Plant Physiol. (1980) 65, 1090–1095
0032-0889/80/65-1090/06/$00.50/0

Kinetics of N^6-(Δ^2-Isopentenyl)Adenosine Degradation in Tobacco Cells

EVIDENCE OF A REGULATORY MECHANISM UNDER THE CONTROL OF CYTOKININS

Received for publication October 9, 1979 and in revised form January 14, 1980

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ABSTRACT

Uptake and degradation of the cytokinin, N^6-(Δ^2-isopentenyl) adenosine, were studied in tobacco cells grown as cell suspensions. Degradation occurs by cleavage of the isopentenyl chain which gives adenylic products. Rate of N^6(Δ^2-isopentenyl)[8-^{14}C]adenosine degradation increases several-fold after a 3- to 4-hour delay when cells have been exposed to a cytokinin. Consequently, only rates of N^6-(Δ^2-isopentenyl)adenosine degradation measured during the first 3 hours of incubation with [8-^{14}C]-N-^{6}(Δ^2-isopentenyl)adenosine are representative of the intrinsic *in vivo* cytokinin degradative activity of tobacco cells. Within these limits, it appears that cytokinin degradative activity is high in cytokinin-autonomous tobacco cells, as indicated by the half life of the supplied N^6(Δ^2 isopentenyl adenosine (about 3 hours) when it is supplied at the physiological concentration of 0.2 micromolar. This cytokinin degradative activity appears to be under the control of cytokinins themselves because N^6-(Δ^2-isopentenyl)adenosine degradative activity is increased several-fold following a 3- to 4-hour delay after these cells have been exposed to a cytokinin.

Studies of cytokinin metabolism in plant cells, tissues and organs have established that adenine and/or adenosine and/or adenylic nucleotides are catabolic products of zeatin (12), BA (3), i^6Ade[1] or i^6Ado (8, 10, 11). These observations indicate that cytokinin degradation occurs by side-chain cleavage in a manner specific for plants because in animal systems i^6Ado is shown to be converted to inosine (5, 6).

Presumably, irreversible inactivation of cytokinins by degradation plays an important role in the expression of their biologic activities in plants, but to our knowledge, no quantitative study of cytokinin degradation in plants has been reported. Pacès *et al.* (11) detected an enzymic activity in crude extracts of tobacco tissues which catalyzed conversion of i^6Ado to adenosine. Whitty and Hall (14) partially purified such an enzymic activity from corn kernels which they termed cytokinin oxidase. This enzymic activity removed the Δ^2-isopentenyl chain as the aldehyde from either N^6-(Δ^2-isopentenyl)-substituted adenine or adenosine (1). It is not known, however, if degradation of cytokinin *in vivo* occurs specifically at the level of the base, riboside or ribotide(s). Occurrence of cytokinin activity in cytokinin-autonomous plant tissues grown *in vitro* has been demonstrated (2, 4, 13). Cytokinin-autonomous cells apparently produce enough cytokinins to meet their own requirements for cell division, either by increased cytokinin biosynthesis or by reduced cytokinin degradation, as compared to cytokinin-requiring cells. In a previous paper (10), we reported that i^6Ado is rapidly degraded in cytokinin-requiring tobacco cells. To evaluate the second hypothesis, we further studied the degradation of i^6Ado supplied to suspensions of cytokinin-requiring and cytokinin-autonomous cells.

We describe herein a simplified assay of i^6Ado degradation *in vivo*. Quantitative aspects of i^6Ado degradation are presented and discussed. The data suggest that cytokinin degradation is promoted by cytokinins themselves.

MATERIALS AND METHODS

Tobacco Cells. Two cell lines of tobacco cells (*Nicotiana tabacum*, Wisconsin 38) originally cloned by Tandeau de Marsac and Jouanneau (13) were used. Cell suspensions were subcultured biweekly in liquid medium according to Jouanneau and Péaud-Lenoël (7). Cells from the cytokinin-independent line (clone 13) were grown in the presence of 0.75 μM 2,4-D, and cells from the cytokinin-requiring line (clone 19) were grown in medium containing 0.2 μM 2,4-D and 0.06 μM BA. Cell population density measurements were made as previously described (10). Except when otherwise stated, 5-day-old 100-ml cultures were used which corresponded to exponentially growing cell suspensions.

