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Identification of Cytokinins of Root Nodules of the Garden Pea, *Pisum sativum* L.[1]

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ABSTRACT

Five cytokinin activities which induced soybean callus proliferation were detected in ethanol extracts of root nodules of the garden pea (*Pisum sativum* L., cv. Little Marvel). The most active factors among them were identified as zeatin and its riboside on the basis of their mobility on thin layer chromatography in three solvent systems. Smaller activities of zeatin ribotide, isopentenyladenine and its riboside were also detected. Cytokinin activity gradually decreased with the cultivation period, but no qualitative change in the active compounds was found.

Tetraploid or higher ploid cells, determined by chromosome counts of mitotic figures, were frequently observed in root nodule tissues of leguminous plants. These polyploid mitoses occasionally showed diplochromosomes (3, 4, 6, 8, 17, 19, 21). Torrey (16, 18) reported that isolated root segments excised from mature root tissues of pea seedlings, when cultured aseptically in synthetic nutrient media provided with the auxin IAA, showed diploid mitoses. The same tissue segments showed numerous polyploid mitoses in addition to diploid mitoses when both IAA and kinetin were added to the culture medium. These mitoses occasionally showed diplochromosomes. It was suggested that rhizobial infection might be analogous to the cytokinin-auxin stimulation, causing endomitotic cortical cells already present in the root to undergo mitosis upon the initiation of nodules (16).

In addition to these cytological observations, it was reported that cytokinins were released into the medium by cultures of both *Rhizobium japonicum* and *R. leguminosarum* (5, 10, 11).

It is generally believed that both auxin and cytokinin are required for cell division in plants (1). It is not unreasonable to suggest that both cytokinin and auxin have an important role in root nodule development. High levels of auxin in root nodules have already been demonstrated (9, 15). The presence of cytokinin in root nodules is scarcely known, except for one brief paper recently published (12).

In this investigation we demonstrated the presence of cytokinins in pea root nodules induced by the infection of *Rhizobium leguminosarum* and identified the chemical nature of the cytokinins.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. cv. 'Little Marvel') were surface-sterilized for 10 min in 10% Pittchlor (commercial sodium hypochlorite) solution, then thoroughly rinsed with distilled H_2O. After soaking in distilled H_2O for 8 hr, fully swollen seeds were germinated in washed quartz sand. On the 5th day selected seedlings were planted in aeroponic culture tanks which were inoculated on the 6th day with a suspension of a pure culture of *Rhizobium leguminosarum*, strain 128 C53 (kindly provided by J. C. Burton of the Nitragin Co., Milwaukee, Wis.). Aeroponic culture permitted easy observation of the growing roots which were continuously bathed in a mist of 1/8 strength Hoagland solution minus nitrogen (22).

Plants were grown in the greenhouse. Nodules on the lateral roots of pea plants at 3 weeks after bacterial inoculation were collected in absolute ethanol precooled with dry ice-acetone or in a freezer (−19 C).

Extraction of Cytokinins. The frozen nodules were collected by filtration on Whatman No. 1 filter paper and thoroughly crushed in a mortar in cold 80% ethanol using 5 ml of solvent/g of nodule tissue. An average of 7.5 g fresh weight of nodule material was extracted for each of the experiments reported (Figs. 1-9). The crushed nodule tissues were filtered and the residue was extracted twice with 80% ethanol, each for 1 hr in a freezer (−19 C). The original absolute ethanol filtrate and the three 80% ethanol extracts were combined and concentrated under reduced pressure below 40 C. After removal of the ethanol, the pH of the aqueous phase was adjusted to 3 with 2 N HCl and extracted with methylene chloride three times. Then the pH of the residual aqueous phase was adjusted to 8 with 2 N NaOH and extracted with 1-butanol three times. The three butanol extracts were combined and taken to dryness *in vacuo*. The resulting residue was subjected to TLC. In the methylene chloride fraction and the water fraction after the extraction with 1-butanol, no cytokinin activity was detected.

Thin Layer Chromatography of the Cytokinins. The 1-butanol fraction was subjected to chromatography on 20 × 20 cm TLC plates. Three developing solvents were employed: water-saturated 1-butanol (A), chloroform-methanol (4:1, v/v) (B), and 3 mM borate buffer (pH 8.4) (C). The adsorbents were silica gel (GF_{254}) in solvent systems A and B and cellulose powder in solvent system C. Thickness of the adsorbent was 400 μm. Each chromatogram was air-dried and cut into 10 equal zones. The silica gel from each zone was scraped off the plate and extracted with methanol. The extracts were evaporated to dryness and tested for cytokinin activity using the soybean callus bioassay. Positions of the cytokinin standards, which were run simultaneously, were determined by UV light absorption.

