Plant Physiol. (1978) 62, 861–865

Cytokinin Biosynthesis in Cultured Rootless Tobacco Plants[1]

Received for publication June 14, 1978 and in revised form August 10, 1978

CHONG-MAW CHEN AND BRYON PETSCHOW
Science Division, University of Wisconsin-Parkside, Kenosha, Wisconsin 53141

ABSTRACT

Biosynthesis of cytokinin in shoots was examined by growing rootless tobacco (*Nicotiana tabacum*) plants *in vitro*. The rootless plants were originated by culturing tobacco callus on a high cytokinin-low auxin medium to induce the formation of plantlets which were then grown on medium without exogenous cytokinin and auxin. The rootless plants supplied with [^{14}C]adenine synthesized ethanol-ethyl acetate-water-soluble radioactive components, portions of which had the same chromatographic and electrophoretic mobilities as N^6-(Δ^2-isopentenyl)adenine, N^6-(Δ^2-isopentenyl)adenosine, 6-(4-hydroxy-3-methyl-2-butenylamino)purine and 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine. The total amount of these four major cytokinins was estimated to be present at a concentration of 14 to 23 nanomoles per kilogram of rootless plant. These data indicate that adenine serves as a precursor of the purine moiety of cytokinin molecules and that the cytokinin biosynthetic sites are also located in the shoot in addition to the presumed root sites.

i^{6}Ade,[2] io^{6}Ade, and closely related derivatives constitute a group of plant hormones, the cytokinins, which promote cell division and differentiation. Precursor of the purine moiety of cytokinins has been shown to be Ade (3, 7). Although there is evidence that cytokinin does exist in the root tip, either free (14) or as constituents of tRNA (1, 9, 14), and that cytokinins from xylem exudate may come from the roots (11, 15), the question of whether the intact root tips and/or other plant parts serve as a primary site of cytokinin biosynthesis remains to be answered.

Information about the biosynthesis of cytokinins in roots comes from indirect evidence based on collection of materials by diffusion and extraction together with the bioassay of substances from root exudates. This evidence has been reviewed recently in some detail by Torrey (17).

This paper reports the growth of rootless tobacco plants and the biosynthesis of i^{6}Ade and its derivatives from [18-^{4}C]Ade supplied to the rootless plants. These plants are capable of synthesizing i^{6}Ade and its derivatives from the purine substrate. These results indicate that cytokinin biosynthesis is not restricted to root sites.

MATERIALS AND METHODS

Chemicals and Tissue Cultures. [8-^{14}C]Ade (50 mCi/mmol) was obtained from New England Nuclear Corporation. Ade, i^{6}Ade, and i^{6}Ado were from Sigma Chemical Co.; *trans*-io^{6}Ade, and *trans*-io^{6}Ado were from Calbiochem; *cis*-io^{6}Ade and *cis*-io^{6}Ado were a generous gift from N. J. Leonard (University of Illinois, Urbana). Cytokinin-dependent tobacco callus (*Nicotiana tabacum*, var. Wis. No. 38) was subcultured routinely on a medium (8) with 4.6 μM kinetin and 11.4 μM IAA.

Analytical Techniques. Biosynthesized cytokinins were partially characterized by Sephadex LH-20 columns, paper electrophoresis (Camag TLE Cell, Whatman No. 3MM paper) with 0.05 M Tris-citrate buffer (pH 3.5) at 22 to 25 C, GLC (6) and paper chromatography (Whatman No. 3MM) in a descending fashion in the following solvent systems (v/v): (A) 2-propanol-water-concentrated NH_4OH (7:2:1); (B) ethyl acetate-1-propanol-water (4:1:2); (C) 95% ethanol-0.1 M $(NH_4)_3BO_3$ (pH 9.0) (1:9); (D) 1-butanol-water-concentrated NH_4OH (86:14:5).

Chromatograms were cut into 1-cm sections and placed in vials containing scintillation fluid (4). Radioactivity was measured in a Nuclear-Chicago Unilux II scintillation system. For liquid samples, an aliquot no more than 0.5 ml was added to 10 ml of Bray's solution (2). Counting efficiency of ^{14}C samples was 74% for paper chromatogram sections and 90% for liquid samples.

