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N^6-(Δ^2-Isopentenyl)adenosine: Its Occurrence as a Free Nucleoside in an Autonomous Strain of Tobacco Tissue[1, 2]

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ABSTRACT

Cytokinins from both the free nucleoside pool and the transfer RNA have been isolated and identified in a habituated strain of tobacco pith callus (*Nicotiana tabacum* [L] var. Wisconsin 38). The transfer RNA of this strain contains both N^6-(Δ^2-isopentenyl)adenosine and N^6-(4-hydroxy-3-methylbut-2-*cis*-enyl) adenosine. The *trans*-hydroxylated derivative is absent from the transfer RNA of this dark-grown tissue. N^6-(Δ^2-Isopentenyl)-adenosine was identified as a component of the free nucleoside pool in concentrations of about 10 micrograms per kilogram of tissue.

The cytokinin-requiring tissue grown in the presence of N^6-furfurylaminopurine contains less than 85 nanograms per kilogram of N^6-(Δ^2-isopentenyl)adenosine. This is the detectable limit under the conditions of the experiment.

Cytokinins are compounds that stimulate cell division and differentiation in plants. A series of cytokinins occurs naturally, of which the archetype molecule is Δ^2-iA[3] (see reviews by Skoog and Armstrong [27] and Fox [12]). The tRNA of every species investigated contains Δ^2-iA and/or closely related derivatives. The tRNA of higher plants, for example, in addition to the archetype molecule, can contain a hydroxylated derivative, *cis*-RZ (see review by Hall [14]). This hydroxylated derivative appears to be restricted to the tRNA of higher plants. In addition, the 2-methylthio derivatives of both the parent compound (Δ^2-iA) and the hydroxylated derivative have been isolated from the tRNA of wheat germ (6).

The *trans*-isomer of ribosylzeatin (*trans*-RZ) and/or its free base and nucleotide have been found as free compounds in chicory roots (5), corn kernels (19, 20), coconut milk (18), and as an excretory product of the fungus, *Rhizopogon roseolus* (21). Its immediate precursor in *R. roseolus* is Δ^2-iA (22), but nothing has been reported about its biosynthesis in higher plants.

The tRNA of a cytokinin-requiring strain of tobacco callus tissue (*Nicotiana tabacum* [L] var. Wisconsin 38) contains Δ^2-iA and ribosylzeatin (7). Chen and Hall (8) showed that the Δ^2-iA of the tRNA of this tissue is synthesized by attachment of a Δ^2-isopentenyl side chain to the preformed tRNA. The requirement for the exogenous cytokinin does not seem to relate directly to this biosynthetic mechanism. In search, therefore, of leads that bear on the mechanism of action of cytokinins we decided to compare the Δ^2-iA content of an autonomous line derived from the Wisconsin 38 strain of tobacco pith callus tissue (11) that grows well in the absence of exogenously added cytokinins. We have found that the tRNA of this strain also contains Δ^2-iA and *cis*-RZ in its tRNA, but in contrast to the cytokinin-requiring strain the autonomous tissue contains a significant level of Δ^2-iA in the free state.

MATERIALS AND METHODS

Tissue Culture Strains. Normal tobacco pith tissue has an absolute requirement of added cytokinins for growth (11). Occasionally this normal tissue spontaneously loses its cytokinin requirement. Such autonomous strains of tissue presumably meet their cytokinin requirement through an alteration in some metabolic process, although the mechanism(s) of this transformation is not known.

The normal cytokinin dependent tobacco pith tissue (*Nicotiana tabacum* [L] var. Wisconsin 38) and the derived autonomous strain used in this study were gifts from J. E. Fox of the University of Kansas. The normal pith tissue was grown on a previously described (11) basal medium containing naphthaleneacetic acid (1 mg per liter) and 6-furfurylaminopurine (kinetin) (1 mg per liter). The autonomous strain was grown on the same basal medium without the growth factors. Tissues were harvested after 4 to 5 weeks growth in the dark.