Cytokinin Bioassay. Methanol extracts from different zones of chromatograms were incorporated into SCF medium (2) without a cytokinin supplement before autoclaving. Under each figure is presented the concentration of nodule extract in the callus medium. For example, in Figure 1 the total extract from 4 g of nodules was separated by TLC and each fraction was added to 100 ml of culture medium and assayed. For assay for cytokinin activity, soybean callus originally isolated from cotyledons of soybean cv. 'Biloxi' was used. Four pieces of soybean callus at 3

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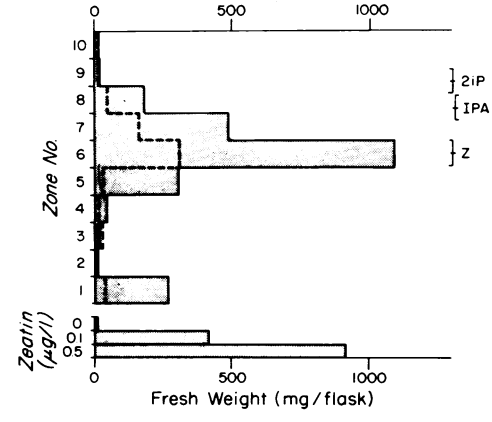
weeks, each approximately 8 mg fresh weight, were inoculated on 20 ml of medium in a 125-ml Erlenmeyer flask. Three to five flasks were used for each treatment. The cultures received 12 hr diffuse light/day and were maintained at 25 and 20 C in light and dark periods, respectively. After 4 weeks the average fresh weight/flask was determined.

RESULTS

Preliminary experiments (Fig. 1) revealed the reduction in cytokinin activity when the butanol extract was precipitated with silver salts by the procedure reported by Short and Torrey (14). Thereafter the precipitation step was excluded. Figure 1 also showed that the chromatogram of cytokinin activity in the butanol fraction precipitated with silver nitrate was roughly parallel to that of the butanol fraction with no treatment. The reduction in cytokinin activity by precipitation with silver salts may result from the loss of cytokinin during the procedure. Loss of 33% of initial zeatin concentration during this procedure was reported (20). This result suggested that the cytokinin was a purine derivative. The main cytokinin activity at zone 6 was similar to authentic zeatin in its migration. A smaller peak of activity was also found at zone 1.

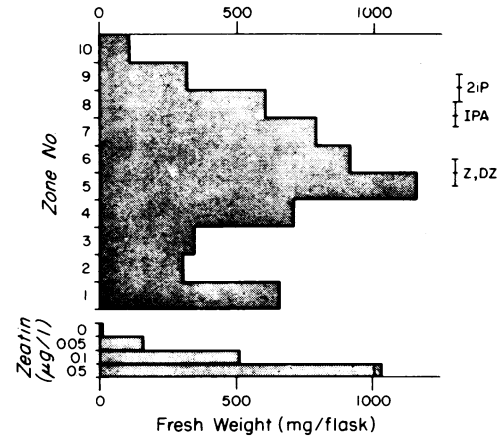
Cytokinin activity at the region (zones 7 and 8) corresponding in position to isopentenyladenine and its riboside appeared more clearly in the two solvent systems A and B, when higher concentrations of butanol extract were used for the detection of cytokinin activity (Figs. 2 and 3). To confirm this result, zones 5 to 7 and zones 8 and 9 in solvent system A were extracted separately by methanol and rechromatographed in solvent system B (Fig. 4).

Activity in zones 5 to 7 in solvent system A was recovered predominantly in zones 4 to 7 in solvent system B. The activity in zones 4 to 7 in solvent system B was similar in its mobility to a synthetic sample of zeatin. Rechromatography of the activity in zones 4 to 7 in solvent system B resulted in two regions exhibiting cytokinin activity in solvent system C (Fig. 5a). The peak of the higher R_F (zone 9) moved with synthetic zeatin riboside, while that of the lower R_F (zones 4 to 6) corresponded to the approximate mobilities of zeatin (Fig. 5a).



