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Cytokinins in Seedling Roots of Pea

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ABSTRACT

The natural occurrence of cytokinins existing both in a free form and as a constituent of transfer RNA was examined in serial segments of young seedling roots of pea. Purified ethanol extracts of root apices were resolved into four factors capable of inducing soybean callus tissue proliferation. The most active factor was identified as zeatin or some closely related compound; it produced polyploid divisions and tracheary element differentiation when tested on cultured pea root segments. The terminal 0- to 1-millimeter root tip contained 43 to 44 times more free cytokinin on a fresh weight or a per cell basis than the next 1- to 5-millimeter root segment. Extracts of more proximal segments behind the tip contained no measurable free cytokinin. Acid hydrolysates of transfer RNA exhibited reproducible cytokinin activity. Bioassays revealed that the predominant amounts of free cytokinin and that present in transfer RNA were restricted to the extreme root tip. There was approximately 27 times more free cytokinin than the amount detected in transfer RNA in root apices.

The occurrence of cytokinins in the xylem sap of decapitated plants (4, 13, 19, 24, 31) and the marked reduction in the amount of cytokinin in bleeding sap when the root system is subjected to water stress (12) or flooding (3) suggests that this hormone may be produced in the root. Pea seedlings are well suited for investigating the natural occurrence of cytokinins in roots, as Carr and Burrows (4) demonstrated that cytokinins are present in the xylem exudate of the field pea. Rogozinska *et al.* (28) and Zwar and Skoog (38) found that extracts from young pea seedlings exhibited cytokinin activity. Furthermore, Babcock and Morris (1) identified N^6-(Δ^2-isopentenyl)adenosine[2] and the *cis*-isomer of zeatin ribonucleoside in tRNA hydrolysates of young seedling roots of pea.

In this investigation we examined the natural occurrence of cytokinins in young seedling roots of Alaska pea, and a comparison was made of the amount of cytokinin existing in a free form to that present in tRNA. This study, by determining the endogenous localization of cytokinins in the root, provides further information concerning the hypothesis that the root is a center of hormone synthesis.

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[2] Abbreviations: IPA: 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine or isopentenyladenosine; 2iP: 6-(3-methyl-2-butenylamino)purine or isopentenyladenine; ZR: 6-(4-hydroxy-3-methyl-2-*cis*butenylamino)-9-β-D-ribofuranyosylpurine or zeatin ribonucleoside; Z: 6-(4-hydroxy-3-methyl-2-*trans*butenylamino)purine or zeatin.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. cv. Alaska) were surface sterilized in 5% Pittchlor (commercial sodium hypochlorite) solution, thoroughly rinsed in sterile water and aseptically germinated in the dark at 23 C. After 3 days about 2000 pea roots were cut into four segments 0 to 1, 1 to 5, 5 to 20, and 20 to 40 mm proximal to the root tip. The excised pea root segments were blotted free of excess water and weighed. Cell number determinations were made by the modification of the tissue maceration technique of Brown and Rickless (2) employed by Fosket and Torrey (7).

