CYTOKININS: FORMATION OF THE NUCLEOSIDE-5'-TRIPHOSPHATE IN TOBACCO AND *ACER* CELLS

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1. Introduction

Cytokinins are N^6-substituted derivatives of adenine which promote cell division in plants. Knowledge of the mechanisms of regulation of the internal levels of these plant hormones and their metabolites will constitute progress in understanding of their mode of action.

Recent studies of the metabolism of cytokinins by various plant systems have shown that on a 'short term basis', the main metabolite formed is the nucleoside-5'-monophosphate [1–5] but in two cases [3,6] the presence of nucleoside-5'-di- and triphosphates has been suspected.

We wish to report the formation of these 5'-ribonucleotides when a naturally occuring cytokinin N^6(Δ^2-isopentenyl) adenosine [7], and an artificial cytokinin N^6-benzyladenine are given to tobacco cells and *Acer pseudoplatanus* cells cultivated in liquid medium.

2. Materials and methods

2.1. *Cytokinins*

N^6 (Δ^2-Isopentenyl) adenosine-[8-^{14}C] (50 μCi/μMole) was synthesized from adenosine-[8-^{14}C] and purified according to Pacès et al. [8]. Synthesis of N^6-benzyladenine-[8-^{14}C] (11.5 μCi/μmole) and N^6-benzyladenine α-^{14}C (0.4 μCi/μmole) has been previously described [9]. Authentic i^6 Ado and i^6 Ade were gifts from Dr R. H. Hall, N^6-benzyladenine was obtained from Sigma and N^6-benzyladenosine was synthesized as described [10].

2.2. *Culture strains*

Suspensions of *Acer pseudoplatanus* cells were grown as previously described [11]. Two cell lines of tobacco cells (Wisconsin 38) were used (cytokinin requiring and non-requiring cell lines) [12].

2.3. *Enzymatic tests*

Calf intestinal alkaline phosphatase and pyruvate kinase were purchased from Boehringer-Mannheim. *Crotalus adamanteus* 5'-nucleotidase and purified potato apyrase grade I with 5'-ATPase, 5'-ADPase, 5'-AMPase activities (2.1, 0.35, 0.03 units/mg of protein respectively) were obtained from Sigma.

Alkaline phosphatase, pyruvate kinase and 5'-nucleotidase assays were performed as described [13]. The apyrase action on the cytokinin nucleoside-5' di- and triphosphates obtained was studied as follows: 250 000 cpm of the nucleotide fraction were incubated in 500 μl of 0.1 M Tris–maleate buffer, pH 6.4, with 0.25 units of enzyme activity based on ATPase activity.

2.4. *Isolation of cytokinin metabolites*

After incubation, the cells were collected by centrifugation, fixed with an equal volume of 1 N cold perchloric acid and sonicated three times for 30 sec. At this point, 300 μg each of ATP, inosine, adenosine and of the corresponding cytokinin base and riboside were added as markers for the column chromatography step.

The markers also prevented losses by adsorption by the cell debris and perchlorate potassium salt pellets. The homogenate was left for 1 hr at 2°C in order to inactivate completely phosphatase activity [14]. After centrifugation the pellet was reextracted twice with an equal volume of cold 1 N perchloric acid. The com-

bined perchloric acid extracts were neutralised to pH 5 with KOH. The mixture was centrifuged and the supernatant was evaporated to a small volume (0.5 ml) at 25°C under reduced pressure. The residual perchlorate potassium salt which had precipitated was washed twice with 0.5 ml of 20% ethanol and discarded. The extract was then made 20% with respect to ethanol and analysed by Sephadex LH_{20} column chromatography. This type of column chromatography is often used for analysis of cytokinins at the level of the base and nucleoside [15].

Bieleski's method [14] using methanol–chloroform–formic acid–water (12:5:1:2, v/v) to inactivate phosphatase activity was also tried. No significant difference could be found with the perchloric acid extraction but the method appeared less convenient as some extracted lipophilic materials interfered on the Sephadex LH_{20} column.