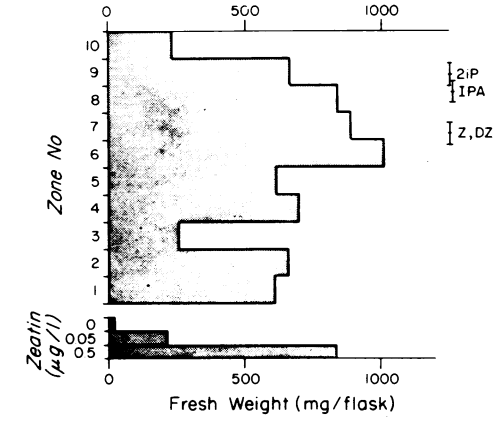
TLC: Silica gel
Solvent system: $CHCl_3$–MeOH (4:1)
Nodules: 3 weeks old, BuOH fraction
Conc. of nodule extract: 40g/l
—— : BuOH fr., no treatment
----- : BuOH fr., $AgNO_3$ treatment
Soybean callus assay: 8 mg/piece, 4 pieces/flask
Culture period: 4 weeks

FIG. 1. Histogram of soybean callus bioassay of butanol fraction after TLC in solvent system B, before (——) and after (- - -) precipitation with $AgNO_3$. Nodule extract was tested at a concentration of 40 g fresh weight/l of medium.



TLC: Silica Gel
Solvent system: $CHCl_3$ – MeOH (4:1)
Nodules: 3 weeks old, Fresh weight; 75g, BuOH fraction
Conc of nodule extract in culture medium; 125g/l
Soybean callus assays: 8mg/piece, 4 pieces/flask
Culture period: 4 weeks

FIG. 2. Histogram of soybean callus bioassay showing cytokinin activity in butanol fraction at a relatively high concentration (125 g fresh weight/l of medium). The thin layer chromatogram was developed with solvent system B.

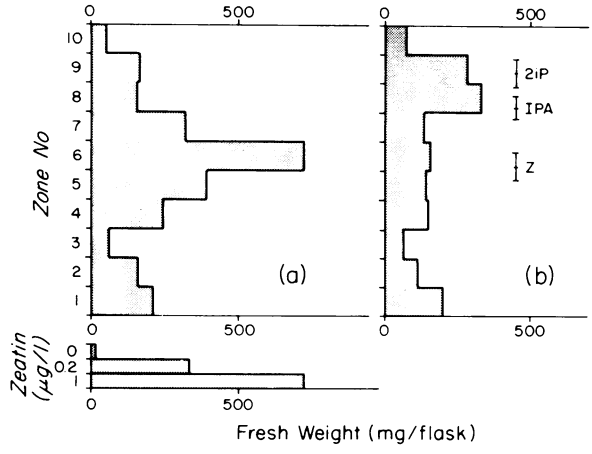


TLC: Silica gel
Solvent system: water – sat n-BuOH
Nodules: 3 weeks old, BuOH fraction
Conc. of nodule extract, 70g/l
Soybean callus assay: 8mg/piece, 4 pieces/flask
Culture period: 4 weeks

FIG. 3. Histogram of soybean callus bioassay of butanol fraction after TLC in solvent system A. Concentration of nodule extract was 70 g fresh weight/l of medium.

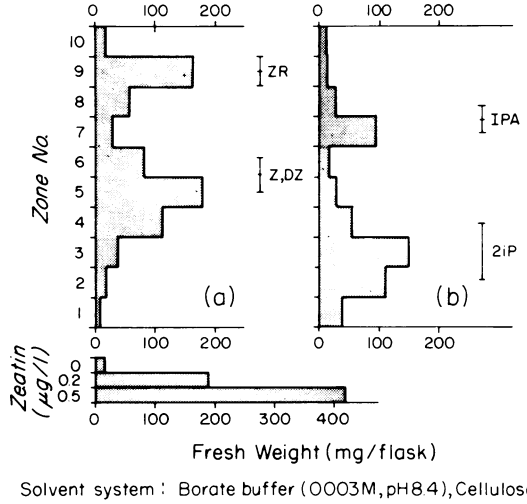
The purified activity in zone 9 in solvent system C (Fig. 5a) was eluted with methanol and divided into two equal portions. One aliquot was hydrolyzed with 0.1 N HCl at 100 C for 60 min. After hydrolysis the solution was made alkaline (pH 8) with NaOH and reextracted with 1-butanol. The other portion was used as a control. As shown in Figure 6, after hydrolysis the activity at zone 9 in solvent system C moved to the lower zone 6 corresponding to an authentic sample of zeatin. This result provided further evidence for the possibility that the cytokinin in zone 9 was zeatin riboside.

