Proc. Nat. Acad. Sci. USA
Vol. 70, No. 3, pp. 658–660, March 1973

Biosynthesis of Cytokinins in Cytokinin-Autotrophic Tobacco Callus

(6-Δ^2-isopentenylaminopurine/zeatin)

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Contributed by Folke Skoog, December 22, 1972

ABSTRACT **A cytokinin-autotrophic strain of tobacco callus contained cytokinin-active compounds with chromatographic mobilities on Sephadex LH-20 corresponding to ribosylzeatin, zeatin, and 6-Δ^2-isopentenylaminopurine. Zeatin, the apparent major cytokinin, was estimated to be present at a concentration of 10^{-4} µmol/kg of tissue. Cytokinin-autotrophic callus supplied with [^{14}C]adenine produced radioactive components with the same chromatographic properties as zeatin and 6-Δ^2-isopentenylaminopurine. These components were not obtained from cytokinin-dependent tissue supplied with [^{14}C]adenine in the same manner.**

Plant tissues that grow *in vitro* in the absence of exogenous cytokinins presumably synthesize adequate endogenous amounts. This paper reports the presence of cytokinins in a cytokinin-autotrophic strain of tobacco callus and the biosynthesis of cytokinins from exogenous adenine by this tissue.

MATERIALS AND METHODS

The cytokinin-autotrophic strain, a variant isolated in 1968 from cytokinin-dependent tobacco callus (*Nicotiana tabacum*, cv Wis. no. 38) and subcultured since then on RM 1965 medium (1) supplemented with 11.4 µM indole-3-acetic acid but without exogenous cytokinin, was kindly provided by R. Y. Schmitz. Cytokinin-dependent tobacco callus that had been subcultured routinely on the same medium supplemented with indole-3-acetic acid and kinetin, as auxin and cytokinin, respectively, and grown for the final passage on this medium with 6-benzylaminopurine (5×10^{-2} µM) instead of kinetin, was used for controls.

Both the normal tobacco callus (1) and the cytokinin-autotrophic strain required auxin and thiamine for continuous growth in subcultures and also needed *myo*-inositol for rapid growth *in vitro* (Table 1).

Cytokinins were extracted as follows: 30 kg of 25-day-old cytokinin-autotrophic tissue were homogenized for 5 min in 95% ethanol (2 liters/kg) to give a final ethanol concentration of 70%, and insoluble material was removed by filtration. After the pH was adjusted to 2.5, the ethanol-soluble fraction was applied to Dowex 50-X4 (H^+) columns (50 ml of resin per kg of tissue), which were eluted with 7.5 liters of water and then with 7.5 liters of 1 N NH_4OH. The NH_4OH eluate

