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Phytohormones, *Rhizobium* Mutants, and Nodulation in Legumes. VII. Identification and Quantification of Cytokinins in Effective and Ineffective Pea Root Nodules Using Radioimmunoassay

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Abstract. Radioimmunoassays (RIA), employing antisera raised in rabbits against bovine serum albumin conjugates of zeatin riboside, dihydrozeatin riboside, and isopentenyladenosine, were used to estimate levels of these cytokinins and their corresponding bases in samples of effective (nitrogen-fixing, Fix^+), ineffective (nonnitrogen-fixing, Fix^-) pea root nodules and uninoculated roots. Assays were done on extracts of nodule tissue, 1–2 g fresh weight, or approximately 10 g fresh weight of root tissue, and high specific activity [^{3}H]zeatin riboside was added during preparation of the extract for use as a recovery marker. Two different purification procedures were employed, each involving several purification steps. High performance liquid chromatography (HPLC) was the final step in both procedures. Fractions from HPLC were analyzed by RIA using the appropriate antiserum. The cytokinins, zeatin, zeatin riboside, dihydrozeatin riboside, isopentenyl adenine, and isopentenyladenosine were detected and quantified in nodule tissue, and similarly, in root tissue (with the exception of zeatin, which we were unable to quantify in root tissue). Cytokinin levels in nodule tissue were higher than those in root tissue. The major cytokinins detected in nodule tissue were zeatin, followed by zeatin riboside and then dihydrozeatin riboside. The levels of zeatin and zeatin riboside estimated in nodules in the present study by RIA were of the same order of magnitude, though tending to be a little higher, than values obtained previously by bioassay. Dihydrozeatin riboside was identified with confidence for the first time in nodule tissue. There was a general decline with age in cytokinin levels in nodules, but no major qualitative change in nodule cytokinins with age. For the *Rhizobium* strains examined, the data did not

indicate a clear correlation between nodule cytokinin levels and the effectiveness of nodules in nitrogen fixation.

For some years, our research group has been interested in the role played by phytohormones (in particular, auxins and cytokinins) in the initiation, development, and maintenance of root nodules (Badenoch-Jones et al. 1982a,b, 1983, 1984a–c). We have a number of mutant strains of *Rhizobium leguminosarum* and *R. trifolii* that are potentially useful for elucidating certain aspects of the legume–*Rhizobium* symbiosis. We have already investigated the metabolism of cytokinins in pea root nodules inoculated with strains of *R. leguminosarum* that form either effective or ineffective root nodules (Badenoch-Jones et al. 1984a,c). No major differences in cytokinin metabolism between the two nodule types were detected, but this did not preclude differences in cytokinin contents. Hence the present study was made to examine cytokinin levels in these two nodule types.

A role for cytokinins in nodule initiation and growth has been implicated, since root nodules are plant regions where accelerated cell division occurs and the major physiological effect of cytokinins is to induce cell division (see Letham 1978). However, progress toward substantiating this has been limited, largely because of analytical difficulties in measuring the low levels of cytokinins in plant tissues, including root nodules. Previous investigations of cytokinin levels in root nodules have employed bioassays (Puppo et al. 1974, Henson and Wheeler 1976, 1977, Syono and Torrey 1976, Syono et al. 1976, Rodriguez-Barrueco et al. 1979, Jaiswal et al. 1981, Wang et al. 1982) or gas chromatography-mass spectrometry (GC-MS) (Wang et al. 1982). However, bioassays are very time-consuming and lack precision (see Letham 1978). Analysis by GC-MS, using deuterium-labeled cytokinins as internal standards (see Brenner 1981), allows unequivocal identification and accurate quantification of cytokinins, but it requires extensive sample purification before analysis, and it lacks the sensitivity required to deal with small samples of tissue. Wang et al. (1982) were not successful in detecting cytokinins in a 0.67-g (dry weight) sample of nodules or a 2-g sample of roots of *Pisum sativum* by GC-MS. It would appear necessary to use much larger amounts of tissue (in the order of 25 g fresh weight) in order to detect cytokinins by this method, but collection of nodules from legumes such as *P. sativum* and *Trifolium repens* is excessively time-consuming. Yet it is nodules from these smaller legumes that are frequently of special interest because of the availability of mutant strains of *R. leguminosarum* and *R. trifolii*. The present study was therefore carried out to assess the usefulness of an alternative method, RIA, for measuring cytokinin levels in root nodules.

Results of studies employing bioassay have suggested the possible presence of the following cytokinins in root nodules: N^6-(Δ^2-isopentenyl)adenine(iP) and [9-β-D-ribofuranosyl-N^6-(Δ^2-isopentenyl)adenine([9R]iP) (Puppo et al. 1974, Syono and Torrey 1976, Jaiswal et al. 1981) and zeatin(6-[4-hydroxy-3-methylbut-*trans*-2-enylamino]purine)(Z) and zeatin riboside (9-β-D-ribofuranosyl zeatin)([9R]Z) (Henson and Wheeler 1976, 1977, Syono and Torrey 1976, Jaiswal et al. 1981). In the present study, emphasis was therefore placed on examining root nodules for these cytokinins. In many of the bioassay studies,

it was possible that dihydrozeatin(6-[4-hydroxy-3-methylbutylamino]purine) ((diH)Z)] and dihydrozeatin riboside ((diH)[9R]Z), respectively, contributed to activity in the fractions designated as Z or [9R]Z. We were therefore also interested in investigating the possibility of the presence of (diH)Z and (diH)[9R]Z in root nodules. It was possible to analyze all the above-mentioned cytokinins by using RIAs that employed, separately, the three following antisera: anti-[9R]Z-serum, anti-(diH)[9R]Z-serum, and anti-[9R]iP-serum.