A Cary model 14 spectrophotometer was used to measure the quantity of purine bases and cytokinins.

Histology. A modified procedure of Johansen (10) was used in histological examination of the rootless plants. The plants harvested from the culture media were rinsed gently in distilled H_2O to remove adherent medium from the stem bases. Stem bases were cut into 1-cm lengths which included any development in the base area except for leaves. The stem bases were dropped immediately into a fixative containing 100 ml of 50% ethanol, 50 ml of formalin, and 50 ml of acetic acid. The fixation was carried out at room temperature for 24 hr.

Dehydration was accomplished using a graded series of ethanol and 1-butyl alcohol washes terminated with 70% ethanol. Infiltration was carried out at 56 C with a series of paraffin-absolute 1-butyl alcohol within a 24-hr period. The infiltrated tissues were then embedded in 100% paraffin in flat paper boats prior to cross-sectioning of stems with an A-O Spencer 820 microtome with injector razor blades. Tissue sections were mounted on slides with Haupt's affixative (10), air-dried, dewaxed in xylene, passed through 95% ethanol, rinsed with double-distilled H_2O, and stained in 1% alcohol-safranin 0 for 4 hr followed by rinsing. Counter-staining was done in fast green-clove oil for 10 sec, followed by a clove oil-absolute ethanol rinse-dehydration. In the final procedure tissue preparations were passed through two xylene rinses and coverslipped.

RESULTS

Growth of Rootless Tobacco Plants. Tobacco callus was subcultured on a high cytokinin-low auxin medium containing 9.4 μM kinetin and 1.14 μM IAA to induce plantlet formation (16). Several plantlets were formed from each callus after 40 to 45 days of incubation. Each plantlet, weighing 0.13 to 0.18 g, was then transplanted to a flask containing 50 ml of "0-1 medium" which lacked both cytokinin and auxin. The transplanted plantlets grew into tobacco plants without roots within 20 to 25 days at room temperature (22–24 C) under fluorescent light. Careful micro-

[1] This work was supported by the National Science Foundation Grant PCM 76-82158 to C.-M. C.

[2] Abbreviations: Ade: adenine; i^{6}Ade: N^6-(Δ^2-isopentenyl)adenine; i^{6}Ado: N^6-(Δ^2-isopentenyl)adenosine; io^{6}Ade: 6-(4-hydroxy-3-methyl-2-butenylamino)purine; io^{6}Ado: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-ribofuranosylpurine.

scopic examination of basal portions of several differentiated shoots confirmed that no root tissue was formed during their cultivation on the 0-1 medium for as long as 48 days (Fig. 1C). However, further incubation of the differentiated plants on the same medium gradually induced root formation, and about 15% of the plants grew roots after transplanted onto the 0-1 medium for about 70 days (Fig. 1D).

Isolation of Biosynthesized Cytokinin. For measurements of cytokinin biosynthesis by the rootless plants, the plants which had been incubated on 0-1 medium for 20 days were again transplanted into fresh 0-1 medium. About 5 nmol (4.7×10^5 cpm) of filtered sterilized [8-^{14}C]Ade was added to each flask of medium before gelation. Alternatively, basal portions of differentiated tobacco plants were removed under sterilized conditions and the rootless