2.5. *Paper chromatography*

Paper chromatography was performed in the descending manner on Whatman No. 1 paper (see table 1 for solvent systems and R_f). Paper electrophoresis was run using a 0.05 M Tris–citrate buffer, pH 3.5, on Whatman 3 MM at 70 V/cm^{-1}.

Table 1
Paper chromatography data

Compound		R_f in solvent systems		
		A	B	C
Adenosine		0.29	0.59	0.54
Inosine		0.06	0.45	0.49
i^6 Ado		0.80	0.89	0.82
i^6 Ade		0.84	0.91	–
	Product 1		0.82	
i^6 Ado				
	Product 2		0.68	
BAPR		0.80		0.82
BAP		0.84		–
i^6 AMP		0.00		0.71
i^6 ADP		–		0.40
i^6 ATP		–		0.13
AMP		–		0.34
ADP		–		0.18
ATP		–		0.08

Solvent systems: A *n*-butanol–NH_3–H_2O, 86:5:11, v/v. B 2-propanol–NH_3–H_2O, 7:2:1, v/v. C *t*-butanol–HCOOH–H_2O, 20:5:8, v/v.

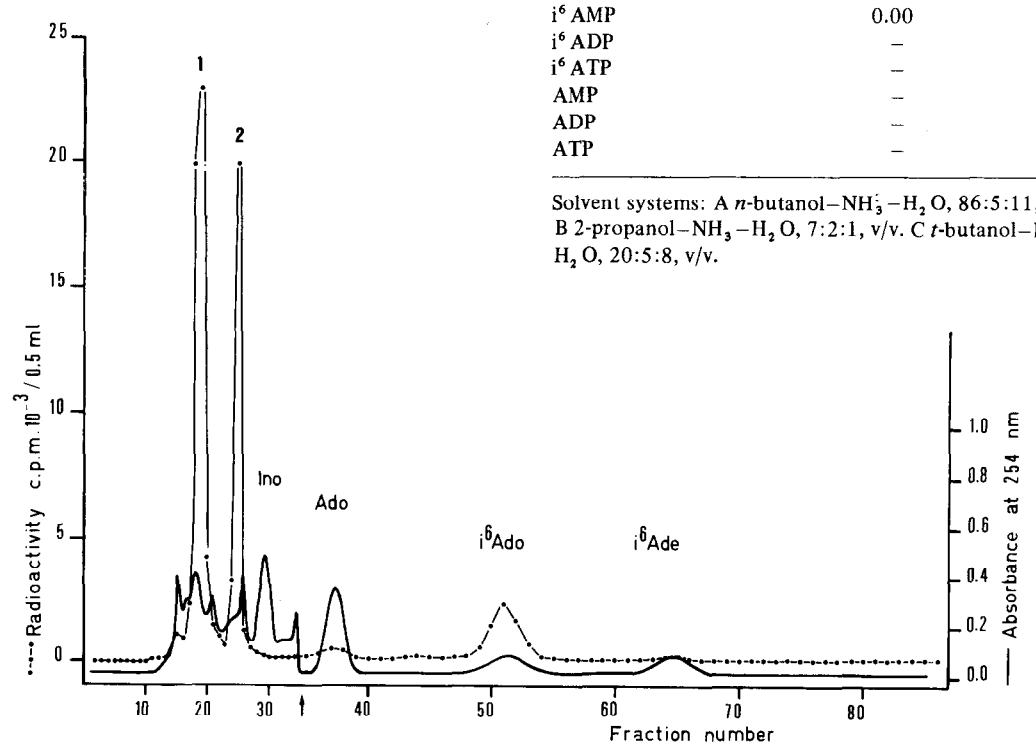

Fig. 1. i^6-Ado metabolites extracted from tobacco cells (see text). Separation by Sephadex LH_{20} column (2.5 × 42 cm) equilibrated and eluted with 20% ethanol–5.5 ml fractions were collected up to fraction 34 (↑), then 16.5 ml fractions were collected. N^6 (Δ^2-isopentenyl)adenosine-5′-diphosphate and triphosphate (peak 1) elute with the common purin nucleotides while N^6 (Δ^2-isopentenyl)adenosine-5′-monophosphate (peak 2) is retained on the column.