We have also examined the cytokinin levels in root tissue, as it has been suggested that nodules contain higher levels of cytokinin than root tissue (Puppo et al. 1974, Henson and Wheeler 1976, 1977), although there is some controversy over this point (Wang et al. 1982).

Before applying RIA to the current problem, we made a study to assess the validity of the method for measuring cytokinins in biological samples. From that study, which employed an anti-[9R]Z-serum (Badenoch-Jones et al. 1984d), we concluded that RIA, although not without limitations, appeared to be a sensitive and powerful technique when used in conjunction with suitable chromatographic procedures for the separation of cross-reactive compounds. In the present study, two different methods were used for the separation of cytokinins present in root nodules, prior to RIA. One of the methods was a modification of that developed in our previous study (Badenoch-Jones et al. 1984d). It was used for examining Z and [9R]Z only, and employed an HPLC step with a Zorbax C8 column. During the current work, we developed another method which enabled the examination of all the following cytokinins in RIAs employing the appropriate antiserum: Z, [9R]Z, (diH)Z, (diH)[9R]Z, iP, and [9R]iP. With this method it was possible to purify a sample as a single entity, with the exception of the final separative step. The present study is one of few studies (MacDonald et al. 1981, Weiler and Spanier 1981, Morris et al. 1982, Akiyoshi et al. 1983) that have applied RIA to the analysis of a practical problem of plant physiology, and the only study that has examined by RIA such a wide range of cytokinins in a tissue.

Materials and Methods

Materials

Cytokinin standards were obtained as follows: Z and [9R]Z were purchased from Calbiochem-Behring Corp. and (diH)Z, iP, and [9R]iP from Sigma Chemical Co. (diH)[9R]Z was prepared by hydrogenation of [9R]Z in methanol solution using platinum oxide at room temperature and under 1 atm of hydrogen. [^{3}H][9R]Z of the highest specific activity available (140.6 GBq mmol^{-1}) was a generous gift from C. H. Hocart. Silica gel 60 PF_{254} was purchased from E. Merck, Darmstadt, FRG. The following dyes were used as markers for thin-layer chromatography (TLC): Nile blue (George T. Gurr, Division of Baird and Tatlock, Romford, England), p-rosaniline (Sigma Chemical Co.), and eosin (BDH Chemicals Ltd., Poole, England).

Bacterial Strains and Plant Culture

The *R. leguminosarum* strains used were an effective strain, ANU897 (Nod^+ Fix^+) (Johnston and Beringer 1976), and an ineffective strain (as indicated by the stunted growth, yellow leaves, and lack of acetylene production in plants inoculated with this strain), ANU203 (Nod^+ Fix^-). Strain ANU203 was constructed (Badenoch-Jones et al. 1983) by transferring the transmissible pea nodulation plasmid (pJB5JI) into strain 202. Strain 202 is a derivative of the naturally occuring *R. trifolii* coryn strain (Chen and Thornton 1940), for which the symbiosis does not continue long enough to achieve much nitrogen fixation (Vincent 1980). This mutant is classed as a nodule persistence (Nop) mutant under the scheme of Vincent (1980).

Strain ANU203 seemed a likely candidate for producing nodules that exhibit an alteration in phytohormone levels or metabolism. Details of the method for growing pea plants (*P. sativum* L. cv Greenfeast) in 250-ml flasks using aseptic techniques have been reported previously (Djordjevic et al. 1982). Quadruplicate samples of approximately 1–2 g fresh weight were collected for each of the following five nodule treatments: nodules harvested at 15 and 30 days after inoculation of plants with strain ANU897, and at 15, 21, and 36 days after inoculation with strain ANU203. There is a 6-day delay in nodulation by strain ANU203 compared with strain ANU897. Roots (triplicate samples of approximately 10 g fresh weight) were collected from uninoculated plants at a time corresponding to the nodule collection made 15 days after inoculation. Nodules and roots were stored in a liquid nitrogen freezer (−198°C) prior to extraction.

Tissue Extraction and Purification

Frozen nodules and roots were weighed and dropped into methanol-water-formic acid (70:25:5, v/v/v) at −20°C (10 ml g^{-1} fresh weight tissue), before thawing. [^{3}H][9R]Z (336 Bq) was added as a recovery marker. After 24 h at −20°C, the sample was homogenized in a small blender. After a further 24 h at −20°C, the homogenate was filtered, resuspended in an equal volume of solvent, and refiltered. The combined filtrates were adjusted to pH 8.2 and partitioned twice with an equal volume of water-saturated n-butanol. The combined butanol extracts were chromatographed by HPLC, using a Bondapak C_{18}/Porasil B column (7.8 × 610 mm; Waters Associates, Milford, MA, USA), under the conditions described by Badenoch-Jones et al. (1984d). Two alternative procedures, A and B (Fig. 1), were used for further purification of the column eluate.