Extraction of Free Cytokinins. Immediately after harvesting, the different fractions of pea root segments were immersed separately in precooled absolute ethanol at −72 C. The frozen segments were collected by filtration on Whatman No. 1 filter paper and homogenized in an all-glass homogenizer in cold 80% (v/v) ethanol. The homogenized tissue was extracted for 3 hr in two portions of 80% ethanol and centrifuged at 10,000*g* for 15 min at 4 C. The original absolute ethanol fraction and the two 80% ethanol extracts were combined and the alcohol evaporated *in vacuo*. The water phase was adjusted to pH 8.0 and extracted with three equal volumes of 1-butanol which were then combined and taken to dryness *in vacuo*. The residue was redissolved in about 100 ml of water and adjusted to pH 2.0 with concentrated H_2SO_4. Fifteen milliliters of cold saturated $AgNO_3$ solution were added, and the mixture constantly was stirred for 12 hr at 4 C and centrifuged at 7000*g* for 15 min. The precipitate was washed with cold 2% $AgNO_3$ solution and stirred with 20 ml of 0.2 N HCl at 50 C for 30 min before being centrifuged at 7000*g*. This procedure was repeated twice. The combined acidic supernatants were adjusted to pH 2.5 with concentrated NaOH and percolated through a Dowex 50W-X8, H^+ (200–400 mesh) column, 1.0 cm × 25.0 cm, which was then washed with 100 ml of water and eluted with 300 ml 3 N NH_4OH. The eluate was evaporated *in vacuo* to about 50 ml and extracted at pH 8.0 with three equal volumes of 1-butanol which were combined and taken to dryness *in vacuo*. The resulting residue was dissolved in 80% ethanol and applied as a streak on Whatman No. 1 paper and developed with solvent system A, 1-butanol-acetic acid-water (12:3:5 v/v). In addition, the following solvents were employed: B: 30 mM borate buffer at pH 8.4; C: ethyl methyl ketone saturated with water; D: 1-butanol-1 N ammonium hydroxide (10:7 v/v); E: 1-butanol saturated with water. Each chromatogram was thoroughly air dried and cut into 10 equal R_F strips and eluted with 80% ethanol. The eluates were reduced in volume and tested for cytokinin activity using the soybean callus bioassay.

Extraction of Cytokinins in tRNA. The fractions of pea root segments were frozen on Dry Ice and homogenized in an all-glass homogenizer in a homogenizing fluid consisting of 80% (w/v) phenol in 0.1 M tris-HCl buffer, pH 7.8, and one volume of 4% sodium lauryl sulfate in 0.1 M tris-HCl buffer, pH 7.8, containing 0.1% 8-hydroxyquinoline as a ribonuclease

inhibitor. The homogenate was centrifuged at 10,000*g* for 15 min and the upper, aqueous phase was removed with a Pasteur pipette. The remaining phenol phase and the protein interface was re-extracted with equal volumes of the homogenizing solutions. After centrifugation the aqueous phase was removed, combined with that of the first extract, and deproteinized twice more by shaking vigorously with an equal volume of 80% (w/v) phenol in 0.1 M tris-HCl buffer, pH 7.8. The nucleic acid present in the aqueous phase was precipitated by the addition of 2.5 volumes of an ice-cold mixture of ethanol, water and 10% NaCl (w/v) in a ratio of 25:10:1, v/v. The mixture was stored at −17 C for not less than 4 hr and usually overnight. The flocculent precipitate that formed was sedimented by centrifugation at 7000*g* for 15 min at 4 C and washed twice by resuspension in the ice-cold ethanol mixture. The nucleic acid residue was dissolved in 0.3 M NaCl in 50 mM sodium phosphate buffer, pH 6.7, and fractionated on methylated albumin kieselguhr columns as described by Short *et al.* (30). The tRNA was collected and precipitated with 2.5 volumes of ice-cold ethanol mixture. The precipitate was redissolved in water and dialyzed against water (two changes) for 24 hr at 4 C. The tRNA content was estimated spectrophotometrically from the absorption at 260 nm, using 1 mg of nucleic acid equivalent to 20 absorbance units (32). To release cytokinins, samples of tRNA were heated in 5 ml of 0.1 N HCl at 100 C for 30 min (20). The hydrolysates were tested for cytokinin activity directly and after fractionation by chromatography on Whatman No. 1 paper with solvent system A. The chromatograms were cut into 10 equal sections and eluted in 80% ethanol. The eluate was reduced in volume and tested for cytokinin activity in the soybean callus bioassay.