Bioassay. Aliquots of column fractions to be bioassayed for cytokinin activity were added to 25-ml flasks and dried *in vacuo* over P_2O_5. A basal medium (11), complete except for cytokinin, was added (10 ml per flask), and the flasks were autoclaved at 18 lb, 121 C for 5 min. A cytokinin requiring strain of soybean cotyledon tissue (*Glycine max* [L.] Merril, var. Acme) was planted (0.5 mg pieces, three per flask), and after 30 days incubation in the dark at room temperature, the increase in fresh weight was compared to controls grown in the presence (1 μM) or absence of kinetin.

tRNA Nucleosides. tRNA was isolated from the autonomous tobacco tissue by a method previously described (9), except that the filtrate was used directly without cold storage. Cytokinin ribonucleotides were released from the tRNA by alkaline hydrolysis (0.3 N KOH for 24 hr at 37 C). The hydrolysate was treated with bacterial alkaline phosphatase (Worthington Biochemicals) at pH 8.6 in 50 mM $MgCl_2$ at 37 C for 24 hr in the presence of toluene to yield the nucleosides.

[1] This work was supported by a grant from the Canadian Cancer Society.

[2] A preliminary report of this work was presented at the 1971 American Society of Plant Physiologists meeting, Asilomar, Calif.

[3] Abbreviations: Δ^2-iA: N^6-(Δ^2-isopentenyl)adenosine; *cis*-RZ: *cis*-ribosylzeatin, [N^6-(4-hydroxy-3-methylbut-2-*cis*-enyl)adenosine]; *trans*-RZ: *trans*-ribosylzeatin [N^6-(4-hydroxy-3-methylbut-2-*trans*-enyl)adenosine]; Δ^3-iA: N^6-(Δ^3-isopentenyl)adenosine; GLC: gas-liquid chromatography; TMS: trimethylsilyl; BSTFA: *N,O*-bis(trimethylsilyl)trifluoracetamide (Regis Chemical Co.).

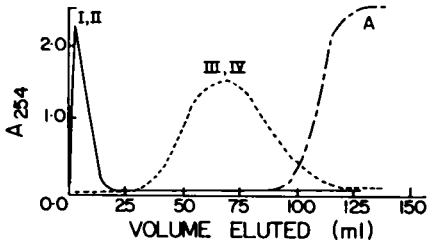


FIG. 1. Partition chromatography of structurally related cytokinin nucleosides on a column of 40 g of Celite 545 utilizing a solvent system of ethyl acetate:1-propanol:H_2O, 4:1:2 (see text for details). The peak numbers correspond to the structures in the text. A is adenosine.

Partition Chromatography. The partition columns were constructed and developed as described by Hall (13) and Robins *et al.* (26). Each column used in the experimental isolations was identical to a standardized column (2 cm diameter precision bore) packed with 40 g of Celite-545[4] coated with 19 ml of the lower phase of ethyl acetate:1-propanol:H_2O, 4:1:2, v/v. The coated Celite was packed into the column in layers by using a Teflon plunger on the end of a steel rod. Samples were dissolved in 4 ml of the lower phase of the solvent, mixed well with 8 g of Celite-545, and packed on top of the column. The column was developed by passage of the upper phase of this solvent at 28 C; flow rate was 30 ml/hr. This partition column is particularly useful for handling samples too crude for paper, thin-layer or gas chromatography and gives reproducible results. Figure 1 shows the separation on the standard column of some purine cytokinins (Scheme I, II, III, and IV) from each other and from adenosine. The non-hydroxylated forms (Δ^2-iA [II] and Δ^3-iA [I]) separate from the hydroxylated derivatives (*cis*- [III] and *trans*- [IV] RZ) and the bulk of the adenosine elutes after *cis*- and *trans*-RZ.

Gel Filtration. Armstrong *et al.* (2) described the use of Sephadex LH-20[5] column chromatography as an analytical and preparative tool for the isolation and identification of cytokinins. We used two standardized column systems. The first system consisted of a column (2.5 cm diameter) of 50 g of Sephadex LH-20 swollen in 35% ethanol. Samples (0.5 ml) were applied to the top of the column and separated by passage of 35% ethanol by gravity flow (28 cm head) at a rate of 0.66 ml per min. The second standardized column system was identical except 20% ethanol was used as the solvent. Elution with 20% ethanol under gravity flow (19 cm head) gave a flow rate of 0.75 ml per min.