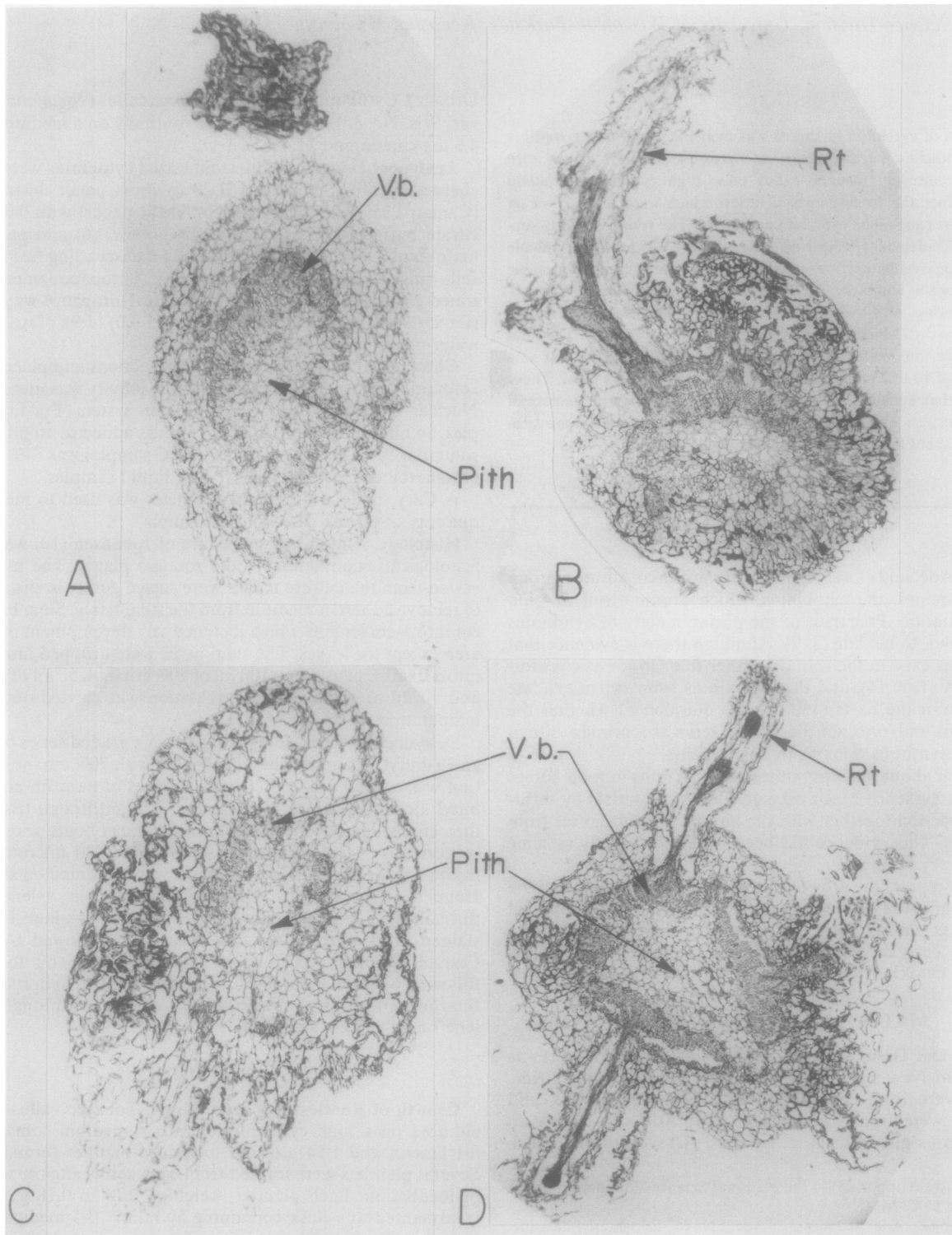

FIG. 1. Transections from stem and root of cultured *N. tabacum*, var. Wis. No. 38. A: stem; B: stem base with growing root; C: rootless stem base, grown on 0-1 medium for 35 days; D: rootless stem base, grown on 0-1 medium for 72 days, showing the formation of roots (Rt) and the presence of vascular bundle (V.b.) (× 17).

shoots were grown on 0-1 medium containing [8-^{14}C]Ade (5 nmol/flask). Each rootless plant or rootless shoot was planted within a flask and 20 flasks were used in each experiment.