Cytokinin Bioassay. Dilutions of ethanol eluates from different sections of chromatograms were incorporated before autoclaving in SCF culture medium (7). For assay of cytokinin activity four pieces of soybean callus tissue, each approximately 8 mg fresh weight, were inoculated on 20 ml of medium in a 125-ml Erlenmeyer flask. The cultures were maintained at 23 C and received 12 hr diffuse warm white light per day. After 4 weeks the average fresh weight per callus piece was determined.

RESULTS

Extractions of Free Cytokinins. Preliminary experiments using crude 80% ethanol extracts of 3-day-old 0 to 1 mm pea root tips revealed no cytokinin activity in the soybean callus bioassay. Purification of crude ethanolic extracts by precipitation with silver salts, ion-exchange chromatography, organic solvent extraction and paper chromatography in solvent A produced a reproducible promotion of growth. Figure 1 represents a typical response of extracts from 0 to 1 mm pea root apices which were bioassayed at a concentration of 10 g fresh weight per liter of medium. Cytokinin activities found at R_F 0.1 to 0.2, R_F 0.4 to 0.5, and R_F 0.7 to 0.8 were designated factors I, II, and III respectively. Factor III was considerably more effective in inducing soybean tissue proliferation than the other two factors, producing a 60-fold increase in fresh weight over the weight of initial explants. The activity of factor III in the soybean bioassay was approximately equivalent to the response given by 50 μg/l kinetin.

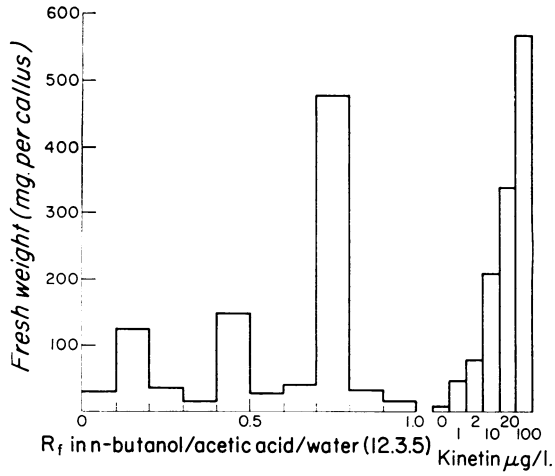

FIG. 1. Histogram of soybean callus bioassay of purified extracts of pea root tips after paper chromatography in solvent system A. The growth response of kinetin standards is illustrated.

Rechromatography of factor III in solvent system B resulted in two regions exhibiting cytokinin activity (Fig. 2A). The most active region produced was at R_F 0.5 to 0.6 and a less active region at R_F 0.8 to 0.9, designated factors IIIa and IIIb respectively. This assay indicated that the predominant activity of extracts from root tips was due to factor IIIa. The more slowly moving active factor corresponds to the approximate mobilities of zeatin and IPA, while the faster moving active region cochromatographs with zeatin ribonucleoside and 2iP (Fig. 2A). Elution of factor IIIa in solvent system B and subsequent development in solvent C produced only one region of activity which cochromatographed with an authentic sample of zeatin (Fig. 2B). Elution of the less active region in solvent B (factor IIIb) and rechromatography in solvent system C resulted in a region of activity which corresponded to the migration of zeatin ribonucleoside in this solvent system (Fig. 2C). The mobilities of factors IIIa and IIIb in solvent system C suggest that zeatin and zeatin ribonucleoside are present in pea root apices.

If one of the cytokinins in pea root tips is zeatin, then it should be labile to potassium permanganate as is synthetic zeatin. Permanganate-treated zeatin is converted into adenine (21), N-(purin-6-yl)glycine (23), and a third unidentified compound (16) and all of these compounds are biologically inactive in cytokinin bioassays at low concentrations. Therefore, the stability of pea root tip cytokinins to potassium permanganate was studied. Ethanolic extracts from 2.2 g fresh weight of 0 to 1 mm pea root apices were purified, as described in "Materials and Methods" section and chromatographed in solvent A. The interval between R_F 0.7 to 0.8 was eluted and rechromatographed in solvent system B. Active factor IIIa (R_F 0.5–0.6, in Fig. 2A) was eluted and divided into two equal portions and reduced to dryness *in vacuo*. Each residue was dissolved in a small quantity of water. In one, potassium permanganate oxidation was carried out (22) and, after 15 min at room temperature, an excess of 95% ethanol was added. The other solution was treated only with ethanol.