The column effluents were monitored for ultraviolet absorption at 254 nm using an Isco UA-2 ultraviolet analyzer with recorder. Teflon and glass tubing were used exclusively.

Figure 2 shows the separation of various cytokinins on the 20% ethanol column. This system resolves a mixture of Δ^2-iA (II) and Δ^3-iA (I), in addition to those separations reported previously (2). The *cis*- (III) and *trans*- (IV) isomers of ribosylzeatin are not resolved with this system.

Gas-Liquid Chromatography. The use of GLC for the separation of purine cytokinins is now well documented (1, 3, 23, 28). The techniques used in this study were similar to those of Alam and Hall (1). All procedures were performed on a Hewlett-Packard Model 402 dual column gas chromatograph equipped with flame-ionization detectors. The detector signals were recorded on a Hewlett-Packard Model 7127A recorder equipped with an integrator for determination of peak areas.

[4] Johns-Manville Corporation brand of diatomaceous earth.
[5] Pharmacia brand of hydroxypropylated dextran gel beads.

For separation of extraction products the columns (0.3 cm diameter × 120 cm U-shaped silanized glass) were packed with 100 to 120 mesh Gas-Chrom Q (Kenscott Ltd., Toronto) coated with DC-11 (Dow-Corning silicone grease) (10% w/w). A carrier gas (He) flow of 35 ml per min at an oven temperature of 250 C (isothermal) was used.

All authentic markers and extraction products were prepared in a uniform manner. Samples were dried *in vacuo* over P_2O_5 in a 0.3-ml reaction vessel, sealed with a Teflon-covered screw-cap and dissolved in 10 μl of dry pyridine injected through the cap. The TMS derivative was prepared by the injection of 20 μl of BSTFA followed by incubation at 65 C for 5 min. Aliquots (1–5 μl) were then withdrawn for injection onto the column. Figure 3 shows the separation of structurally related cytokinins on this column.

Samples were collected for further analysis in capillary tube traps, cooled by liquid nitrogen, introduced into the collection port through a Teflon sleeve. The TMS derivatives were hydrolyzed to the original nucleoside with 0.01 N acetic acid for 10 min at 27 C prior to mass spectral analysis.

Mass Spectroscopy. Mass spectroscopy was carried out on a

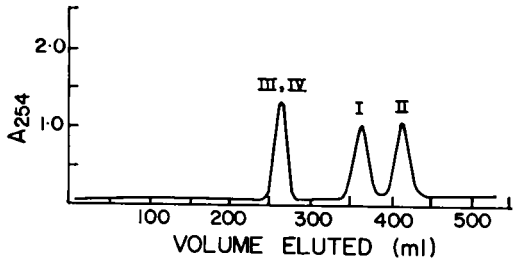
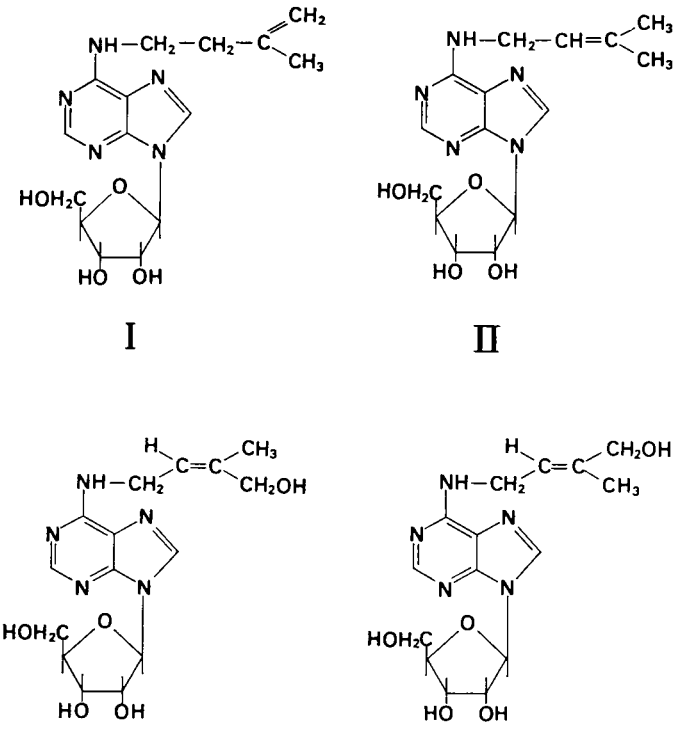