Procedure A. Following HPLC on the Bondapak C_{18}/Porasil B column, one nodule sample from each treatment was chromatographed on a Zorbax C8 column (9.4 × 250 mm; DuPont Company, Wilmington, DE, USA) prior to analysis of Z and [9R]Z. The solvents and flow rate for the Zorbax C8 column were as described by Badenoch-Jones et al. (1984d). The Zorbax C8 column enables separation of several cytokinins that cross-react with anti-[9R]Z-serum—namely, zeatin-9-glucoside ([9G]Z); lupinic acid (L-β-[6-(4-hydroxy-3-

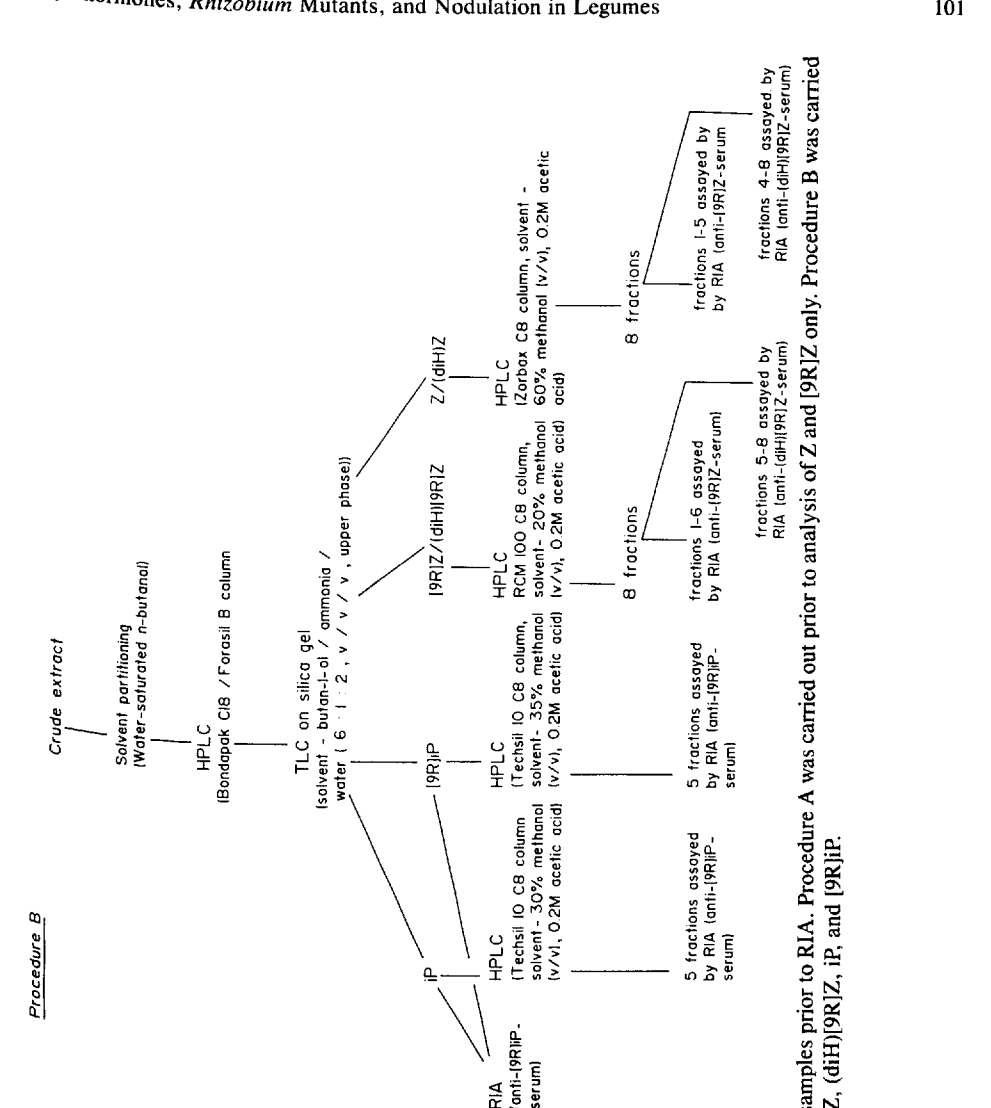

Fig. 1. Procedures used for purification of samples prior to RIA. Procedure A was carried out prior to analysis of Z and [9R]Z only. Procedure B was carried out prior to the analysis of Z, [9R]Z, (diH)Z, (diH)[9R]Z, iP, and [9R]iP.

methylbut-*trans*-2-enylamino)-purin-9-yl]-alanine) ([9A1a]Z); [9R]Z; (diH)-[9R]Z; Z; and (diH)Z (Badenoch-Jones et al. 1984d). Following the injection of each nodule sample onto the Zorbax C8 column, 13–18 HPLC fractions were collected. The aim was to collect putative Z and [9R]Z as discrete fractions at the elution times of the authentic standards. Fractions were assayed by RIA employing the anti-[9R]Z-serum.

Procedure B. Three nodule samples from each treatment and three root samples were purified by procedure B prior to analysis for the following cytokinins: Z, [9R]Z, (diH)Z, (diH)[9R]Z, iP, and [9R]iP. Following HPLC on the Bondapak C_{18}/Porasil B column, extracts were chromatographed by TLC using 0.5-mm thick layers of silica gel 60 PF_{254} and butan-1-ol/14N ammonia/water (6:1:2, v/v/v, upper phase) as solvent. After TLC, cytokinins were located with reference to authentic standards, which were parallel-chromatographed and visualized under UV light (254 nm), and to the following dyes which were added to the sample prior to chromatography: Nile blue, p-rosaniline, and eosin. The Rf values for standards and dyes were as follows: Z and (diH)Z (0.53), [9R]Z and (diH)[9R]Z (0.33), iP (0.62), [9R]iP (0.40), Nile blue (0.67), p-rosaniline (0.48), and eosin (0.26). Chromatogram zones were packed into columns and eluted exhaustively with ethanol/water/acetic acid (25:24:1, v/v/v). All samples were further purified by HPLC prior to RIA, with the exception of iP and [9R]iP samples derived from one nodule sample of each treatment.