Both solutions were evaporated to dryness, the residues were subsequently redissolved in 80% ethanol, and their biological activity was tested at a concentration of 10 g fresh weight/liter of medium in a pea root segment system (34), employing the experimental methods of Phillips and Torrey (26). Torrey (34) demonstrated that only cytokinins stimulate polyploid mitoses in cortical cells of cultured pea root segments, and Torrey and Fosket (36) found that a portion of these polyploid cells differentiate to form tracheary elements in the presence of cytokinin. This system was used to determine the effect of potassium permanganate treatment on the ability of a naturally occurring cytokinin in pea root tips to stimulate polyploid mitoses and tracheary element differentiation in the cortex of cultured pea root segments. The data in Table I show that both factor

IIIa and 10 μg/l zeatin promoted polyploid divisions and tracheary element differentiation in the pea segment system. However, the biological activity of factor IIIa was destroyed by permanganate treatment, which confirms that this naturally occurring cytokinin may be zeatin or some closely related compound.

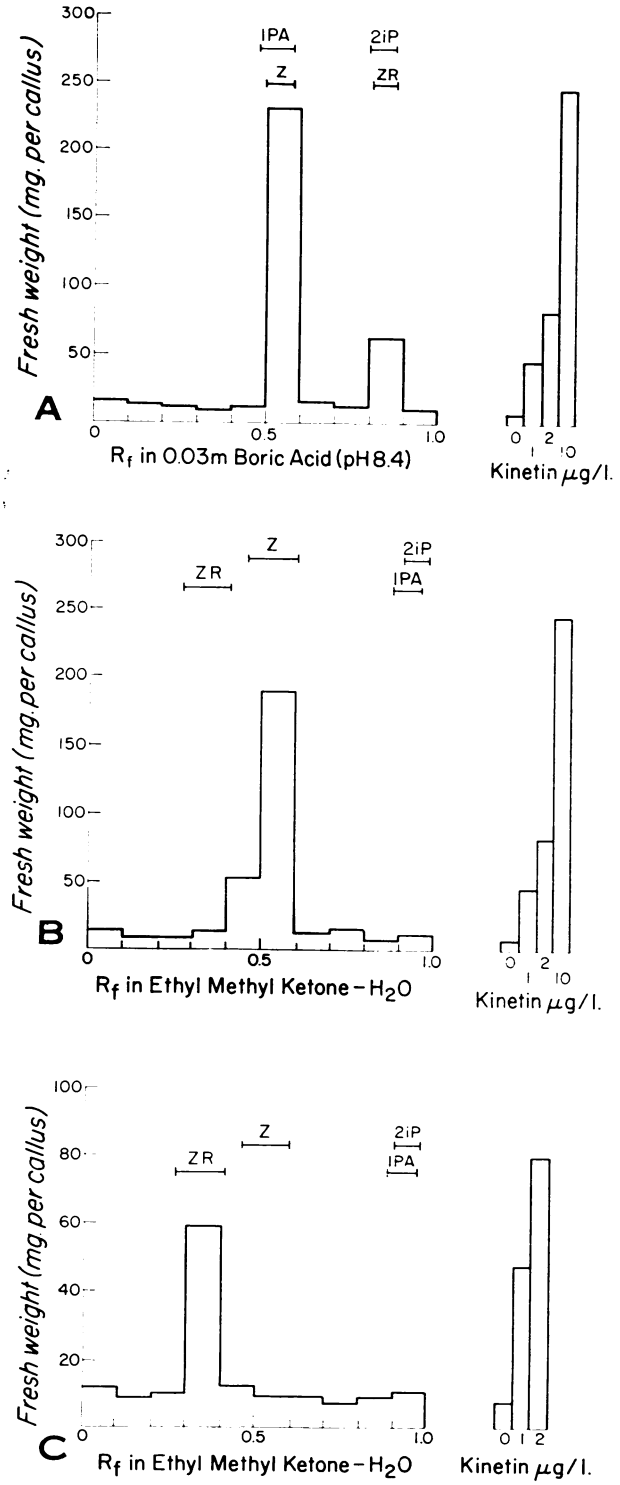

FIG. 2. Distribution of activity of factor III obtained by paper chromatography of extracts of pea root tips in solvent A on subsequent rechromatography in different solvent systems. A: Bioassay of factor III after chromatography in solvent system B; B: subsequent chromatography in solvent system C of the material between R_F 0.5 to 0.6 in solvent B; C: activity of material eluted at R_F 0.8 to 0.9 in solvent B after rechromatography in solvent C.

Table I. *Effect of Permanganate Treatment on the Ability of a Naturally Occurring Cytokinin in Pea Root Tips to Initiate Polyploid Mitoses and Tracheary Element Differentiation in Cultured Pea Root Segments*