Cytokinins. N^6-(Δ^2-isopentenyl)[8-^{14}C]adenosine with a specific radioactivity of 46.5 μCi/μmol. was synthesized from [8-^{14}C]adenosine according to Pacès *et al.* (11) and purified by Sephadex LH-20 column chromatography. It was stored as aqueous mM solution at −18 C and repurified periodically. All experiments were achieved with [8-^{14}C]i^6Ado of a radiochemical purity better than 99%. N^6-Benzyl-[8-^{14}C]adenine with a specific radioactivity of 20 μCi/μmol was purchased from Amersham (England). Authentic i^6Ado and i^6Ade were gifts from R. H. Hall. BA was from Sigma.

Incubation Conditions. Depending upon the volume of cell suspension required, several 100-ml cell cultures were pooled when necessary. Cell population density of the cell suspension was adjusted to about 10^5 cells/ml by adding or removing the required volume of medium after measurement of the wet packed cell volume of cells of 10-ml aliquots of the cell suspension collected in 12-ml graduated conical centrifuge tubes and centrifuged for 5 min at 700*g*. Approximately 10^5 cells of tobacco cell line 13 corresponded to a wet packed cell volume of 0.12 ml and a fresh weight of 40–45 mg. In the case of cells from cell line 19, 10^5 cells corresponded to 0.08 ml wet packed cells and 25 mg fresh weight. Labeled i^6Ado was generally added at a final concentration of 1 μM to 100-ml aliquots of cell suspensions agitated in 1-liter Fourneau flasks placed in a water bath reciprocal shaker (26 C, 75

[1] Abbreviations: i^6Ade: N^6-(Δ^2-isopentenyl)adenine; i^6Ado: N^6-(Δ^2-isopentenyl)adenosine; i^6AMP, i^6ADP, i^6ATP: N^6-(Δ^2-isopentenyl)adenosine-5′-mono-, di-, and triphosphate; Ado: adenosine; Ade: adenine.

cycles/min). Its absorption was calculated from the disappearance of radioactivity in the culture medium.

Radioactivity Measurements. All radioactivity measurements were made by liquid scintillation spectrometry. Aliquots from culture medium or from fractions obtained after Sephadex LH-20 column chromatography were counted in 6 ml of Bray's solution. Radioactivity in the paper chromatograms was measured as follows: the chromatogram strip 2–3 cm wide was cut in 1-cm-wide bands and each piece was put in a counting vial with 6 ml toluene containing 5 g/liter PPO and 0.3 g/liter POPOP. Radioactivity was measured with an Intertechnique spectrometer, model SL 4220. In the case of culture media, counting efficiency was automatically monitored and corrected by the channel ratio method and results were directly expressed in dpm.

Extraction, Separation and Characterization of i^6Ado Metabolites[2]. Cells were fixed and extracted with 1 N cold $HClO_4$ and separation of i^6Ado metabolites was achieved by Sephadex LH-20 column chromatography according to a procedure previously described (8, 9). Characterization and identification of these metabolites were reported in previous communications (8–10). Briefly, i^6AMP, Ado, Ade, i^6Ado, and i^6Ade were separated from each other by this first chromatographic step. The first radioactive fraction eluted from the column contained i^6ADP, i^6ATP, adenylic nucleotides and a small amount of zeatin ribotide(s) which were dephosphorylated with potato apyrase and 5′-nucleotidase (10). The resulting nucleoside mixture was resolved by paper chromatography as previously described (9, 10). The relative amounts of adenosine and i^6Ado allowed the calculation of the initial amounts of i^6ADP + i^6ATP and adenylic nucleotides. Ribosylzeatin was not considered as it represented less than 2% of this fraction.

Direct I^6Ado Degradation Assay. We had shown previously (9) that 7-glucosylation of i^6Ade prevents removal of the Δ^2-isopentenyl chain, therefore, we preferred to measure the degradation of i^6Ado which was shown to be not significantly converted to N^6-(Δ^2-isopentenyl)adenine-7-glucoside.