Authentic samples of zeatin and dihydrozeatin could not be separated by the three solvent systems A, B, and C. If the cytokinin activity in zones 4 to 7 in solvent system B (Fig. 5a) is zeatin and its riboside, it should be labile to potassium permanganate as is synthetic zeatin. The active factor in zones 4 to 7 in solvent system B (Fig. 5a) was eluted and divided into two equal



TLC: Silica gel
Solvent system: $CHCl_3$ - MeOH (4:1)
Nodule extract : 3 weeks old, n-BuOH fraction → TLC : H_2O sat - n-BuOH, Zone 5~7 (a) ; Zone 8and9 (b)
Conc. of extract : 35g FWE/l (a) , 70g FWE/l (b)

FIG. 4. Soybean callus assay of the sample at zones 5 to 7 (a) and zones 8 and 9 (b) in Fig. 3 after rechromatography in solvent system B. Nodule extract was tested at concentrations of 35 (a) and 70 g (b) fresh weight/l of medium.

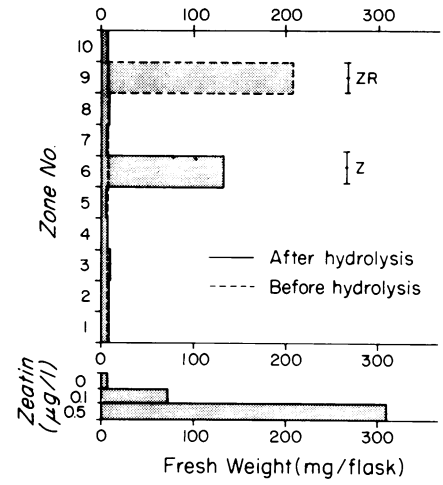


Solvent system : Borate buffer (0003M, pH8.4), Cellulose powder
3 Week old nodules:
(a) n-BuOH layer → TLC Silica gel H_2O sat - n-BuOH, Zone 5~7 → $CHCl_3$ - MeOH (4:1), Zone 4~7 ; 70g FWE/l medium
(b) n-BuOH layer → TLC Silica gel H_2O sat - n-BuOH, Zone 4 → $CHCl_3$ - MeOH (4:1), Zone 8,9 ; 140g FWE/l medium

FIG. 5. Soybean callus assay of the sample at zones 4 to 7 in Fig. 4a and zones 8 and 9 in Fig. 4b after rechromatography in solvent system C. Concentrations of extract were 70 and 140 g fresh weight/l of medium, in Fig. 5, a and b, respectively.

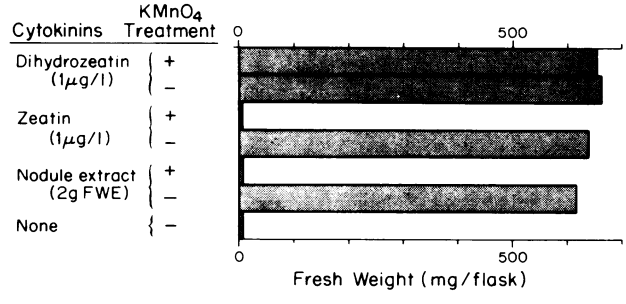
portions and evaporated to dryness *in vacuo*. Each residue was dissolved in a small quantity of water. In one, potassium permanganate oxidation was carried out, and after 15 min at room temperature, an excess of 95% ethanol was added. The other solution was treated only with ethanol. Authentic samples of zeatin and dihydrozeatin were also treated with potassium permanganate separately.

Both zeatin and nodule cytokinins in zones 4 to 7 in solvent system B (Fig. 5a) were labile to potassium permanganate treatment, while authentic samples of dihydrozeatin were stable to this treatment (Fig. 7). Thus, we concluded that cytokinins in zones 4 to 7 in Figure 5a were zeatin and its riboside.



Solvent system: 0.003M Borate buffer (pH 8.4), Cellulose powder
Nodules : 3 weeks old, Conc. of extract, 70g FWE/l
Purification : TLC Silica gel H_2O sat - n-BuOH, Zone 5~7 → $CHCl_3$ - MeOH (4:1), Zone 4~7
Hydrolysis : 0.1N HCl, 100°C 1 hr.