Polyploid mitoses: + = 10 to 20 division figures/slide; tracheary elements: + = present, – = absent.

Treatment	Polyploid Mitoses	Tracheary Elements
Factor IIIa (10 g/l)	+	+
Factor IIIa treated with $KMnO_4$	–	–
10 μg/l Zeatin	+	+
Basal medium	–	–

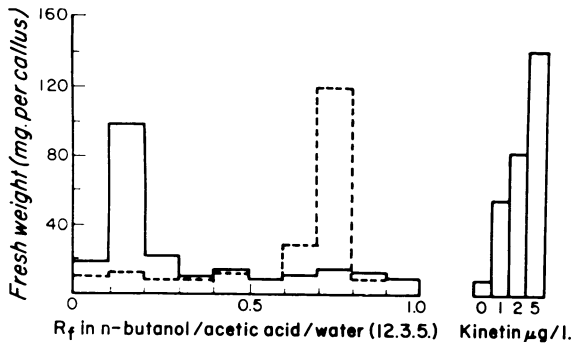

FIG. 3. Histogram of soybean callus bioassay of chromatograms developed in solvent system A. Broken line represents factor I eluted from a previous chromatogram and treated with phosphatase; solid line represents factor I treated with boiled enzyme solution.

Figure 1 shows that the region of activity in the soybean callus test at R_F 0.4 to 0.5 (factor II) in solvent A was approximately equivalent to the growth induced by 4 μg/liter kinetin. Elution of factor II and subsequent chromatography in solvent systems D and E produced a single band of activity at R_F 0.2 to 0.3 and R_F 0.3 to 0.4 respectively in these solvents.

The chromatographic mobility of factor I at R_F 0.1 to 0.2 in Figure 1 suggests that this compound may be a nucleotide and this possibility was investigated. The interval between R_F 0.1 to 0.2 in solvent A of extracts from pea root apices was eluted and divided into two equal portions. One aliquot was treated with 0.1% chicken intestine alkaline phosphatase in 0.1 M tris and 10 mM $MgCl_2$ at pH 8.2 for 3 hr at 37 C. The other portion was used as a control and was incubated with boiled enzyme solution. Both treatments were chromatographed in solvent A and tested in the soybean callus assay at a concentration of 10 g fresh weight/liter of medium. Phosphatase treatment destroyed factor I but produced a more mobile active compound at R_F 0.7 to 0.8 in solvent system A (Fig. 3). This result is consistent with the notion that factor I is a nucleotide.