Inasmuch as there were at least 10 different products (base, riboside and riboside-5′-monophosphate, di- and triphosphates of the cytokinin and the purine, respectively) without considering zeatin ribotide(s), and no convenient technique was available to separate AMP, ADP, ATP, and the corresponding i^6Ado nucleotides, we chose to convert all i^6Ado metabolites to the riboside level prior to analysis by using the phosphatase activities present in tobacco cells. i^6Ado and i^6Ade are easily separated from adenosine and adenine by paper chromatography.

The protocol follows. At intervals after addition of [8^{14}C]i^6Ado, 1- or 2-ml aliquots of cell suspensions were injected into 5 ml of ice-cold water, then the mixture was filtered through a GF/A filter using a Millipore filtration device. Cells were scraped off the filter and homogenized at 4 C in a Potter Elvehjem homogenizer with 2 ml of 0.01 M citrate-NaOH (pH 5.5) containing 1 μmol mercaptoethanol, 100 μg Ado, and 100 μg i^6Ado/ml. The homogenate was transferred to a 6-ml disposable test tube and stored overnight at −18 C.

Next day, the homogenates were incubated at 35 C for 8 h in order to convert all cytokinin metabolites and purine metabolites to either i^6Ado and i^6Ade or Ado and Ade. Incubation was stopped by addition of 2 volumes of 95% ethanol. After centrifugation, the pellet was reextracted two times with 1 ml 70% ethanol. The pellet which contained about 2% of the total radioactivity of the homogenate was discarded. The combined ethanolic supernatants were evaporated to almost dryness in a rotary film evaporator under reduced pressure and the residue was dissolved in 200 μl 50% ethanol. These extracts were chromatographed. As a control to check the validity of the procedure we made cell homogenates of both lines and added [8-^{14}C]AMP. After 8 h of incubation at 35 C, 98% of the substrate was converted to adenine. In contrast, when [8-^{14}C]i^6Ado was added under these conditions, 97% remained unchanged.

Cytokinin degradation was measured by the quantity of degradation products formed in tobacco cells calculated from the total amount of intracellular radioactivity plus the corresponding percentage of radioactivity on the chromatogram strip which was no longer associated with i^6Ado.

RESULTS

Kinetics of I^6Ado Uptake and Degradation in Tobacco Cells. Figure 1 represents the time course of i^6Ado uptake and degradation in tobacco cells in a typical experiment. During the first 3 h, i^6Ado accumulated in the cells. Degradation continuously increased with time after the first 3 h, as shown by the amount of degradation products, while the rate of uptake decreased. As a result of these two phenomena, i^6Ado accumulated at a slower rate than during the initial phase and finally the intracellular pool of i^6Ado and i^6Ado metabolites began to decrease when rate of degradation exceeded rate of uptake towards the end of the incubation period.

In order to assess i^6Ado degradative activity *versus* time, rates of i^6Ado degradation were determined graphically from the tangent to the curve which represents the amount of degradation products formed *versus* time. During the first 3–4 h of incubation, the i^6Ado degradation rate increased in relation to the amount of intracellular i^6Ado (Fig. 2). Towards the end of the incubation, however, the rate of degradation unexpectedly doubled although