After various periods of incubation at room temperature under fluorescent light, plants were harvested and any residual medium attached to the plants was wiped off with Kimwipe paper. The average fresh weight for each rootless plant grown for 17 to 28 days was from 0.33 to 1.63 g, and for each rootless shoot incubated for 23 and 24 days was from 0.59 to 0.64 g, respectively. When the uptake of [8-^{14}C]Ade was compared, the rootless shoots incubated for 24 days had about 2.8-fold greater uptake of radioactivity (0.54 nmol/g fresh weight) than the rootless plants grown for the same period of time (0.19 nmol/g fresh weight).

Radioactive materials in the plants were extracted with 50 and 95% ethanol (20 ml ethanol/g fresh tissue) and with ethyl acetate-water (5:1, v/v; 15 ml/g tissue). Insoluble material was removed by centrifugation. The ethanol, ethyl acetate, and water-soluble fractions were combined, reduced to less than 2 ml in a flash evaporator (38 C), and the final volume was adjusted to 2 ml with water. Aliquots of the extracts were removed and radioactivity was determined using Bray's solution (2). The radioactive materials appearing in the ethanol-ethyl acetate-water-soluble fraction from the rootless plants were about 3 to 5% of the total radioactivity in the medium for the growth period of 17 to 28 days, and from the rootless shoots were about 7% after 24-day incubation.

In order to separate biosynthesized cytokinins, the radioactive materials extracted were initially chromatographed on a Sephadex LH-20 column and eluted with 35% ethanol. Figure 2 shows a separation pattern of the radioactive materials obtained from rootless plants grown 20 days in the presence of [^{14}C]Ade. The results were compared with the relative mobilities of authentic samples (Table I). The three pooled fractions (Fig. 2, F-1 to F-3) from Sephadex LH-20 column were further purified by Whatman No. 3MM paper chromatography. The paper chromatographic comparison with control samples (Table II) led to the localization of i^{6}Ade, i^{6}Ado, io^{6}Ade, and io^{6}Ado which were found in F-2. Other ^{14}C-labeled samples eluted from the Sephadex LH-20 column (F-1 and F-3) have not been identified.

Figure 3A shows a further separation of the pooled F-2 fraction on paper in solvent A system. Four major radioactive bands were obtained. The relative ^{14}C radioactivity of the four major radioactive bands, A-1 through A-4, amounted to 29.9, 9.5, 47.9, and 12.7%, respectively, of the total radioactive counts. The radioactive bands were cut from the paper and eluted with 50% ethanol. The eluates were analyzed by paper chromatography in various solvent systems. The A-4 band, where the major species of cytokinins were expected to be located according to the R_F values of control samples, was further separated into four bands in solvent B system (Fig. 3B). Radioactive materials in the A-1 to A-3 bands were also analyzed by paper chromatography in solvent systems B, C, and D. No known species of cytokinins were found in these radioactive samples.

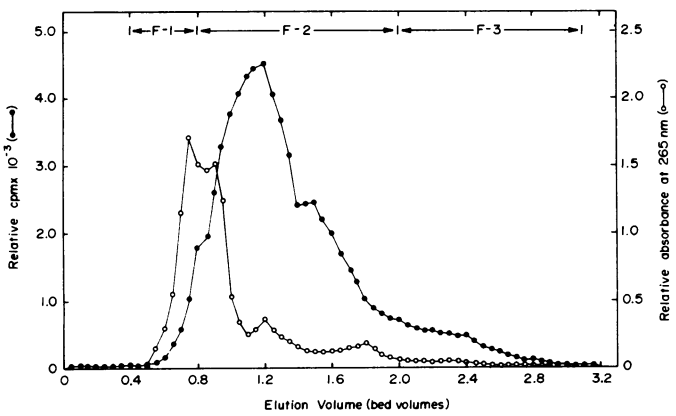

FIG. 2. Elution profile of radioactive materials extracted from cultured rootless plants on Sephadex LH-20 column. Rootless tobacco plants, grown on 0-1 medium containing [^{14}C]Ade for 20 days, were extracted with ethanol and ethyl acetate-water. The ethanol-ethyl acetate-water-soluble fraction (3 ml) was applied onto a column (1.5 × 20 cm) previously equilibrated with 35% ethanol. The radioactive materials were eluted with the same ethanol solution. Elution volume 1.0 represents 1 column volume.