Bioassay of extracts from seedling pea root segments cut 1 to 5 mm proximal to the root tip revealed only one region of activity at R_F 0.7 to 0.8 in solvent A (Fig. 4). The growth response was less than that given by 2 μg/liter kinetin. Unlike the active factor at the same R_F in root tip extracts, rechromatography of this region in solvents B and C produced a single band of activity which cochromatographed with zeatin. Extracts from pea root segments excised proximal to the 0 to 5 mm region did not reproducibly induce any proliferation in the soybean bioassay.

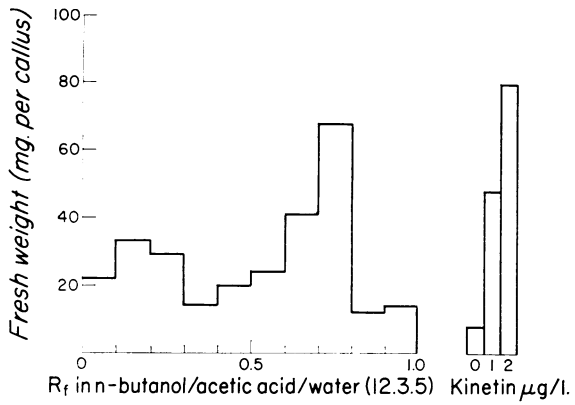

FIG. 4. Histogram of soybean callus bioassay of extracts from seedling pea root segments cut 1 to 5 mm proximal to the root tip. Extract was tested at a concentration of 10 g fresh wt/liter of medium; the response given by kinetin standards is shown.

Table II. *Amount of Free Cytokinin in Extracts of Serial Pea Root Segments as Determined by the Soybean Callus Assay*

All extracts were tested at a concentration of 10 g fresh wt/liter of medium.

Position of Segment Excised from Root Tip	Kinetin Equivalents per 10 g Fresh Wt Tissue	Kinetin Equivalents per 10^8 Cells
	μg	
0–1 mm	59.6	9.7
1–5 mm	1.4	0.2
5–20 mm	0	0
20–40 mm	0	0

Table II illustrates the results of an experiment in which the quantitative distribution of free cytokinin was determined in serial pea root segments. The cytokinin content, expressed as μg kinetin equivalents per 10 g fresh weight of tissue, was greatest in the 0 to 1 mm root tip. The quantity present was about 43 times that found in the segment cut 1 to 5 mm behind the root apex. Cell number determinations showed 3×10^4 cells per 0 to 1 mm root tip as compared to 17×10^4 in the 1 to 5 mm segment. From these data the cell number per 10 g fresh weight of serial pea root segments was calculated and showed that when the cytokinin content was expressed relative to cell number, there was about 44 times more cytokinin in the cells of the root apex than in the segment immediately behind the tip.

Extractions of Cytokinins from tRNA. Soybean callus bioassays of acid hydrolysates of tRNA extracted from 0 to 1 mm pea root tips showed a single region of cytokinin activity at R_F 0.8 to 0.9 in solvent system A (Fig. 5). When the tRNA hydrolysate was tested at a concentration of 10 mg/liter, the growth response was approximately equivalent to that produced by 3 μg kinetin/liter. A qualitatively similar pattern of cytokinin activity was found in tRNA hydrolysates from other portions of the root on paper chromatography in solvent A. Table III presents the results of an experiment in which cytokinin activity was determined in tRNA hydrolysates in successive pea root segments. The data indicate that tRNA content per unit fresh weight of tissue was greatest in the 0 to 1 mm root tip and declined in successive segments excised proximal to the apex. However, the amount of cytokinin detected in tRNA hydrolysates was virtually constant in the pea root segments examined. From these results it was possible to calculate the amount of cytokinin in tRNA per 10 g fresh weight of tissue (Table III). These values afford a direct comparison to the amount of free cytokinin found in pea root segments (Table II). Comparison of the results in Tables II and III show that there were 2.2 μg kinetin equivalents in tRNA hydrolysates compared to 59.6 μg kinetin equivalents of free cytokinin per 10 g of 0 to 1 mm root tips. Therefore, in an equivalent amount of root tips there was about 27 times more cytokinin existing in a free form than was present in tRNA. Comparable amounts of free cytokinin to that in tRNA were found in pea root segments excised 1 to 5 mm proximal to the root tip (Tables II and III). Approximately similar amounts of cytokinin were found in tRNA hydrolysates from pea root segments excised 5 to 20 mm and 20 to 40 mm behind the root tip (Table III). In contrast, no free cytokinin was detected in these portions of the root (Table II).