FIG. 6. Histogram of soybean callus bioassay of the sample at zones 8 and 9 in Fig. 5a after rechromatography in solvent system C. Extract was tested at a concentration of 70 g fresh weight/l of medium, before (- - -) and after (——) hydrolysis with 0.1 N HCl at 100 C for 1 hr.



$KMnO_4$ treatment : Room temperature , 15 min
Nodule extract : 3 week old nodules ; TLC, Silica gel, H_2O sat - n-BuOH, Zone 5~7 → $CHCl_3$ - MeOH (4:1), Zone 4~7

FIG. 7. Effect of permanganate treatment on authentic samples of zeatin and dihydrozeatin and the samples of extract at zones 4 to 7 in Fig. 4a. Activity was determined by soybean callus bioassay. Nodule extract was used at a concentration of 25 g fresh weight/l of medium.

Activity in zones 8 and 9 in solvent system A was recovered in zones 8 and 9 in solvent system B, although smaller peaks of activity were detected in the lower regions (zones 4 to 6 and zones 1 and 2). The activity in zones 8 and 9 was similar to authentic samples of isopentenyladenine and its riboside in its mobility again (Fig. 4b). Rechromatography of the activity in zones 8 and 9 in solvent system B exhibited cytokinin activity in two regions in solvent system C (Fig. 5b). The more slowly moving active factor corresponded to the approximate mobility of 2iP[3], while the faster moving active region co-chromatographed with the authentic sample of IPA (Fig. 5b).

Cytokinin activity in zones 1 and 2, because of its low mobility in solvent system A, was suspected of being a nucleotide. To confirm this possibility, the region containing this cytokinin was scraped off the plate and eluted with H_2O and treated with alkaline phosphatase. The reaction mixture consisted of the

[3] Abbreviations: 2iP: isopentenyladenine; IPA: isopentenyladenosine; Z: zeatin; ZR: zeatin riboside; DZ: dihydrozeatin.

following components: 0.5-ml extract of cytokinin, 0.05 ml of 0.1 M $MgCl_2$, 0.4 ml of 0.1 M tris buffer (pH 8.5), and 0.07 mg of chicken intestine alkaline phosphatase (14). This reaction mixture was incubated for 3 hr at 37 C. A control was treated similarly, except that the boiled enzyme was used. After the 3-hr reaction period, the reaction mixture was extracted with 1-butanol and chromatographed in solvent system B.

The cytokinin activity found in zone 1 in the boiled enzyme treatment (Fig. 8a) completely disappeared and the cytokinin activity corresponding to zeatin riboside (zone 5) increased in extracts treated with enzyme. This result showed the presence of cytokinin nucleotide, probably zeatin ribotide. No attempt was made to determine whether or not the other two peaks of cytokinin activity (zone 5 and zone 10 in Fig. 8a) were artifacts produced during procedures.

Quantitative changes of cytokinins in relation to nodule development was also examined (Fig. 9). The cytokinin activity was the highest in 2-week-old nodules, and gradually decreased with age. However, no fundamental qualitative change in cytokinins was observed. The main cytokinin activity was found in zones 5 and 6 in solvent system B, corresponding in their mobility to authentic samples of zeatin and its riboside (Fig. 9).

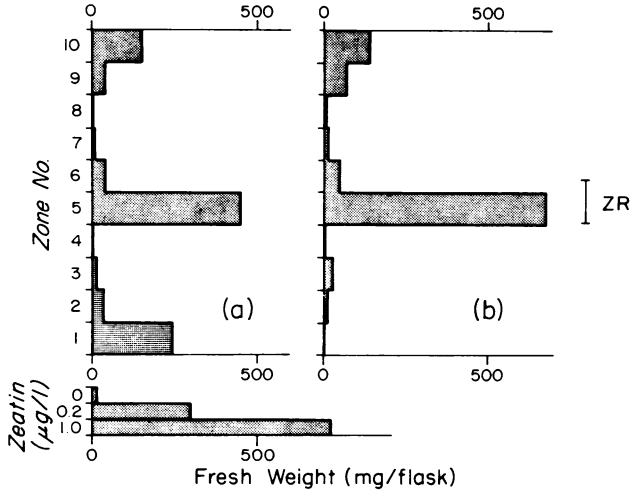


FIG. 8. Histogram of soybean callus bioassay of chromatogram developed in solvent system B. The samples of extract (70 g fresh weight/l of medium) at zones 1 and 2 in Fig. 3 were tested after treatment with alkaline phosphatase (a) and with boiled enzyme (b).

