g] 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, following TLC on Camag silica-gel.

[h] Root without accompanying nodules.

Table 2. The contribution of various cytokinin nucleotides to radioactivity extracted from root and nodule tissue after application of [^{3}H][9R]Z via the root tip. Acidic washes from cellulose phosphate columns were hydrolyzed with alkaline phosphatase, butanol-extracted, and analyzed by TLC (Merck silica-gel, solvent B). The radioactivity co-chromatographing with Ados and [9R]Z is expressed as a percentage of total dpm in the acidic wash.

Strain of *Rhizobium*	Time of labeling (dai)[b]	Duration of labeling period (days)	Tissue	Position of vial containing [^{3}H][9R]Z[c]	Ados	[9R]Z
ANU897[a]	16	8	Root	A	16.7	5.2
				B	11.4	5.1
			Nodule	A	7.1	4.8
				B	8.0	4.0
ANU203[a]	16	8	Root	A	15.8	10.4
			Nodule		12.9	7.1
ANU203	22	8	Root	A	24.5	9.1
			Nodule		8.8	4.3
ANU897	16	2	Root	A	21.4	24.6
			Nodule		16.1	11.4
ANU203	22	2	Root	A	11.1	28.7
			Nodule		16.8	11.5
ANU203	22	5	Root	A	10.5	12.0
			Nodule		2.3	3.2

[a] Data are means of two experiments carried out on separate occasions.
[b] dai = days after inoculation.
[c] A = at root tip; B = within 10 mm of root nodules nearest the root tip.

averaging 23% and 17%, respectively, and the contribution of Ade and Z nucleotides to the extracted radioactivity in the acidic wash was calculated (Table 2). Nucleotides of Z, and particularly of Ade, were metabolites of [9R]Z in both root and nodule tissue, but were more prominent in the former.

Discussion

Results from the present study indicate that radioactivity applied via the root tip as [9R]Z moves from the root tip to the root nodules. A complex of metabolites was detected in the nodules, including metabolites with an intact Z moiety. Previous studies (Badenoch-Jones et al. 1983a) in which the nodules were labeled directly with [9R]Z, established that nodules themselves have the capacity to metabolize [9R]Z to the complex of metabolites detected in nodules in the present study. Hence, in the current experiments, these metabolites may have been formed from [9R]Z received by the nodules; however, the possibility that some or all of the metabolites were formed in root tissue and then transported to the nodules cannot be eliminated. Although data from the present

study indicate that root nodules receive cytokinin from the root tip, the possibility that nodules simultaneously synthesize cytokinins is not precluded.

Several trends observed in the present experiments were similar to those observed previously when nodule or leaf tissue was labeled directly (Badenoch-Jones et al. 1983a). First, no more than a few percent of ^3H in nodule tissue remained as [9R]Z, and although 8 d was the standard labeling period, the extensive metabolism of [9R]Z was observed when harvests were made only 2 or 5 d after labeling. Second, a relatively large proportion of ^3H in the cellulose phosphate NH_4OH eluates of root and nodule tissue was not detected in known cytokinin metabolites. Third, a quite considerable proportion of ^3H in the alkaline phosphatase-treated acidic wash from cellulose phosphate columns co-chromatographed with Ados and [9R]Z, indicating the occurrence of nucleotides of Ade and Z as metabolites of [9R]Z in the tissues examined. Nucleotides of Ade generally represented a greater proportion of ^3H, in both root and nodule tissue, than the nucleotides of Z, while the nucleotides of both Ade and Z generally represented a greater proportion of ^3H in root extracts than in nodule extracts. Finally, a qualitatively similar spectrum of metabolites in both root and nodule tissue was detected in the two series of experiments, including products of side-chain cleavage, of reduction and glucosylation, and of deribosylation. However, there were some quantitative differences in the metabolites formed in the two series of experiments. [9Ala]Z, for example, if present at all, was only a minor metabolite in nodules supplied with [9R]Z via the root tip, but was a more major metabolite when nodules were labeled directly. A greater percentage of ^3H was present as O-glucosides in nodule tissue when [^3H][9R]Z was received from the root tip as opposed to most experiments involving direct application; this may reflect the more physiological conditions in the former experiments.

As observed previously (Badenoch-Jones et al. 1983a), there were few major differences between root and nodule tissue in their metabolism of [^3H][9R]Z, and differences between effective and ineffective nodules were minor, with the possible exception of the O-glucosides, and in particular (OG)[9R]Z, which appeared to be a more prominent metabolite in effective nodules than in ineffective nodules. It is noteworthy that it was this O-glucoside that was a major metabolite when [^3H][9R]Z of high specific activity was applied in physiological quantities directly to root nodules formed by strain ANU897 (Badenoch-Jones et al. 1983a).

In summary, data from the present experiments, which were an attempt to maintain conditions as close to the physiological state as possible, largely confirm the data from previous experiments in which nodule and leaf tissue were labeled directly, although cytokinin metabolism is clearly influenced to some extent by experimental conditions. It can again be concluded that for nodules formed by strain ANU203, the defect in nitrogen fixation is not associated with a major alteration in cytokinin metabolism.

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