DISCUSSION

Elaborate purification of ethanolic extracts from seedling pea roots was necessary in order to demonstrate cytokinin activity in the soybean callus bioassay. The failure to detect free cytokinins in crude ethanol extracts was probably due to the presence of endogenous inhibitors in the pea root. Torrey (33) reported the presence in seedling pea roots of inhibitors of auxin-induced lateral root initiation of pea. More recently, Shibaoka and Thimann (29) demonstrated that methanolic extracts of 0 to 4 mm pea root tips contain high levels of cytokinin antagonists. Incomplete removal of these endogenous in-

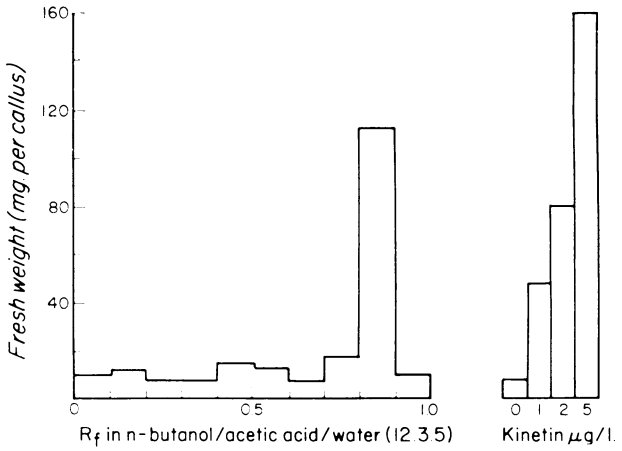

FIG. 5. Histogram of soybean callus bioassay of an acid hydrolysate of tRNA extracted from 0 to 1 mm pea root tips. The hydrolysate was tested at a concentration of 10 mg/liter of medium and the growth response given by standard amounts of kinetin is shown.

Table III. *Quantitative Distribution of tRNA and of Cytokinin Present in tRNA Hydrolysates Extracted from Excised Pea Root Segments Cut at Increasing Distances from the Root Tip*

Acid hydrolysates of tRNA were tested at a concentration of 10 mg RNA/liter of test medium.

Position of Segment Excised from Root Tip	tRNA per 10 g Fresh Wt Tissue	Kinetin Equivalents per 10 mg tRNA Hydrolysates	Kinetin Equivalents in tRNA Hydrolysates per 10 g Fresh Wt Tissue
mm	*mg*	*μg*	
0–1	8.42	2.7	2.2
1–5	3.31	3.1	1.0
5–20	0.9	1.9	0.2
20–40	0.7	2.2	0.2

hibitors in their studies may have resulted in the absence of detectable cytokinin activity in extracts of these roots. However, Shibaoka and Thimann (29) suggested the possibility that cytokinins in pea roots were in a methanol-insoluble form.

Extracts of pea root apices soluble in cold ethanol have been resolved into four factors capable of inducing soybean callus tissue proliferation. On the basis of paper chromatographic evidence and lability to potassium permanganate treatment, one of these (factor IIIa) may be zeatin or some closely related derivative. Another compound (factor IIIb) cochromatographs with zeatin ribonucleoside in two solvent systems.