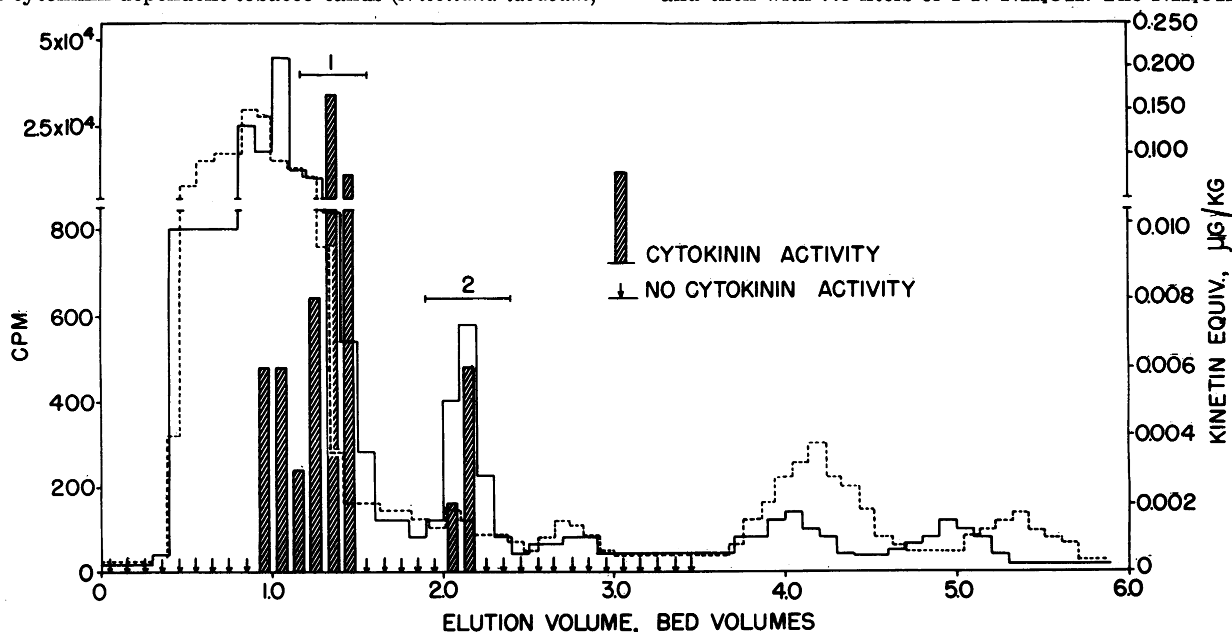

FIG. 1. Fractionation of cytokinins from cytokinin-autotrophic tissue on a Sephadex LH-20 column. *Bars* indicate cytokinin activity in the tobacco bioassay. Column (25 g, 2.2 × 32.7 cm) was eluted with 35% ethanol; sample volume was 2 ml. *Solid line* shows radioactive products from cytokinin-autotrophic tissue (17.1 g) incubated for 5 hr with 2 µM [^{14}C]adenine. Column (5 g, 1.0 × 26 cm) was eluted with 35% ethanol; sample volume was 2 ml. The *dashed line* shows radioactive products from cytokinin-dependent tissue (10.3 g) incubated and fractionated in the same way. *Bars 1* and *2* correspond to the elution volumes of zeatin and 6-Δ^2-isopentenylaminopurine, respectively, from the smaller column.

was evaporated at 37° to 3.5 liters, the pH was adjusted to 2.5, and the resulting solution was passed through Dowex 50-X4 (H^+). The columns were eluted in turn with 7.5 liters of water, 5 liters of 70% ethanol, and 7.5 liters of 1 N NH_4OH. The dried residue of the ammonia eluate was extracted with water-saturated ethyl acetate. Cytokinin-active compounds in this extract were separated on a Sephadex LH-20 column with 35% ethanol as eluent (2).

For measurements of adenine utilization in cytokinin biosynthesis, tissue from 25- to 27-day-old cultures was transferred from stock medium to 50 ml of liquid RM 1965 medium with 11.4 μM indole-3-acetic acid and specified amounts of [8-^{14}C]adenine (New England Nuclear Corp., 50 Ci/mol). The tissue was incubated on a reciprocal shaker and then extracted with ethanol as described above. The ethanol extract was chromatographed on a Sephadex LH-20 column eluted with 35% ethanol (2).