FIG. 2. Elution profile of a mixture of cytokinins (see text for structures) on a column of Sephadex LH-20 (50 g, 37 × 2.5 cm) eluted with 20% ethanol at a flow rate of 0.75 ml per min.



CEC 21 Model B-110 mass spectrometer at 70 ev. The samples were introduced by a direct insertion probe.

Ion-Exchange Chromatography. The collection of purine base and ribonucleoside cytokinins by ion-exchange chromatography was described by Miller (20, 21), who also recognized the possibility of degradation arising from the production of heat during elution steps (20). To overcome these problems in our study, special techniques were used to prevent the accumulation of heat during elution.

Free cytokinins were collected by passing an ethanolic solution (60%), pH 5.5, through a column of Dowex-50W × 8, 50 to 100 mesh in the cold (4 C). After loading, the resin was removed from the column and transferred to a beaker chilled by ice. The resin was washed with ice cold 50% ethanol, and the washings were discarded. A solution of 3 N NH_4OH at 0 C was added slowly while stirring constantly to minimize heat accumulation. The temperature of the resin was maintained below 5 C. When the resin was neutralized, it was poured back into the column and eluted with an additional quantity of cold 3 N NH_4OH. All basic effluents were collected. If this procedure was not followed, the heat generated during neutralization of the resin would cause hydrolysis of purine nucleosides.

Solvents. All solvents used in this study were distilled within 2 weeks of use. Chromatographic solvents were mixed within 24 hr of use.

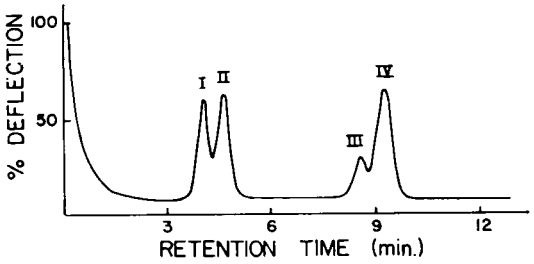


FIG. 3. GLC separation of a mixture of TMS derivatives of structurally related cytokinins (peak numbers correspond to structures in text) on a column of 10% DC-11 on 100 to 200 mesh Gas Chrom Q in a 0.3- × 120-cm glass column at an oven temperature of 250 C (isothermal) with a carrier gas (He) flow of 35 ml per min.

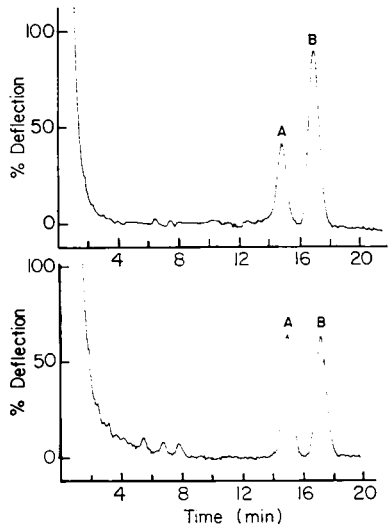


FIG. 4. Gas chromatographic separation of *cis*- and *trans*-isomers of commercial RZ (top) on a glass column (0.3 nm × 6 ft) of 5% DC-11 on Gas Chrom Q (100–200 mesh), carrier (He) flow at 100 ml per min at 252 C, isothermal. Bottom shows commercial sample coinjected with *cis*-RZ from TLC separation.