The purification step involving silver precipitation in acidic solution is generally regarded as definitive for free purines. Based on chromatographic evidence described above, precipitation of 9-substituted purines seems to have occurred in these experiments. Such precipitation of ribonucleosides by silver at an acid pH has been reported before (9, 15). It is assumed that under the conditions employed in these experiments, precipitation of 9-substituted purine derivatives was probably complete, since no cytokinin activity was detected in extracts which were made of the soluble fraction in acidic silver nitrate solutions. The chromatographic mobility of factor II differs from those of the known naturally occurring free cytokinins. Factor II appears to be a purine derivative as it is precipitated by silver ions at an acidic pH. It is also extracted into 1-butanol from aqueous solutions at pH 8. The properties of factor II are similar to those of an unidentified cytokinin isolated from sweet corn (17), apple fruitlets (18), and seeds of pumpkin (9) and watermelon (27). We have found a compound with similar properties in pea root callus tissue cultures. Therefore it is proposed that factor II is an unidentified cytokinin. The susceptibility of factor I to phosphatase treatment suggests that it is the ribotide of factor III. In this investigation no attempt has been made to identify the biologically active acid hydrolysis product of tRNA of seedling pea roots. Hall and Srivastava (11) demonstrated that acid hydrolysis of IPA, which is known to occur in the tRNA of garden peas (10) and pea roots (1), results in the formation of two degradation products, one of which, 6-N(3-hydroxy-3-methylbutyl amino) adenine, is a potent cytokinin. Therefore it is possible that this active compound is produced on acid hydrolysis of the tRNA of seedling pea roots.

The quantitative distribution of cytokinins in pea roots showed that the greatest amount of cytokinin existing in a free form and present in tRNA was found in the highly meristematic 0 to 1 mm root tip. Considerably smaller amounts of cytokinin were found in the segments cut 1 to 5 mm behind the root tip which contained fewer meristematic cells. These results are in agreement with those of Weiss and Vaadia (37) who found that in sunflower seedlings free cytokinins were restricted to the youngest portions of the root tip. Additional support for the hypothesis that root apices may be the site of cytokinin synthesis in roots is provided by the work of Burrows and Carr (3). These workers demonstrated that there was a dramatic reduction in the cytokinin content of the xylem sap when the root systems were flooded. This reduction was coincident with an increase in the number of blackened root apices which were unable to reduce tetrazolium and were presumably dead.

The origin and specific pathways of cytokinin biosynthesis, and also its site of production in the root tip, are unclear. Goldacre (8) obtained evidence for the production of a cytokinin-like substance by the dividing cells of unemerged root initials in isolated flax roots, and proposed that cytokinin production may be a normal accompaniment of cell division. Furthermore, Torrey (35) suggested that the quiescent zone may be the site of highest cytokinin concentration in the root tip. If this is so, then the lack of cell division in this zone could be explained by a supra-optimal cytokinin concentration. Although it seems probable that the quiescent zone and the surrounding meristematic tissue is the site of free cytokinin production in the seedling pea root, direct evidence for this conclusion is lacking.

The relationship between free cytokinin and that present in tRNA is unresolved. In a recent review Kende (14) stated that the experimental evidence available suggests that free cytokinins are not involved in any direct way with the formation of cytokinins in tRNA. There is strong evidence (5, 6, 25) that cytokinins in tRNA are synthesized by the attachment of the isopentenyl group to preformed tRNA. It is also presumed that mevalonate is the precursor of the isoprenoid side chain of free cytokinins. Chen and Hall (5) proposed that normal catabolism of tRNA would release its biologically active constituents, and that regulation of its degradation may affect the level of free cytokinins in the cell. Our data show that there was approximately 27 times more cytokinin in a free form than was present in tRNA in the 0 to 1 mm root tip. Therefore, it would be desirable to determine the rate of turnover in the pea root apex of tRNA species which contain cytokinins. This would resolve whether the large amount of free cytokinin in this region is produced by tRNA catabolism or by a separate biosynthetic pathway.

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