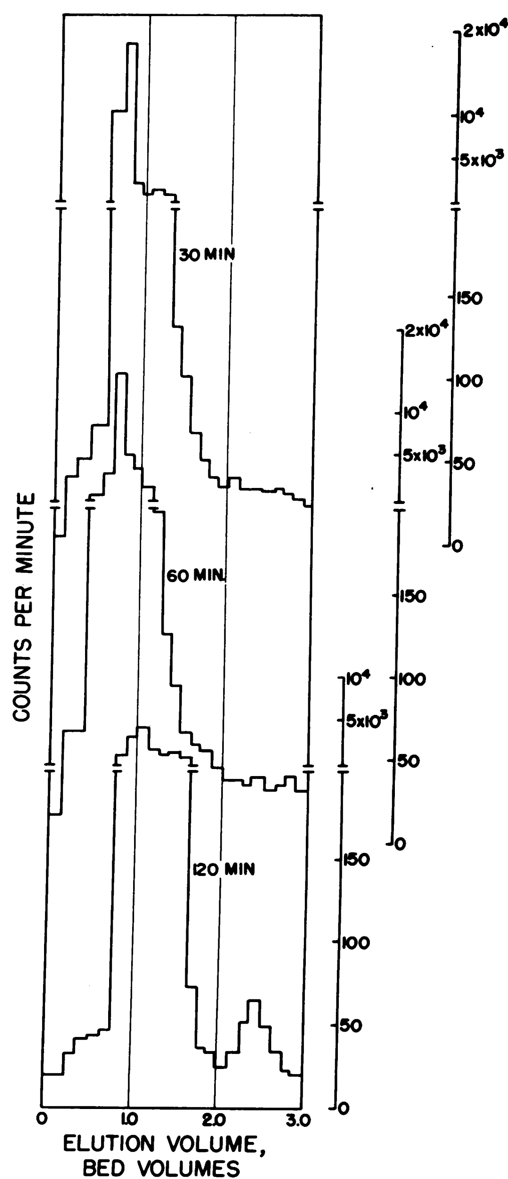

FIG. 2. Time course of cytokinin biosynthesis. Incubation media contained 0.4 μM [^{14}C]adenine. Other experimental conditions were identical to those described in the legend to Fig. 1. 30 min, 4.0 g; 1 hr, 3.4 g; 2 hr, 3.8 g.

TABLE 1. *Fresh weight yields of cytokinin-autotrophic tobacco callus on media with and without growth factors* (growth period 1/29–3/10/71).

Added growth factors (μM)				
Indole-3-acetic acid	Thiamine	*myo*-Inositol	6-Δ^2-Isopentenylaminopurine	Yield* (g per flask fresh wt)
0	0	0	0	0.03
0	1.2	550	0	0.02
11.4	0	550	0	0.85
11.4	1.2	0	0	3.08
11.4	1.2	550	0	9.45
0	0	0	0.5	0.04

* Mean of 10 flasks.

RESULTS

Cytokinin synthesis in tobacco callus tissue

The bioassays showed cytokinin activity in two regions of the Sephadex LH-20 chromatogram of cytokinin-autotrophic tissue (Fig. 1). The first active region, from 0.9 to 1.5 bed volumes, contained more than 90% of the cytokinin activity in the extract. It had a minor peak between 0.9 and 1.2 bed volumes, the elution volume of ribosylzeatin, and a major peak between 1.3 and 1.5 bed volumes, the elution volume of zeatin. The second cytokinin-active region, from 2.0 to 2.2 bed volumes, corresponded to the elution volume of 6-Δ^2-isopentenylaminopurine. Since no cytokinin activity was detected in the region from 3.5 to 6.0 bed volumes in a preliminary experiment, these fractions were not assayed.

The distinct peak of radioactivity corresponding to the elution volume of 6-Δ^2-isopentenylaminopurine in the profile from cytokinin-autotrophic tissue that was not found in the profile from cytokinin-dependent tissue indicates that this cytokinin was synthesized from the labeled adenine by the autotrophic tissue. In spite of the lack of resolution in the region corresponding to zeatin, the marked difference between the radioactivity profiles of cytokinin-autotrophic and cyto-

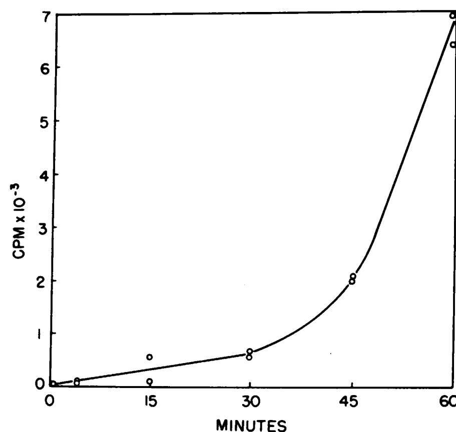

FIG. 3. Synthesis of trichloroacetic acid-insoluble compounds in cytokinin-autotrophic tissue. 20 g of tissue incubated with 2 μM [^{14}C]adenine was treated as described in the *Methods*. The ethanol-insoluble fraction suspended in Tris buffer (pH 7.6) was mixed with an equal volume of cold 5% trichloroacetic acid, and the radioactivity in the precipitate was determined.