Cytokinin Standards. Starks Associates (Buflalo) synthesized the Δ^2-iA; Δ^3-iA and *trans*-RZ were a gift from Dr. H. Vorbrügen of the Schering AG. The synthesis of purine ring labeled [8-^{14}C] Δ^2-iA is described elsewhere (24). A sample of ribosylzeatin was purchased from Calbiochem. Babcock and Morris (3) previously demonstrated that this commercial preparation is a mixture of 30% *cis*- and 70% *trans*-RZ. Our sample also separated into two components by GLC (Fig. 4). The mass spectra of the two fractions were each consistent with published spectra of ribosylzeatin, differing only in the minor peaks at *m/e* 192, 228, and 331. These differences were attributed by Hall (15) as being due to the *cis* or *trans* configuration of the hydroxyl. In order to confirm our configurational assignments of these two compounds, we compared their relative R_F values on silica gel thin layers developed in 9:1, chloroform:methanol. Playtis and Leonard (25) reported that the *cis* isomer migrated slightly faster than the *trans* isomer in this system. Our GLC peak A (Fig. 4, top) migrated slightly ahead of peak B, R_F values 0.28 and 0.21 respectively. Conversely, GLC analysis of the components of the original sample separated first by TLC, revealed that the isomer with the higher R_F enhanced peak A of the commercial sample (Fig. 4, bottom) thus establishing our GLC peak A as the *cis*-isomer of ribosylzeatin.

Comparison of the areas encompassed by peaks A and B of the commercial sample of ribosylzeatin confirm the previous report (3) that this sample consists of 30% *cis*- and 70% *trans*-RZ.

EXPERIMENTAL

Cytokinins in Autonomous Tobacco Pith Tissue. Dark-grown autonomous tobacco tissue (10 kg) was homogenized in a Waring Blendor at high speed for 1 min at 0 to 4 C with 50 liters of 95% ethanol. The precipitate and cell debris were separated from the ethanolic solution by filtration. The collected solids served as a source of tRNA, and the ethanolic solution was used for determination of free cytokinins.

tRNA. tRNA (900 $A_{260\,nm}$ units) was isolated from the solids by a method previously described (9). This isolation was nonquantitative, and the product was contaminated with carbohydrate and various nucleic acid fragments. Alkaline hydrolysis released the nucleotides which were degraded to nucleosides by the action of bacterial alkaline phosphatase. Cytokinin nucleosides were separated from the bulk of the common nucleosides by partition chromatography on Celite-545.

The Celite fraction corresponding to the position of Δ^2-iA was collected and purified on a column of Sephadex LH-20 in 35% ethanol. Individual fractions were analyzed by gas chromatography as described, and in those fractions where Δ^2-iA is known to elute, a compound was found having a GLC retention time identical to that of an authentic sample of Δ^2-iA (Fig. 5). Based on a comparison of peak area with standard samples, the tRNA sample corresponds to about 12 μg of Δ^2-iA in the Sephadex LH-20 fraction. A dropping base line on the GLC profile in the area of Δ^2-iA prevented quantitative assessment.

In view of the fact that the elution characteristics of Δ^2-iA on both the Celite-545 and Sephadex LH-20 columns were previously standardized, we considered these chromatographic data sufficient demonstration of the identity of Δ^2-iA in the tRNA.

Cis-RZ was detected in the second fraction from the Celite-545 column. The material in this fraction was analyzed as above on a Sephadex LH-20 column. Individual fractions

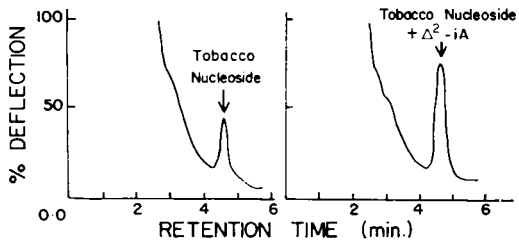


FIG. 5. Gas chromatographic detection of Δ^2-iA from tRNA hydrolysate. Left: GC of presumed Δ^2-iA from tRNA hydrolysate; right: coinjection of authentic Δ^2-iA with sample from left. See text and Figure 3 for operating parameters.