The following columns were used for HPLC: a Techsil 10 C8 column (8.0 × 250 mm; HPLC Technology Ltd., Cheshire, UK) for the purification of iP and [9R]iP, a Zorbax C8 column for the separation of Z and (diH)Z, and an RCM 100 C8 column (8 × 100 mm; Waters Associates, Milford, MA, USA) for the separation of [9R]Z and (diH)[9R]Z. The solvent used was methanol in 0.2 M acetic acid, at a flow rate of 4.0 ml min^{-1}. The proportion of methanol (v/v) was 20% for the separation of [9R]Z and (diH)[9R]Z, 30% for chromatography of iP, 35% for chromatography of [9R]iP, and 60% for separation of Z and (diH)Z. Following injection of the putative iP or [9R]iP samples onto the Techsil column, five HPLC fractions were collected. Following injection of the putative Z/(diH)Z mixture onto the Zorbax C8 column and the putative [9R]Z/(diH)[9R]Z mixture onto the RCM 100 C8 column, eight HPLC fractions were collected. The aim was to collect the putative cytokinins as discrete fractions at the elution times of the corresponding authentic standards: iP and [9R]iP each in fraction 3, Z in fraction 3, (diH)Z in fraction 6, [9R]Z in fraction 4, and (diH)[9R]Z in fraction 7. All fractions off the Techsil column were assayed by RIA employing the anti-[9R]iP serum. Fractions 1–5 of the Z/(diH)Z separation and fractions 1–6 of the [9R]Z/(diH)[9R]Z separation were assayed by RIA employing the anti-[9R]Z serum. Fractions 4–8 of the Z/(diH)Z separation and fractions 5–8 of the [9R]Z/(diH)[9R]Z separation were assayed by RIA employing the anti-(diH)[9R]Z-serum.

Radioimmunoassay

The antisera have been characterized previously: anti-[9R]Z-serum

(Badenoch-Jones et al. 1984d), and anti-[9R]iP-serum and anti-(diH)[9R]Z-serum (Badenoch-Jones et al. J Plant Growth Regul, in press). The RIA was based on that developed by Weiler (1980), with minor modifications (Badenoch-Jones et al. 1984d). Eluates from TLC zones or fractions from HPLC were evaporated to dryness and dissolved in a known volume (normally 325 or 350 μl) of the buffer used in RIA (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4). Triplicate 50-μl aliquots of all zones or fractions were assayed. Further aliquots (10 and 5 μl) of the fractions expected to contain cytokinins were also assayed, each in triplicate. An aliquot (5%) of each fraction (of the [9R]Z series) was taken in duplicate for the measurement of radioactivity in order to estimate recoveries. A computer program based on that of Brooker et al. (1979) was used for assay evaluation and computation of results.

The [9R]Z content per unit fresh weight of nodule tissue was estimated from the activity in RIA of the fraction containing radioactivity ([^{3}H][9R]Z) or, if there was a spillover of [^{3}H][9R]Z into a second fraction, from the fraction containing the most radioactivity. The appropriate correction was made for the recovery of [^{3}H][9R]Z. Levels of other cytokinins were calculated from the activity (in the appropriate RIA) of fraction(s) collected at the retention times of authentic standards and on the assumption that recovery for these cytokinins was the same as the total recovery of [^{3}H][9R]Z for that sample. For Z and iP, the previously determined values for the molar cross-reactivity of these cytokinins with the appropriate antibody—42% (Badenoch-Jones et al. 1984d) and 19.4% (Badenoch-Jones et al. J Plant Growth Regul, in press), respectively—were used. A correction factor of 2.02 was used to calculate the concentration of the naturally occurring isomer of (diH)[9R]Z when (±)(diH)[9R]Z was used as the standard.

Results

Purification Procedure A

In most samples, the fractions at the elution times of authentic [9R]Z and Z exhibited high activity. Activity in the [9R]Z and Z fractions was lowest in the nodules formed by strain ANU203 and harvested 36 days after inoculation. For two of the five nodule treatments examined, the results of RIA on all of the fractions collected off the Zorbax C8 column are shown in Fig. 2. When activity was detected in fractions that were assayed at more than one dilution, the sample dilution curves were parallel to the standard curve (for an example, see Fig. 3), suggesting that the samples were not subject to interference from substances that altered the reaction of the compounds being assayed with the antiserum. In Table 1 the estimates of the [9R]Z and Z content of nodules are shown. In all samples (i.e., for both nodule types and at each age) the Z content was greater than the [9R]Z content. In some samples, RIA activity was detected in the fraction at the elution time of authentic [9G]Z, a cytokinin that cross-reacts with the antiserum (Badenoch-Jones et al. 1984d) and also partitions to some extent into n-butanol (Horgan 1978). In contrast, two other cyto-