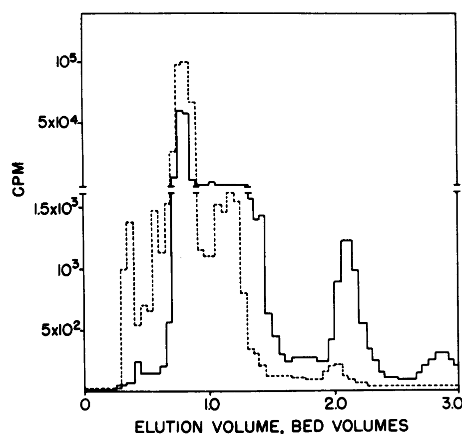

FIG. 4. Inhibition of cytokinin synthesis by actinomycin D. Incub tion media contained 4 μM [^{14}C]adenine. Other conditions were the same as those desc ibed in the legend to Fig. 1. Control flask (*solid line*) had 12.3 g of tissue; treated flask (*dashed line*) contained 15 mg/liter of actinomycin D and 12.9 g of tissue

kinin-dependent tissues indicates that also zeatin was synthesized from labeled adenine by the autotrophic tissue.

Time course of cytokinin biosynthesis

As shown in Fig. 2, labeled cytokinin became detectable in the second hour of incubation. Even when the concentration of labeled adenine and the quantity of tissue were both increased 5-fold, there still was no detectable free cytokinin after 1 hr (unpublished results). Fig. 3 shows that labeled RNA appears earlier than labeled free cytokinin, which rules out the possibility that the time lag in cytokinin synthesis was due solely to poor penetration of adenine into the cells.

Inhibitory effect of actinomycin

Actinomycin D (15 mg/liter) inhibited synthesis of cytokinin and reduced the radioactivity in the RNA to about 1/3 the amount in controls. The absence of peaks corresponding to the two cytokinins in tissue treated with actinomycin D is clearly shown in Fig. 4. The total radioactivity in RNA from tissue homogenate treated with phenol and fractionated with 1 M NaCl (3) was 10,920 cpm in the tRNA-enriched fraction and 13,870 cpm in the rRNA fraction for tissue treated with actinomycin D. The corresponding values for the control tissue were 27,400 cpm and 43,180 cpm, respectively.

DISCUSSION

The presence of zeatin and 6-Δ^2-isopentenylaminopurine was indicated by the biological activity as well as by radioactivity localized in the elution volume corresponding to the authentic compounds. Estimates of the tissue contents based on bioassays of 30 kg of cytokinin-autotrophic tissue grown on semisolid medium gave values of about 10^{-4} μmol/kg zeatin and 10^{-5} μmol/kg 6-Δ^2-isopentenylaminopurine. Estimates based on radioactivity measurements on tissues together with incubation medium after 5 hr of exposure to radioactive adenine gave values up to 10^{-3} μmol of 6-Δ^2-isopentenylaminopurine per kg of tissue. In each case the amounts were too minute for rigorous chemical identification. Both cytokinins have been reported earlier as constituents of tRNA in cytokinin-dependent (4) and cytokinin-autotrophic (5) tobacco callus. N^6-Δ^2-isopentenyladenosine was not found but has been reported for another cytokinin-autotrophic strain of tobacco callus (6). The presence of ribosylzeatin was suggested by biological activity in the region corresponding to the elution of the authentic compound but could not be confirmed in the radioactivity profiles. Possibly it was masked by high counts from other adenine derivatives eluting in the same region. These results indicate the presence of cytokinin-active purines in amounts adequate for the growth of the autotrophic strain of tobacco callus but do not provide an answer to the question of whether or not the free cytokinins are produced by the suggested pathway that includes synthesis and degradation of cytokinin-containing tRNAs (7).

This work was supported in part by NSF Grant GB-35260X.

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