3. Results

3.1. *i^6Ado nucleotides in tobacco cells*

8-^{14}C-i^6 Ado was given at a concentration of 10^{-6} M for 3 hr to 25 ml of tobacco cells suspension (cytokinin requiring strain previously grown on i^6 Ado at the concentration of 6.25×10^{-8} M and used at the cell density of 600 000 cells ml^{-1}). The profile obtained on LH_{20} chromatography is shown in fig. 1.

3.1.1. Peak 1

The material from peak 1 was treated as follows: (a) After alkaline phosphatase action it gave i^6 Ado* and 3–4% of inosine separated by paper chromatography. The recovered 8-^{14}C-i^6 Ado was characterized as such by its two characteristic degradation products [16] obtained upon mild acid hydrolysis together with 200 μg of authentic i^6 Ado; (b) peak 1 contained two main products separated by paper chromatography and paper electrophoresis. The relative mobilities observed (fig. 2a) compared to those of ATP and ADP were suggesting the peak 1 contained 55% of i^6 ATP and 41% of i^6 ADP; (c) this mixture was submitted to the apyrase action and a transient accumulation of diphosphate and monophosphate nucleotides (fig. 3) was observed. The degradation proceeded slowly to the ribonucleoside level because the commercial apyrase preparation contained some 5′-monophosphatase activity. Although the apyrase is unspecific for the organic moiety, its action indicates the presence of triphosphate and diphosphate i^6 Ado nucleotide esters in peak 1; (d) this mixture was also incubated with pyruvate kinase in the presence of phosphoenol pyruvate. The data (fig. 2b) show that the diphosphate nucleotide was fully phosphorylated to the triphosphate level.

3.1.2. Peak 2

The material from peak 2 was treated with alkaline phosphatase and *Crotalus adamanteus* 5′-nucleotidase and gave i^6 Ado in both cases. The product from peak 2 was homogenous after paper chromatography in several solvent systems and had the same mobility as AMP on paper electrophoresis.

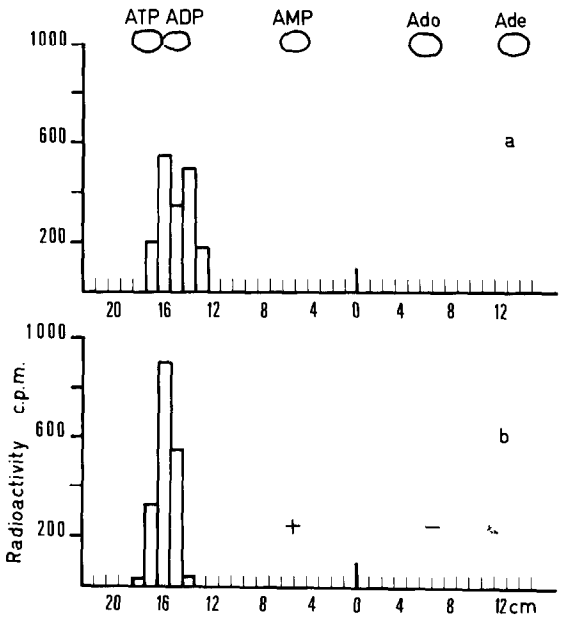

Fig. 2. Electrophoretic separation at pH 3.5 of N^6 (Δ^2-isopentenyl)adenosine-nucleotides: a) separation of the mixture of i^6 ADP and i^6 ATP from peak 1 (fig. 1); b) i^6 ADP is converted to i^6 ATP after 10 mn of incubation with pyruvate kinase in the presence of phosphoenolpyruvate (see Materials and methods).

All these criteria indicate that the nucleotides obtained were respectively the 5′-triphosphate, diphosphate and monophosphate nucleotides of i^6 Ado. The yield of these nucleotides represented 77% of the total extracted radioactivity. This was probably underestimated as i^6 Ado still represented 18% of the radioactivity and might have not been fully taken up as the cells were not washed prior to perchloric acid extraction. The base represented less than 0.5% of the total radioactivity. A similar pattern was observed in the non cytokinin-requiring tobacco cells.