DISCUSSION

The existence of high cytokinin activity in pea root nodules was clearly demonstrated. The presence of cytokinins in the root nodules of *Phaseolus vulgaris* infected by *Rhizobium phaseoli* was also reported recently (12). The cytokinins found predominantly in pea root nodules infected by *R. leguminosarum* were zeatin and its riboside and ribotide. Small amounts of isopentenyladenine and its riboside were also detected. The presence of cytokinins in root nodules and their changing concentration during nodule development (Fig. 9) strongly support the idea (16) that cytokinins play a critical role in nodule development, particularly in relation to the meristematic nature of the nodule, *i.e.* its cell division activity and also to the distinctive polyploid state of the dividing nuclear population.

Of particular interest is the question concerning the origin of the cytokinins present and active during nodule initiation and development. Earlier analyses of cytokinins in pea root meristems (14) showed that zeatin and its riboside and ribotide were the major free cytokinins occurring in the uninfected root apex. The cytokinins produced by *Rhizobium leguminosarum* in pure cultures were different. According to Phillips and Torrey (11) *R. leguminosarum* produces a different cytokinin than that produced by *R. japonicum*; from chromatographic mobilities one would assign the activity to 2-iP or IPA. More recently, Giannattasis and Coppola (5) reported that *R. leguminosarum* releases IPA and two unknown cytokinins into the medium. From the results presented here, developing root nodules of pea contain two groups of cytokinins, *viz.* zeatin and its immediate derivatives and 2-iP and its derivatives. These two compounds are

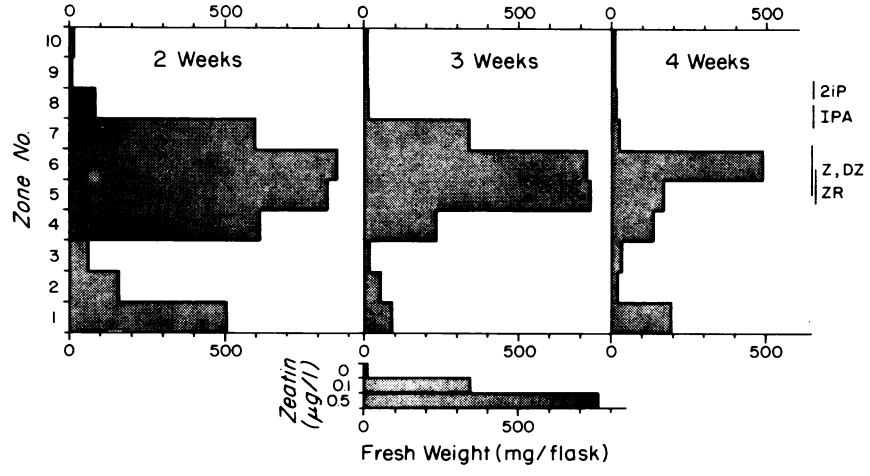


FIG. 9. Time course of changes in cytokinin activities in root nodules of pea: (a) 2 weeks, (b) 3 weeks, and (c) 4 weeks. Activity was determined by soybean callus bioassay. The extract at a concentration of 25 g fresh weight/l of medium was developed with solvent system B.

closely related and 2-iP may serve as precursor to zeatin (7). From Figures 3 and 4 one can calculate that the amount of 2-iP and its riboside in 3-week-old nodules of pea was only about one-seventh that of zeatin and its derivatives.

Several possible explanations can be proposed concerning the cytokinin content of pea root nodules. (*a*) The host tissue, especially the meristematic region of the nodule is the source of zeatin and its derivatives while the invading bacteria and bacteroids produce 2-iP and its derivatives. Both function in stimulating DNA synthesis and cell division. (*b*) The bacteria produce 2-iP which is converted by the host tissue to zeatin and its derivatives and this latter cytokinin stimulates cell division in the nodule apex. (*c*) The initial infection by *Rhizobium* is stimulated by the bacterial cytokinin, 2-iP and thereafter the host tissue forms plant root cytokinin (zeatin). The idea that the bacterial cytokinin may be different from that produced by the host is stengthened by the observation (unpublished) that another strain of *R. leguminosarum* which produces ineffective nodules leads to the formation of yet another unidentified but distinctive cytokinin. The problem of the origin of the cytokinins in the nodule is an intriguing problem remaining to be solved. The possibility clearly exists that symbiosis in nodule development extends even to the contribution of hormones from each partner during nodule development.

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