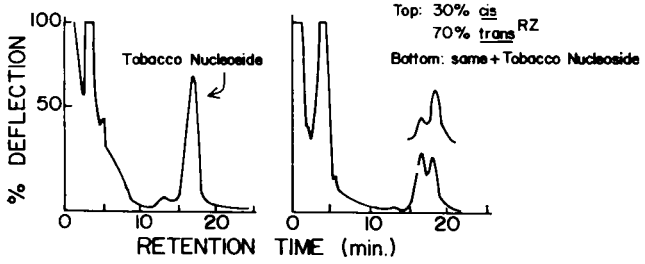


FIG. 6. Gas chromatographic detection of *cis*-RZ from tRNA hydrolysate. Left: GC of presumed *cis*-RZ from tRNA hydrolysate; right: Coinjection of presumed *cis*-RZ with a mixture of *cis*- and *trans*-RZ.

were analyzed by gas chromatography and, in the position of the elution profile where ribosylzeatin is known to elute, a compound was found having a GLC retention time identical to that of *cis*-RZ (Fig. 6). Coinjection of the tobacco nucleoside with an authentic *cis, trans* mixture of ribosylzeatin shows that the tRNA nucleoside is the *cis*-isomer (Fig. 6, right). The mass spectrum of this nucleoside is identical with that of previously characterized *cis*-RZ (15), with an apparent molecular ion at *m/e* 351 and other diagnostic peaks at *m/e* 334, 320, 262, 248, 219, 202, 188, 160, 148, and 136. The total amount of this hydroxylated derivative recovered from the hydrolysate was 30 μg, as determined by comparison of the gas chromatographic peak area with standards containing known quantities of *cis*-RZ.

The Soluble Fraction. The initial phase of the extraction procedure involved the collection of ribonucleoside and purine base cytokinins by passage of the cold ethanol extract obtained above through a Dowex 50 column (900-ml bed volume) as described in "Materials and Methods."

The ammonium hydroxide eluate (4 liters) was evaporated to a volume of 300 ml in a rotary evaporator *in vacuo*. The aqueous solution was extracted five times with equal volumes of water saturated *n*-butanol, and the alcohol soluble material was fractionated by partition chromatography on the standard Celite-545 column described above. The first 110 ml of the eluate was collected. The contents of the fraction were refractionated on a Sephadex LH-20 column (Fig. 7). Aliquots of the individual Sephadex fractions were analyzed for cytokinin activity using the micro-soybean bioassay. The areas of cytokinin activity, superimposed against an elution profile, are shown in Figure 7. The first area (fractions 10–12) was not investigated further. The second area (fractions 16–18) has a mobility in this system similar to that of ribosylzeatin, although its presence could not be confirmed by gas chromatography.

The third and major region of cytokinin activity (fractions 24 to 29) has an elution volume similar to that of Δ^2-iA. This area was refractionated on a second column of Sephadex LH-20 using 20% ethanol as the eluent (Fig. 8). The elution profile shown in Figure 8 is again consistent with the presence of Δ^2-iA. Further, the cytokinin activity is destroyed by permanganate oxidation, indicating the presence of a double bond in the molecule.

An aliquot of the fraction having cytokinin activity was analyzed by gas chromatography (Fig. 9). The result shows that a compound is present having a retention time identical to that of authentic Δ^2-iA. The TMS derivative collected from the gas chromatograph was hydrolyzed and a mass spectrum obtained. The tobacco-soluble cytokinin has a mass spectrum (Fig. 10) identical to that of Δ^2-iA (26). By comparison of the GLC peak areas with those of Δ^2-iA standards, a total of 50 μg of Δ^2-iA