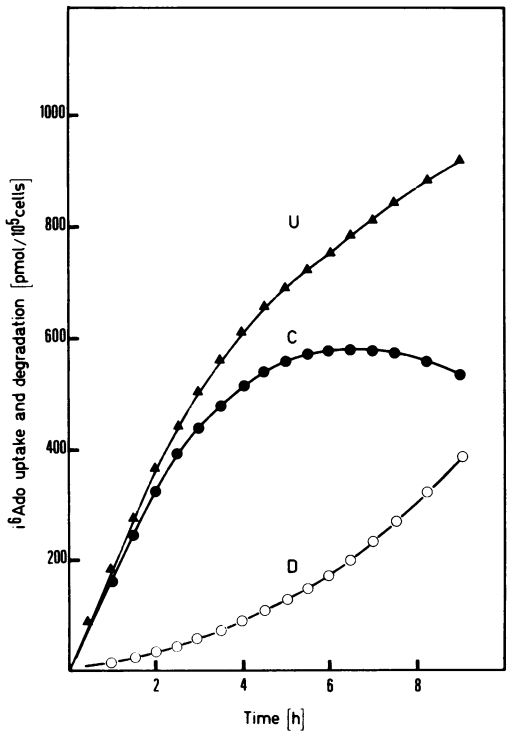

FIG. 1. Time course of N^6(Δ^2-isopentenyl)adenosine uptake (U) and degradation (D) in tobacco cells. [8-^{14}C]i^6Ado was supplied at a concentration of 1 μM to a tobacco cell suspension (cytokinin-autonomous cells, 0.95 × 10^5 cells/ml). Results are expressed in pmol/10^5 cells. Curve C corresponds to i^6Ado metabolites which retained the Δ^2-isopentenyl chain (i^6ATP, i^6ADP, i^6AMP and i^6Ade). Curve D corresponds to i^6Ado catabolites resulting from Δ^2-isopentenyl chain cleavage (see Fig. 3, part C).

[2] Throughout this paper, "i^6Ado metabolites" refers only to metabolites which have kept the Δ^2-isopentenyl chain: i^6Ado, i^6Ade, and corresponding nucleotides. Metabolites which have lost the Δ^2-isopentenyl chain are referred to as "degradation products" or i^6Ado catabolites.

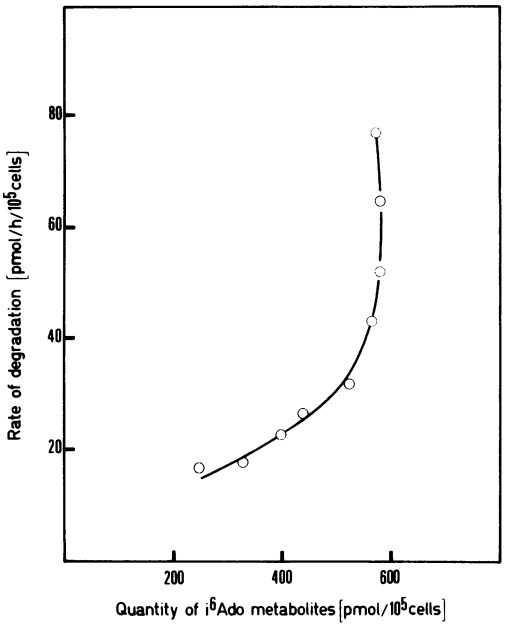

FIG. 2. Relation between the rate of N^6-(Δ^2-isopentenyl)adenosine degradation in tobacco cells and the quantity of cellular i^6Ado and i^6Ado metabolites (corresponding nucleotides and base). Data are derived from the experiment described in Figure 1.

the level of total i^6Ado in the cells remained almost constant (Fig. 3, C). Similar kinetics of i^6Ado uptake and degradation were observed when i^6Ado was supplied to tobacco cell suspensions at concentrations of 0.2 and 0.5 μM, respectively. In all cases, during the first 3 h of incubation (Fig. 3), i^6Ado degradative activity increased in relation to the cellular accumulation of i^6Ado followed by a severalfold stimulation of i^6Ado degradative activity which was independent of the absolute amount of i^6Ado and i^6Ado metabolites present in cells but possibly in relation to the duration of the incubation. However, maximal and constant i^6Ado degradation rates observed after 7–8 h of incubation correlated with the intracellular levels of cytokinin although the relation was less than linearly proportional (Table I).

Effect of Preincubation with I^6Ado upon Kinetics of [8-^{14}C]-I^6Ado Degradation. To confirm that incubation of the cells with i^6Ado resulted in increased degradative activity, we preincubated the cell suspensions with low concentrations of unlabeled i^6Ado before addition of [8-^{14}C]i^6Ado.