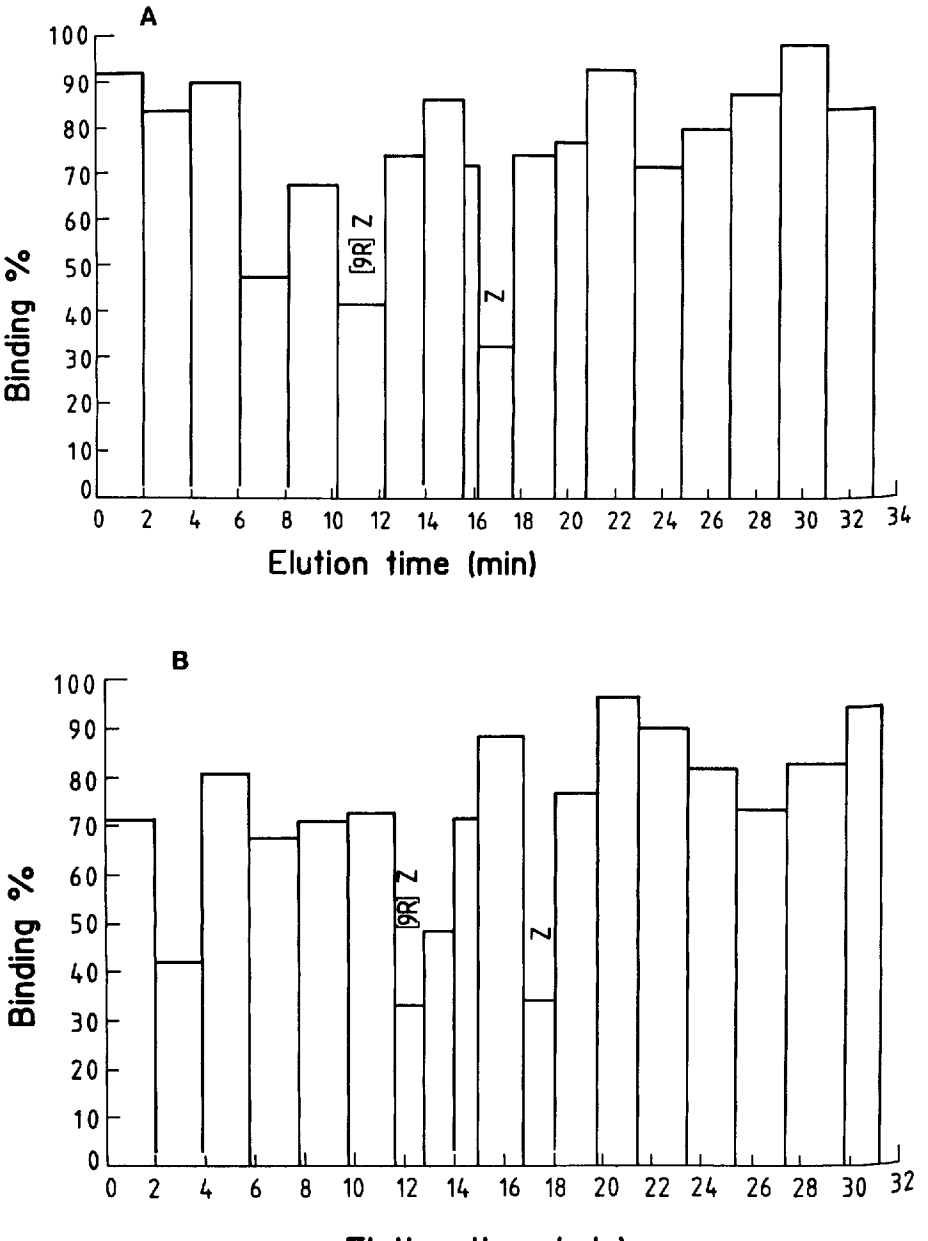

Fig. 2. RIA activity (expressed as percent binding of tracer to antibody relative to binding of tracer to antibody in the absence of [9R]Z) for 50-μl aliquots of the final volume (300 μl) of fractions eluted from the Zorbax C8 column. The fractions collected at the elution times of authentic Z and [9R]Z standards are indicated. The volume of most fractions was approximately 4.8 ml. Fractions were evaporated to dryness and dissolved in 300 μl of the buffer used in RIA. (A) Nodule sample, strain ANU897, 30 days after inoculation; corresponds to 447 mg tissue. (B) Nodule sample, strain ANU203, 15 days after inoculation; corresponds to 78 mg tissue.

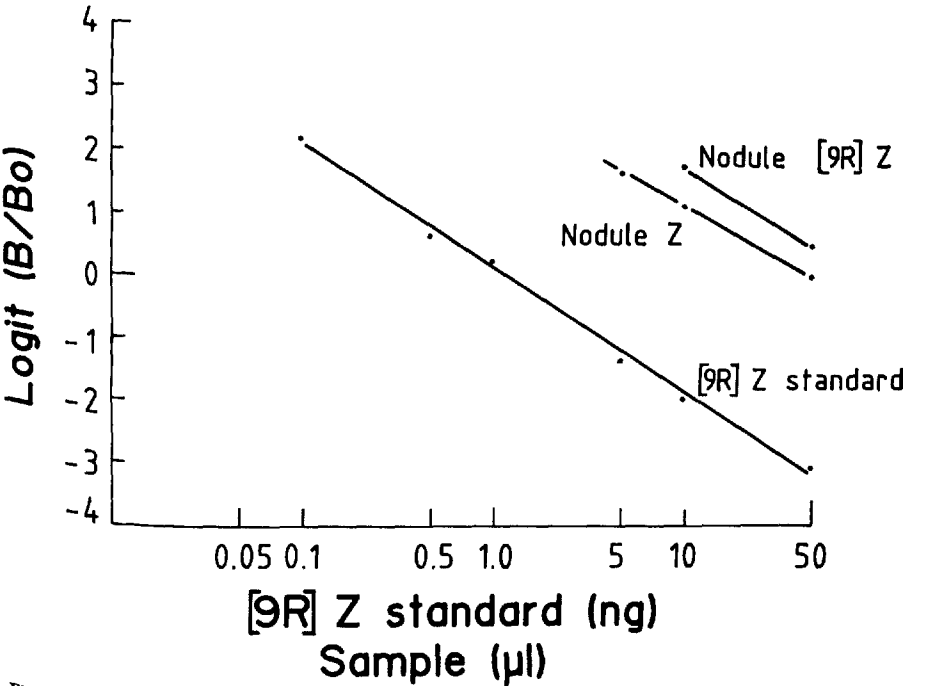

Fig. 3. Logit transformation of standard curve for [9R]Z and of dilution curves for fractions from the Zorbax C8 column collected at the elution times of authentic [9R]Z and Z, following injection of a nodule sample (strain ANU897, 15 days after inoculation). B, binding of tracer to antibody in the presence of [9R]Z standard, or sample; Bo, binding of tracer to antibody in the absence of [9R]Z.

kinins that cross-react with the antiserum, [9A1a]Z and Z nucleotide (Badenoch-Jones et al. 1984d), have very low partition coefficients into n-butanol (unpublished data). The low cross-reactivities of (diH)[9R]Z and (diH)Z (1.9% and 1.3%, respectively; Badenoch-Jones et al. 1984d) with the antiserum make it difficult to detect these compounds in samples assayed by RIA employing anti-[9R]Z-serum. It was therefore not unexpected that the fractions at the elution times of these compounds (i.e., 2 fractions after the corresponding unsaturated compound), or of [9A1a]Z or Z nucleotide, contained little activity. However, in some samples, RIA activity was detected in fractions in which no known cytokinins elute.