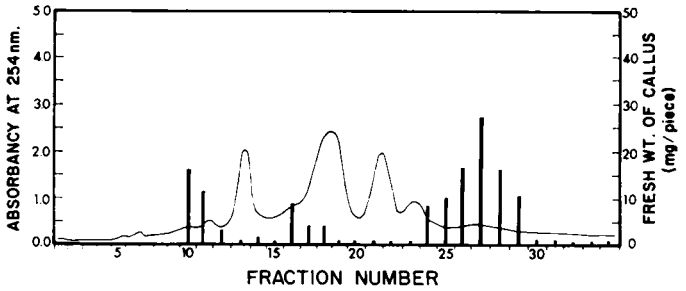


FIG. 7. Sephadex LH-20 separation of ethanol soluble nucleosides and bases. The column (50 g, 35 × 2.5 cm) was eluted with 35% ethanol at 0.66 ml per min. Twelve-ml fractions were collected. The solid line indicates ultraviolet absorbancy and vertical bars indicate cytokinin activity, tested at a level equivalent to 100 kg tissue per liter medium in a micro-soybean assay.

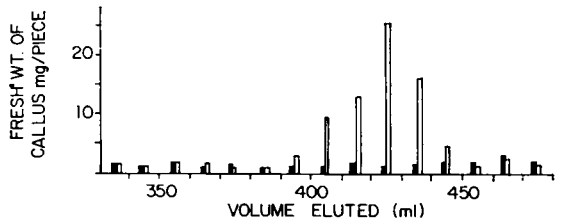


FIG. 8. Sephadex LH-20 profile of major cytokinin from Figure 7. The column (50 g, 37 × 2.5 cm) was eluted with 20% ethanol at 0.75 ml per min. Open bars indicate cytokinin activity before permanganate oxidation, solid bars represent cytokinin activity after permanganate oxidation.

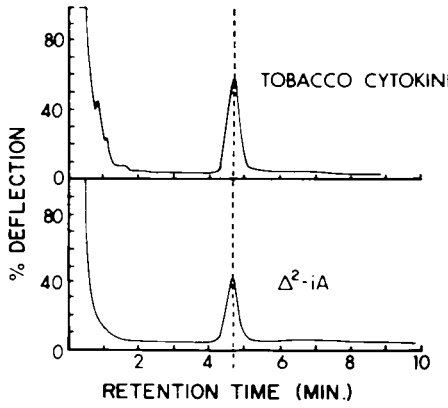


FIG. 9. Gas chromatographic detection of Δ^2-iA from the soluble fraction of tobacco. See text and Figure 3 for operating parameters. Top: GC analysis of Sephadex fractions having cytokinin activity (Fig. 8); bottom: retention time of authentic Δ^2-iA.

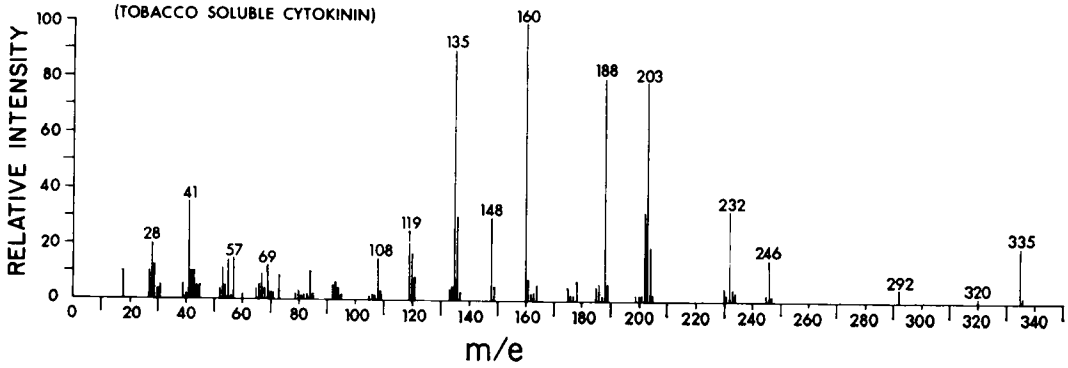


FIG. 10. Mass spectrum of tobacco soluble cytokinin from Figure 9, top. Spectrum obtained at 70 ev, direct inlet, 185 C. The molecular ion is *m/e* 335, with loss of CH_3 at 320, loss of $C(CH_3)_2$ and H at 292. The free base is 203, loss of CH_3 at 188 and adenine at 135. The most abundant species, 160 is due to loss of ribosyl and $C(CH_3)_2$ from Δ^2-iA.