Table I. Relative mobilities of cytokinins and purine derivatives on Sephadex LH-20 columns[a]

Compound	Relative mobility
Ade	1.36
Ado	1.20
i^{6}Ade	1.82
i^{6}Ado	1.70
io^{6}Ade	1.30
io^{6}Ado	1.15

[a]The value 1.0 represents an elution volume equivalent to one column volume. The values given are compiled from several different columns.

Table II. R_F values of cytokinins and purine derivatives[a]

Compound	R_F in various solvents			
	A	B	C	D
i^{6}Ade	0.84	0.88	0.57	0.86
i^{6}Ado	0.85	0.87	0.79	0.83
io^{6}Ade	0.77	0.62	0.63	0.70
io^{6}Ado	0.79	0.41	0.80	0.61
Ade	0.47	0.33	0.42	0.35
Ado	0.52	0.18	0.68	0.30
Guanine	0.12	0.02	0.31	0.02
Guanosine	0.21	0.04	0.56	0.03
Hypoxanthine	0.36	0.12	0.61	0.11

[a]The R_F values were obtained on Whatman No. 3 paper in descending fashion at room temperature. Solvent systems (v/v): (A) 2-propanol-water-concn NH_4OH (7:2:1); (B) ethylacetate-1-propanol-water (4:1:2); (C) 95% ethanol-0.1 M $(NH_4)_3BO_3$, pH 9.0,(1:9); (D) 1-butanol-water-concn NH_4OH (86:14:5).

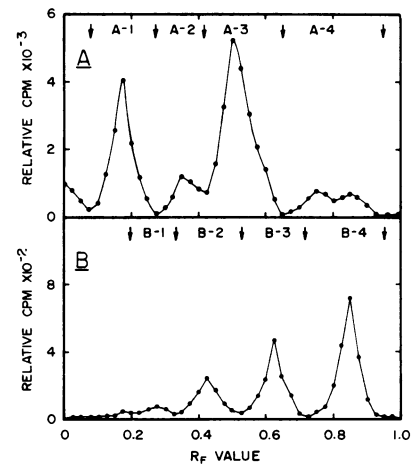

FIG. 3. A: paper chromatographic separation of pooled radioactive fraction F-2 obtained from Sephadex LH-20 column (Fig. 2) in solvent A system. B: further separation of the eluate of radioactive band A-4 (Fig. 3A) in solvent B.

Identification of Cytokinins. The purified suspected cytokinin samples were analyzed by paper and GLC as well as paper electrophoresis. Comparison of the paper chromatographic mobilities of the purified B-1 to B-4 radioactive materials (Fig. 3B) with control samples in solvents A-D (Table II) led to the partial

characterization of io^{6}Ado (B-2), io^{6}Ade (B-3), i^{6}Ade (B-4), and i^{6}Ado (B-4). The compound in B-1 was not identified. The B-4 sample was further separated into three radioactive peaks in the solvent C system by paper chromatographic analysis (Fig. 4). The chromatographic mobility of the C-1 sample corresponded to i^{6}Ade and the C-2 sample was identical to i^{6}Ado. The C-3 sample remains to be identified.

For a further identification, each purified ^{14}C-labeled sample was subjected to GLC analysis. The retention time of the B-2 sample is identical to *trans*-io^{6}Ado and the B-3 sample corresponds to *trans*-io^{6}Ade (Table III). No *cis*-io^{6}Ado or *cis*-io^{6}Ade could be detected in the GLC analysis. Similarly, the chromatographic mobility of the C-1 sample and C-2 sample corresponds to i^{6}Ade and i^{6}Ado, respectively. The purified radioactive samples were also characterized by paper electrophoresis. Each of the partially identified cytokinin samples (about 800–1,000 cpm) was mixed with the corresponding unlabeled authentic cytokinin and spotted on Whatman No. 3MM paper for electrophoresis. The identity of each purified radioactive sample obtained from the electrophoretic analysis (Table IV) agreed with the results derived from GLC characterization. Paper electrophoretic analysis did not distinguish the *cis*- and *trans*-isomers of io^{6}Ade or io^{6}Ado. The quantities of each isolated cytokinin species among the total purified cytokinin samples, analyzed by paper chromatography in three solvent systems (A–C), indicated that i^{6}Ade is about 23 to 32%; i^{6}Ado, 12 to 18%; io^{6}Ade, 26 to 37%; and io^{6}Ado amounted to 9 to 17%. These four cytokinins were also obtained from rootless shoot labeled with [^{14}C]adenine. The biosynthesis of cytokinins was not suppressed by exogenous kinetin (4.6 μM) and auxin (11.4 μM) in the incubation medium.