3.2. *i^6Ado nucleotides in Acer cells*

These cells are not cytokinin dependant. Under the incubation conditions described above, a peak of i^6 ADP and i^6 ATP was obtained after Sephadex LH_{20} column chromatography. In this case, several nonnucleotidic products were observed but not characterized. The

Abbreviations: BAP: N^6-benzyladenine; BAPR: N^6-benzyladenosine; i^6 Ade: N^6 (Δ^2-isopentenyl)adenine; i^6 Ado: N^6 (Δ^2-isopentenyl) adenosine; i^6 AMP: N^6 (Δ^2-isopentenyl) adenosine-5′-monophosphate, etc.; Ado: adenosine; Ino: inosine.

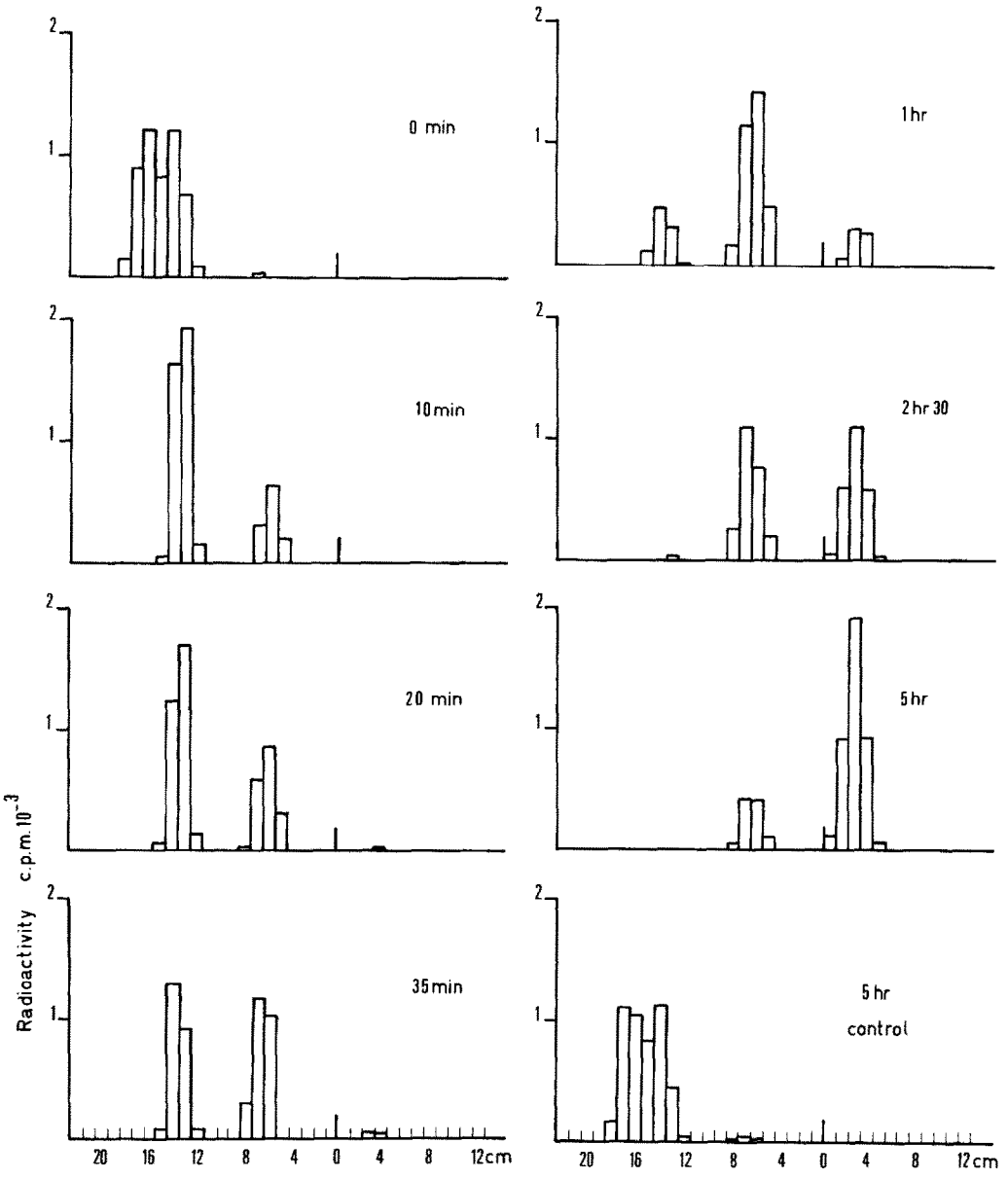

Fig. 3. Electrophoretic separation at pH 3.5 of the products obtained from i^6ATP and i^6ADP incubated in the presence of apyrase (see Material and methods). Control: incubation without enzyme.

pooled nucleotidic fractions represented only 60% of the extracted radioactivity, with the following distribution: i^6AMP 72%, i^6ADP and i^6ATP 16%; inosine 5'-nucleotides 12%.

3.3. *Benzyladenosine 5'-nucleotides*

The metabolism of N^6-benzyladenine was studied under the same conditions using tobacco cells. After 3 hr of incubation, a similar pattern of metabolites was observed for the purine ring or for the side-chain labelled benzyladenine. 30% of the extracted radioactivity remained as the free base, but no benzyladenosine was detected. 6-benzyl-AMP represented 28% and 6-benzyl ADP with 6-benzyl ATP 6% of the recovered radioactivity.

The fact that a nucleotidic fraction phosphorylated at the di- and triphosphate level was obtained with side-chain labelled benzyladenine is another criteria for the existence of cytokinin nucleotides di- and triphosphate, as no interference with labelled classical purine nucleotides could be possible.

4. Discussion

These results showed that exogenously supplied cytokinins (BAP, i^6Ado), either at the base or nucleoside levels were metabolised to 5′-nucleotides. The existence in vivo of cytokinin nucleosides-5′mono- di- and triphosphate was demonstrated by the following criteria: (i) sensitivity to alkaline phosphatase and 5′-nucleotidase giving