Purification Procedure B

The estimates of cytokinins made following purification of samples by procedure B are also shown in Table 1. Recovery of [^{3}H][9R]Z averaged 37.7% for all samples. Considerable [9R]Z, (diH)[9R]Z, iP, and [9R]iP were detected in nodule samples, but (diH)Z was not detected in any sample. We attempted to collect each cytokinin in a discrete fraction. For [9R]iP and iP, this appeared to

be achieved, as indicated by the detection of activity in RIA in only the single expected fraction. However, for [9R]Z, Z, and (diH)[9R]Z, this was not always achieved, and these cytokinins frequently eluted considerably earlier than authentic standards. This was particularly a problem with root samples, which contained a greater fresh weight of tissue than nodule samples.

iP and [9R]iP were separated following the TLC step. For one sample from each treatment, iP and [9R]iP were assayed immediately after the TLC step (i.e., without subjecting the sample to purification by HPLC). Only the zones containing putative iP and [9R]iP were assayed. Assuming the same recovery as that of [^{3}H][9R]Z after TLC (approximately 50%), estimates of iP and [9R]iP from samples analyzed after the TLC step were similar to estimates from samples purified by HPLC (Table 1).

The data for iP and [9R]iP were less variable than the data for [9R]Z, and considerably less variable than the data for Z and (diH)[9R]Z. This probably reflects the better quality of the HPLC procedures for iP and [9R]iP than for Z, [9R]Z, and (diH)[9R]Z. The greater accuracy of the [9R]Z estimates than the Z and (diH)[9R]Z estimates probably reflects the availability of a recovery marker for [9R]Z. Estimates of Z and [9R]Z were somewhat lower following procedure A than procedure B, particularly for nodules formed by ANU203 at 21 and 36 days after inoculation.

In the nodules formed by both strains, there was, for most cytokinins, a trend toward a decline in cytokinin level with increasing time after inoculation. However, there was no fundamental qualitative change in cytokinins with age. The major cytokinin detected in nodule tissue was Z, followed by [9R]Z and then (diH)[9R]Z. For most cytokinins, nodules contained considerably higher levels than uninoculated roots. In the case of (diH)[9R]Z, the estimate obtained for roots was not particularly low, relative to the estimates for nodules, but had a high standard error. Cytokinin contents of the ineffective nodules were found to be of the same order of magnitude as those of the effective nodules.

Discussion

In the current study we have used RIA to examine, both qualitatively and quantitatively, the cytokinins present in a number of pea root and nodule samples. We have obtained evidence for the occurrence of the following cytokinins in pea root nodules: Z, [9R]Z, (diH)[9R]Z, iP, and [9R]iP. Although there is considerable potential for improvements in the purification procedures that are combined with RIA, the sensitivity of RIA and its greater precision than bioassay probably mean that this is the most accurate and, in terms of the range of cytokinins investigated, the most extensive study of cytokinin levels in pea root nodules to have been carried out.

The identification of the cytokinins in the present study is almost certainly less equivocal than identifications made in previous studies employing bioassays. We have previously emphasized the importance of combining appropriate separative procedures with RIA (Badenoch-Jones et al. 1984d). To estimate Z, [9R]Z, (diH)Z, (diH)[9R]Z, iP, and [9R]iP, concurrently, in root

Table 1. *Cytokinin levels in pea root nodules estimated by radioimmunoassay.*

Strain of *Rhizobium*	Age of nodules (days after inoculation)	Cytokinins (ng g^{-1} fresh weight tissue)[b]							
		[9R]Z	[9R]Z[a]	Z	Z[a]	(diH)[9R]Z	(diH)Z	[9R]iP	iP
ANU897 (Nod$^+$ Fix$^+$)	15	35 ± 20.9	15	45	41	59 ± 29.5	ND	16[c] (range,19) 7[d]	21[c] (range,0) 26[d]
ANU897	30	32 ± 19.2	12[e] (range,4)	16	31[e] (range,9)	18 ± 5.5	ND	4[c] (range,6) 9[d]	8[c] (range,6) 48[d]
ANU203 (Nod$^+$ Fix$^-$)	15	99 ± 47.6	82[e] (range,25)	NA	145[e] (range,6)	8 ± 3.0	ND	7[c] (range,2) 3[d]	16[c] (range,5) 20[d]
ANU203	21	38 ± 14.9	3	56[c] (range,68)	193	34 ± 10.7	ND	6[c] (range,2) 10[d]	14[c] (range,4) 8[d]
ANU203	36	22 ± 4.7	1	NA	11	8 ± 3.2	ND	11[c] (range,10) 2[d]	9[c] (range,3) 21[d]
Uninoculated root		1.1 ± 0.2	NA	NA	NA	18 ± 4.5	ND	0.5 ± 0.14	1.2 ± 0.05