was recovered from the ethanol-soluble pool of the autonomous tobacco. This nucleoside was detected by gas chromatography on six separate lots of autonomous tobacco, on amounts of tissue ranging from 250 g to 15 kg. In four lots of tissue exceeding 8 kg per lot, the amount of Δ^2-iA recovered ranged from 4 to 5.5 μg per kg of tissue. To estimate the yield, 10,000 dpm of [8-^{14}C]Δ^2-iA (specific radioactivity 5 mc per mmole) (24) was added to the ethanolic extract from 8 kg of tissue, and extracted in the same manner. On the basis of radioactivity (4800 dpm) recovered from the Sephadex LH-20 column (20% ethanol), we estimate the recovery to be 50% for larger lots of tissue. A similar procedure resulted in recovery of only 20% of the added radiolabeled Δ^2-iA from smaller lots of tissue (250 g).

Based on this estimate of the recovery, the concentration of Δ^2-iA in the soluble pool is 5 to 10 μg per kg of tissue (wet weight), equivalent to an average intracellular concentration of about 30 nM.

Soluble Fraction of Normal Tissue. The normal cytokinin-requiring tobacco pith callus (11.5 kg, grown in the presence of 0.5 mg/l 6-furfurylaminopurine) was treated in a manner identical to that used for the autonomous tissue. Gas chromatographic analysis of the corresponding Sephadex LH-20 fractions revealed the presence of a small amount of material having a retention time identical to Δ^2-iA. The amount present was insufficient to obtain a mass spectrum. If this material is indeed Δ^2-iA, then the peak area is equivalent to 0.2 μg, or about 17 ng per kg of tissue. Assuming the poorest yield observed above (20%), the concentration of free Δ^2-iA in the normal tissue could not exceed 85 ng per kg of tissue, which is equivalent to an average intracellular concentration of 0.25 nM.

DISCUSSION

The occurrence of free cytokinins in plant tissue has been the subject of considerable research, since it is felt that production and possible circulation of this hormone is necessary for growth and development of plant tissue (see reviews by Kende [16] and Skoog and Armstrong [27]). The only free cytokinins found in plant tissue to date are the hydroxylated derivatives of Δ^2-iA: *trans*-RZ and the corresponding base and ribonucleotide and dihydrozeatin. These compounds have been isolated from developing fruit (17–19) and roots of chicory plants (5). The occurrence of Δ^2-iA in the free state in the autonomous strain of tobacco tissue represents a departure from these previous findings.

The estimated level of Δ^2-iA in the autonomous tissue is about 30 nM (assuming it is evenly distributed in the tissue) and this would be of sufficient magnitude to provide the cytokinin requirements (based on the exogenous needs of the normal tissue). The genotype of this tissue is probably the same as that of the normal tissue; in any case, the normal tissue must contain the genetic information necessary for production of the endogenous cytokinin (*i.e.*, Δ^2-iA), but this information is suppressed.

This interpretation does not necessarily imply that it is a question of production or nonproduction of Δ^2-iA. It could be a question of degree. We were able to obtain an indication that Δ^2-iA is present in the normal parent tobacco strain. The amount found was at the limit of our detection procedure, so we cannot be sure of this result. Nevertheless, the level would be less than 1% of the level in the autonomous strain.

The term "normal tissue" is used to define the strain of pith tissue Wisconsin 38 that requires cytokinin. It may not be normal in the sense that it resembles the tissue of an intact plant. Nevertheless, this tissue and its autonomous variant represents an experimental model that may provide some clues as to the significance of cytokinins in growth and development. Braun (4), referring to the crown gall system, comments that production in greater than regulatory amounts of growth substances could account for unregulated and autonomous proliferation.

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