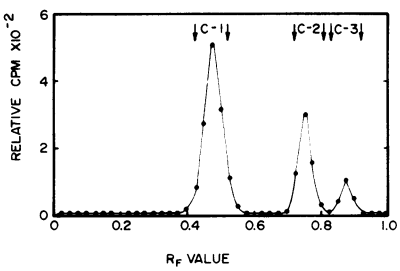

FIG. 4. Further separation of radioactive band B-4 (Fig. 3B) on Whatman No. 3MM paper, solvent C.

Table III. Retention time of trimethylsilyl cytokinins and related compounds

Compound	Retention time (min.)	Relative retention time*
Guanosine	19.50	1.00
Guanine	3.00	0.15
Ade	1.26	0.06
Ado	9.02	0.46
	11.00	0.56
Inosine	9.36	0.48
Hypoxanthine	1.50	0.08
i^{6}Ade	2.75	0.14
i^{6}Ado	21.70	1.11
cis-io^{6}Ade	5.67	0.29
trans-io^{6}Ade	6.93	0.35
cis-io^{6}Ado	40.95	2.10
trans-io^{6}Ado	57.40	2.94
[^{14}C]B-2 sample	57.35	2.94
[^{14}C]B-3 sample	6.91	0.35
[^{14}C]C-1 sample	2.76	0.14
[^{14}C]C-2 sample	21.73	1.11

*Relative retention time refers to the retention time with respect to guanosine. Chromatographic conditions: a 0.3 x 300 cm aluminum column of 2% QF-1 on 80–100 mesh gas chrom Q. Helium flow rate 100 ml/min. Temperature: column at 200 C, inlet at 300 C, and flame ionization detector at 300 C.

Table IV. The relative electrophoretic mobility of cytokinins and some related compounds

Compound	Relative electrophoretic mobility*
Hypoxanthine	-1.0
Ade	-6.4
Ado	-3.2
i^{6}Ade	-4.6
i^{6}Ado	-4.2
cis and trans io^{6}Ade	-5.0
cis and trans io^{6}Ado	-3.6

*Relative electrophoretic mobility refers to the mobility with respect to hypoxanthine. Electrophoresis was carried out on Whatman 3 MM paper in 0.05 M tris-citrate buffer, pH 3.5, for 2 hr at 260 V.

DISCUSSION

The results of this study show that rootless plants and rootless shoots grown in culture medium are capable of synthesizing cytokinins using Ade as a substrate. Our preliminary experiments also indicate that other purine derivatives, such as guanine and hypoxanthine can also serve as substrates for cytokinin biosynthesis. The free cytokinins extracted from rootless plants may be derived from the turnover of cytokinin containing tRNA (5, 12), and/or from the tRNA-free biosynthetic pathway (3) in which purine derivatives combine with isopentenyl side chain to form cytokinins. Although the precursor of the isopentenyl side chain of cytokinin in tRNA has been shown to be mevalonate in higher plants (5, 13), the origin of the isopentenyl side chain for tRNA-free cytokinin biosynthesis in plant systems remains to be determined.

Although our results support the view that cytokinin biosynthesis is not restricted to roots, the anatomical data do not rigorously exclude the possibility of root primordia participation in cytokinin biosynthesis. The finding that cytokinin can also be extracted from rootless shoots in which stem bases were removed suggests that shoots may be functional in cytokinin biosynthesis.