Values are mean ± SEM.
NA, not assayed.
ND, not detected.
[a] Purification by procedure A (see Materials and Methods). Mean and range of two assays following separate injections onto the Zorbax C8 column.
[b] Recoveries of cytokinins were estimated as described in Materials and Methods. A molar cross-reactivity value of 42% for Z with the anti-[9R]Z-serum (Badenoch-Jones et al. 1984d) was used to calculate the concentration of Z. A molar cross-reactivity value of 19.4% for iP with the anti-[9R]iP-serum was used to calculate the concentration of iP, and a correction factor of 2.02 was used to calculate the concentration of the naturally occurring isomer of (diH)[9R]Z when (±)(diH)[9R]Z was used as the standard (Badenoch-Jones et al. J Plant Growth Regul, in press).
[c] Mean and range of two values.
[d] Sample assayed by RIA immediately following TLC (i.e., without final HPLC step). A recovery of 50% was assumed.
[e] Mean and range of two values (samples were subjected twice to chromatography on the Zorbax C8 column).

nodule samples, it was necessary to separate these cytokinins by more complex chromatographic procedures (procedure B) than those used when estimating Z and [9R]Z only (procedure A). Results from the current work have revealed the importance of using a recovery marker, both as a means of estimating losses during sample purification and as an indicator of chromatographic performance.

It may be preferable to avoid the use of ammoniacal TLC solvents prior to HPLC in the purification of samples for RIA analysis. This study has revealed that when samples are purified by such procedures, it is difficult to collect the cytokinins Z, [9R]Z, and (diH)[9R]Z as single discrete HPLC fractions, and these cytokinins frequently eluted considerably earlier than authentic standards. These difficulties were not encountered with HPLC of samples containing iP and [9R]iP. This work suggests that it may not be necessary to subject samples containing putative iP and [9R]iP eluted from TLC plates to HPLC prior to RIA, especially if appropriate recovery markers can be synthesized. However, more detailed studies will need to be carried out before recommendations can be made on using TLC as a final step in purification, particularly as changes in the solvent systems are envisaged.

No unequivocal identifications of cytokinins in root nodules have ever been reported, but the following tentative identifications and estimates of approximate levels have been made, based on bioassay, although generally no account was taken of losses during sample purification. For *Vicia faba* nodules, Henson and Wheeler (1976) estimated 55 μg and 11 μg kinetin equivalents (KE) kg^{-1} fresh weight tissue for [9R]Z and Z, respectively and, for nodules of a range of nonlegumes, 8–49 μg KE kg^{-1} fresh weight of tissue for Z/[9R]Z/(diH)Z/(diH)[9R]Z (Henson and Wheeler 1977). For *Phaseolus mungo* nodules, Jaiswal et al. (1981) estimated approximately 21 μg Z and 17 μg [9R]Z kg^{-1} fresh weight tissue, and for *P. sativum* nodules, Syono and Torrey (1976) estimated approximately 4–26 μg Z/[9R]Z kg^{-1} fresh weight tissue, with approximately equal amounts of Z and [9R]Z. In nodules of *Phaseolus vulgaris*, Puppo et al. (1974) did not detect Z or [9R]Z but did detect iP and [9R]iP. Syono and Torrey (1976) and Jaiswal et al. (1981) detected iP and [9R]iP in the nodules they examined, but at levels lower than those of Z and [9R]Z.

The present study provides good evidence to support the results from these previous studies employing bioassay, in which Z, [9R]Z, iP, and [9R]iP were tentatively identified in root nodules. The present study also identified (diH)[9R]Z in pea root nodules. The values of root nodule cytokinin contents as estimated by RIA in the current study were of the same order of magnitude, though tending to be a little higher, than the above values obtained by bioassay. It is of interest to note that in a recent study (Ernst et al. 1983) in which estimates of iP of an anise cell line were made concurrently by GC-MS, RIA, and bioassay, the former two techniques gave results that correlated well, but bioassay gave values reduced by a factor of 4. This reduction was attributed to possible interference by contaminants in bioassay and to losses during sample purification.

For the *Rhizobium* strains examined in this study, there would not appear to be a clear correlation between nodule cytokinin levels and the effectiveness of nodules in nitrogen fixation. Our data support the conclusion reached by sev-

eral workers (Puppo et al. 1974, Henson and Wheeler 1976, 1977) that cytokinin levels in nodule tissue are higher than those in root tissue, a result that implicates cytokinins as playing a role in nodule maintenance and/or initiation. Because of the lower cytokinin levels in root tissue, purification of extracts prior to RIA analysis was more difficult for root than nodule tissue, and results were correspondingly more variable for root than nodule tissue. It should also be borne in mind that our data probably overestimate the cytokinin levels in the root tissue that is adjacent to the nodules. This is because our measurements were made on the whole root system, including the root tips, and it has been shown that cytokinin levels vary in different parts of the root. Short and Torrey (1972), for example, found that the terminal 0- to 1-mm root tip contained 43–44 times more cytokinin on a fresh-weight basis than the next 1- to 5-mm root segment, but they detected no measurable free cytokinin in segments further from the tip.

The general decline with age in cytokinin levels in nodules that we observed is in agreement with the results of Syono and Torrey (1976) and Syono et al. (1976). The lack of any major qualitative change in nodule cytokinins with age was also a consistent feature of these studies. A detailed study of cytokinin levels in nodules, from nodule initiation to nodule senescence, may prove useful for elucidating the role of cytokinins in nodule development. Such a study would be feasible with the availability of RIA. The present study has demonstrated the usefulness of RIA for analysis of cytokinins where only a small amount of tissue is available. The antisera employed in the current work could also be used for analyses other than RIA—for example, in cytokinin localization studies at the light or electron microscope level. Now that it is possible to locate the position on the root where a nodule will form following spot inoculation (Bhuvaneswari et al. 1980), the anticytokinin sera could be used to examine whether there is an increase in cytokinin activity at the site of nodule initiation.