back the cytokinin nucleoside; (ii) electrophoretic mobilities of 3 anionic products at pH 3.5 similar to the electrophoretic mobilities of AMP, ADP and ATP; (iii) sensitivity of the triphosphate and diphosphate nucleotides to apyrase; (iv) phosphorylation of the diphosphate nucleotide to the triphosphate level in presence of pyruvate kinase. In the case of BAP, labelled either in the side-chain or the purine ring, the same chromatographic profiles were obtained.

The isolation of the di- and triphosphate cytokinin nucleosides was made possible by the use of column chromatography analysis which separated the cytokinin nucleoside 5′-monophosphate from all the common purine nucleotides and by the use of strains of the Tobacco cells which did not degrade appreciably the N^6-side-chain. *Acer* cells, in comparison, appear to remove the side-chain. These observations may explain why the di- and triphosphate nucleotides could not be isolated in a previous study [4] in our laboratory.

The existence in vivo of cytokinin nucleoside 5′-triphosphate is of theoretical importance as they could be incorporated in tRNA molecules. There has been some controversy upon the possible incorporation [17,18] of cytokinins in tRNA, although evidence against incorporation has been presented [19]. Cytokinins do exist in specific tRNA molecules in which they occur adjacent to the 3′ end of the anticodon [20]. Since it was shown that their biosynthesis is part of the general process of maturation of tRNA molecules (the isopentenyl group is attached in situ to the specific adenosine residue [21] the incorporation of cytokinins into tRNA molecules seems unlikely.

Nevertheless, our results show the importance of cytokinin nucleotides. Most of the isolation procedure and identification of free cytokinins in plants or plant tissues (see review by Hall [22]) have been made at the base or nucleoside levels, but not enough attention has been paid to cytokinin nucleotides, although ribosyl-zeatin-5′-monophosphate has been isolated [23].

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