Based upon experiments repeated four times using ^{14}C Ade as a substrate following 17 to 28 days of incubation, the amounts of radioactive cytokinins appearing in the ethanol-ethyl acetate-water-soluble fractions ranged from 14 to 23 pmol/g of plant or about 3 to 5% of the total radioactive materials in the soluble fractions. These values are minimal since the dilution factor resulting from endogenously synthesized adenine and cytokinins is unknown.

Cytokinins occur in high levels in meristematic tissues (17). It remains to be determined whether the actively dividing meristematic tissues of the rootless plants serve as the major site for cytokinin biosynthesis. If these tissues are indeed the cytokinin biosynthetic site, then all actively dividing plant cells may be the source of cytokinins. The rootless plants appear to offer an excellent source of materials from which to study plant hormone biosynthetic sites as does the quiescent center in the root (18).

Acknowledgment—The authors wish to thank N. J. Leonard of the University of Illinois, Urbana, for gifts of *cis*-io^{6}Ade and *cis*-io^{6}Ado.

LITERATURE CITED

1. BABCOCK DF, RO MORRIS 1970 Quantitative measurement of isoprenoid nucleosides in transfer ribonucleic acid. Biochemistry 9: 3701-3705
2. BRAY G 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal Biochem 1: 279–285
3. CHEN C-M, RL ECKERT 1976 Evidence for the biosynthesis of transfer RNA-free cytokinin. FEBS Lett 64: 429–434
4. CHEN C-M, RL ECKERT 1977 Phosphorylation of cytokinin by adenosine kinase from wheat germ. Plant Physiol 59: 443–447
5. CHEN C-M, RH HALL 1969 Biosynthesis of N^6-(Δ^2-isopentenyl)adenosine in the transfer ribonucleic acid of cultured tobacco pith tissue. Phytochemistry 8: 1687–1695
6. CHEN C-M, GF HARTNELL, OC SMITH, WS FRANCIS 1974 Quantitative measurement of

hypermodified adenosine ribonucleoside in plant tissues. Anal Biochem 60: 441–448
7. Einset JW, F Skoog 1973 Biosynthesis of cytokinins in cytokinin-autotrophic tobacco callus. Proc Nat Acad Sci USA 70: 658–660
8. Fox JE 1964 Indoleacetic acid-kinetin antagonism in certain tissue culture systems. Plant Cell Physiol 5: 251–254
9. Hall RH 1970 N^6-(Δ^2-Isopentenyl)adenosine: chemical reactions, biosynthesis, metabolism and significance to the structure and function of tRNA. *In* JN Davidson, WE Cohn, Eds, Progress in Nucleic Acid Research and Molecular Biology, Vol 10. Academic Press, New York, pp 57–86
10. Johansen DA 1940 Plant Microtechnique. McGraw-Hill, New York, pp 126–154
11. Kende H, D Sitton 1967 The physiological significance of kinetin and gibberellin-like root hormones. Ann NY Acad Sci 144: 235–243
12. Klemen F, D Klämbt 1974 Half-life of sRNA from primary roots of *Zea mays*. A contribution to the cytokinin production. Physiol Plant 31: 186–188
13. Murai N, DG Armstrong, F Skoog 1975 Incorporation of mevalonic acid into ribosylzeatin in tobacco callus ribonucleic acid preparations. Plant Physiol 55: 853–858
14. Short KC, JG Torrey 1972 Cytokinins in seedling roots of pea. Plant Physiol 49: 155–160
15. Skene KGM 1968 Increase in the level of cytokinins in the bleeding sap of *Vitis vinifera* L. after CCC treatment. Science 159: 1477–1478
16. Skoog F, CO Miller 1957 Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp Soc Exp Biol 11: 118–131
17. Torrey JG 1976 Root hormones and plant growth. Annu Rev Plant Physiol 27: 435–459
18. Torrey JG, Feldman LJ 1977 The organization and function of the root apex. Am Sci 65: 334–344