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References

Akiyoshi DE, Morris RO, Hinz R, et al. (1983) Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the tDNA. Proc Natl Acad Sci USA 80:407–411

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

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

Badenoch-Jones J, Rolfe BG, Letham DS (1983) Phytohormones, *Rhizobium* mutants, and nodulation in legumes. III. Auxin metabolism in effective and ineffective pea root nodules. Plant Physiol 43:347–352

Badenoch-Jones J, Rolfe BG, Letham DS (1984a) Phytohormones, *Rhizobium* mutants, and nodu-

lation in legumes. V. Cytokinin metabolism in effective and ineffective pea root nodules. Plant Physiol 74:239–246

Badenoch-Jones J, Summons RE, Rolfe BG, Letham DS (1984b) Phytohormones, *Rhizobium* mutants, and nodulation in legumes. IV. Auxin metabolites in pea root nodules. J Plant Growth Regul 3:23–39

Badenoch-Jones J, Rolfe BG, Letham DS (1984c) Phytohormones, *Rhizobium* mutants, and nodulation in legumes. VI. Metabolism of zeatin riboside applied via the tips of nodulated pea roots. J Plant Growth Regul 3:41–49

Badenoch-Jones J, Letham DS, Parker CW, Rolfe BG (1984d) Quantitation of cytokinins in biological samples using antibodies against zeatin riboside. Plant Physiol 75:1117–1125

Brenner ML (1981) Modern methods for plant growth substance analysis. Annu Rev Plant Physiol 32:511–538

Brooker G, Harper JF, Terasaki WL, Moylan RD (1979) Radioimmunoassay of cyclic AMP and cyclic GMP. In: Brooker G, Greengard PG, Robison GA (eds) Advances in cyclic nucleotide research. Raven Press, New York, pp 1–31

Bhuvaneswari TV, Turgeon G, Bauer WD (1980) Early stages in the infection of soybean (*Glycine max* L. Merr.) by *Rhizobium japonicum*. I. Localization of infectible root cells. Plant Physiol 66:1027–1031

Chen HK, Thornton HG (1940) The structure of "ineffective" nodules and its influence on nitrogen fixation. Proc R Soc Lond (Biol) 129:208–229

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

Ernst D, Schäfer W, Oesterhelt D (1983) Isolation and quantitation of isopentenyladenosine in an anise cell culture by single-ion monitoring, radioimmunoassay and bioassay. Planta 159:216–221

Henson IE, Wheeler CT (1976) Hormones in plants bearing nitrogen-fixing root nodules: The distribution of cytokinins in *Vicia faba* L. New Phytol 76:433–439

Henson IE, Wheeler CT (1977) Hormones in plants bearing nitrogen-fixing root nodules: Cytokinin levels in roots and root nodules of some nonleguminous plants. Z Pflanzenphysiol 84:179–182

Horgan R (1978) Analytical procedures for cytokinins. In: Hillman JR (ed) Isolation of plant growth substances, Society for Experimental Biology Seminar Series 4. Cambridge University Press, Cambridge, U.K., pp 97–114

Jaiswal V, Rizvi SJH, Mukerji D, Mathur SN (1981) Cytokinins in root nodules of *Phaseolus mungo*. Ann Bot 48:301–305

Johnston AWB, Beringer JE (1976) Pea nodules containing more than one *Rhizobium* species. Nature 263:502–504

Letham DS (1978) Cytokinins. In: Letham DS, Goodwin PB, Higgins TJV (eds) Phytohormones and related compounds—a comprehensive treatise, Vol I. Elsevier North-Holland, Amsterdam, pp 205–263

MacDonald EMS, Akiyoshi DE, Morris RO (1981) Combined high performance liquid chromatography—radioimmunoassay for cytokinins. J Chromatogr 214:101–109

Morris RO, Akiyoshi DE, MacDonald EMS, Morris JW, Regier DA, Zaerr JB (1982) Cytokinin metabolism in relation to tumor induction by *Agrobacterium tumefaciens* In: Wareing PF (ed) Plant growth substances. Academic Press, London, pp 175–183

Puppo A, Rigaud J, Barthe P (1974) Sur la présence de cytokinines dans les nodules de *Phaseolus vulgaris* L. CR Acad Sci Paris (Ser D) 279:2029–2032

Rodriguez-Barrueco C, Miguel C, Palni LMS (1979) Cytokinins in root nodules of the nitrogen-fixing non-legume *Myrica gale* L. Z Pflanzenphysiol Bd 95S:275–278

Short KC, Torrey JG (1972) Cytokinins in seedling roots of pea. Plant Physiol 49:155–160

Syono K, Torrey JG (1976) Identification of cytokinins of root nodules of the garden pea, *Pisum sativum* L. Plant Physiol 57:602–606

Syono K, Newcomb W, Torrey JG (1976) Cytokinin production in relation to the development of pea root nodules. Can J Bot 54:2155–2162

Vincent JM (1980) Factors controlling the legume-*Rhizobium* symbiosis. In: Newton WE, Orme-Johnson WH (eds) Nitrogen fixation, Vol 2. University Park Press, Baltimore, pp 103–129
Wang T, Wood EA, Brewin NJ (1982) Growth regulators, *Rhizobium* and nodulation in peas. The cytokinin content of a wild-type and a Ti-plasmid-containing strain of *R. leguminosarum*. Planta 155:350–355
Weiler EW (1980) Radioimmunoassays for trans-zeatin and related cytokinins. Planta 149:155–162
Weiler EW, Spanier K (1981) Phytohormones in the formation of crown gall tumors. Planta 153:326–337