Two identical cell suspensions, A and B, of tobacco cells (cell line 13, 10^5 cells/ml) were used. At time zero, nothing was added to A, unlabeled i^6Ado (0.33 μM) was added to B. After 3 h of preincubation, [8-^{14}C]i^6Ado was added to both cell suspensions to a concentration of 1 μM and its uptake and degradation measured. Figure 4 shows the comparative time course of [8-^{14}C]i^6Ado absorption and degradation. During the first 2 h of incubation, preincubation with i^6Ado did not significantly modify rates of [8-^{14}C]i^6Ado uptake (147 pmol/h·10^5 cells for the control cells *versus* 136 pmol/h·10^5 cells for preincubated cells). In i^6Ado preincubated cells, however, i^6Ado degradative activity was 3-fold greater than in control cells as shown in Table II. This stimulation of i^6Ado degradation is minimal because the measured degradative activity in preincubated cells was less than actual because of the isotopic dilution. Towards the end of the incubation time, comparable maximal rates of i^6Ado degradative activity were observed (80–85 pmol/h·10^5 cells) because stimulation of i^6Ado degradation activity described in Figures 1 and 3 occurred in the control cell suspension.

Similar results were obtained in repeated experiments and

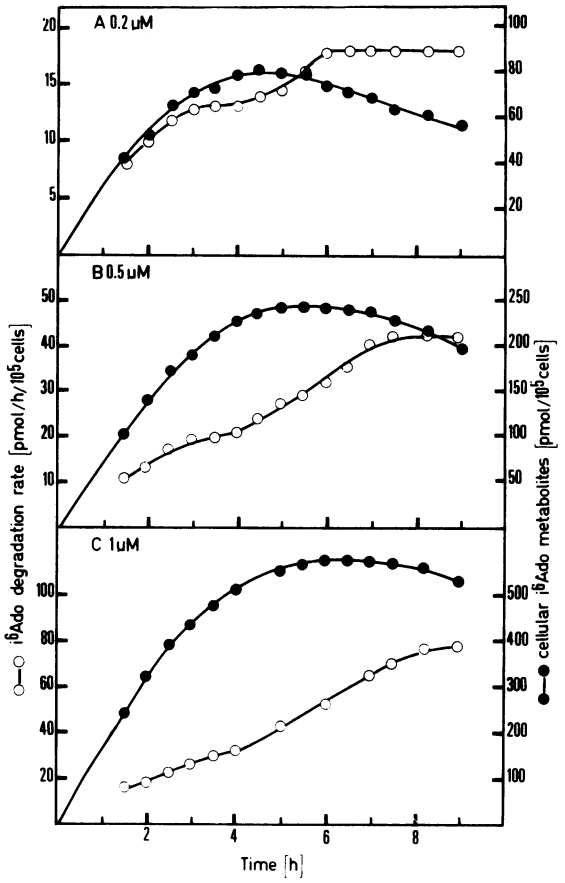

FIG. 3. Evolution with time of the rate of N^6-(Δ^2-isopentenyl)adenosine degradation and of metabolites accumulation in cytokinin-autonomous tobacco cells (0.95 × 10^5 cells/ml) supplied with [8-^{14}C]i^6Ado at concentrations of 0.2 μM (A), 0.5 μM (B) and 1 μM (C).

Table I. *i^6Ado Degradative Activity in Cytokinin-requiring Tobacco Cells, in Relation to Quantities of i^6Ado Metabolites*

Influence of incubation time and concentration of supplied [8-^{14}C]i^6Ado (data from experiment described in Fig. 3).

Incubation Time	[8-^{14}C]i^6Ado Concentration	Intracellular Quantities of Cytokinin	Rate of Degradation
h	*µM*	*pmol/10^5 cells*	*pmol/10^5 cells·h*
2.5	0.2	66	12
	0.5	173	17
	1	395	23
7	0.2	75	18
	0.5	238	40
	1	578	65

stimulation of *in vivo* i^6Ado degradation activity was also observed when tobacco cells were preincubated with BA (Fig. 5 and Table II).

DISCUSSION

When [8-^{14}C]i^6Ado is added to the culture medium of a tobacco cell suspension, it is rapidly absorbed by the cells and phosphorylated. It is also rapidly degraded by removal of the isopentenyl chain which gives adenylic products. Rate of degradation increases severalfold during the incubation period and our results clearly demonstrate that this is due to two distinct phenomena. First, the rate increases as the quantity of intracellular i^6Ado metabolites

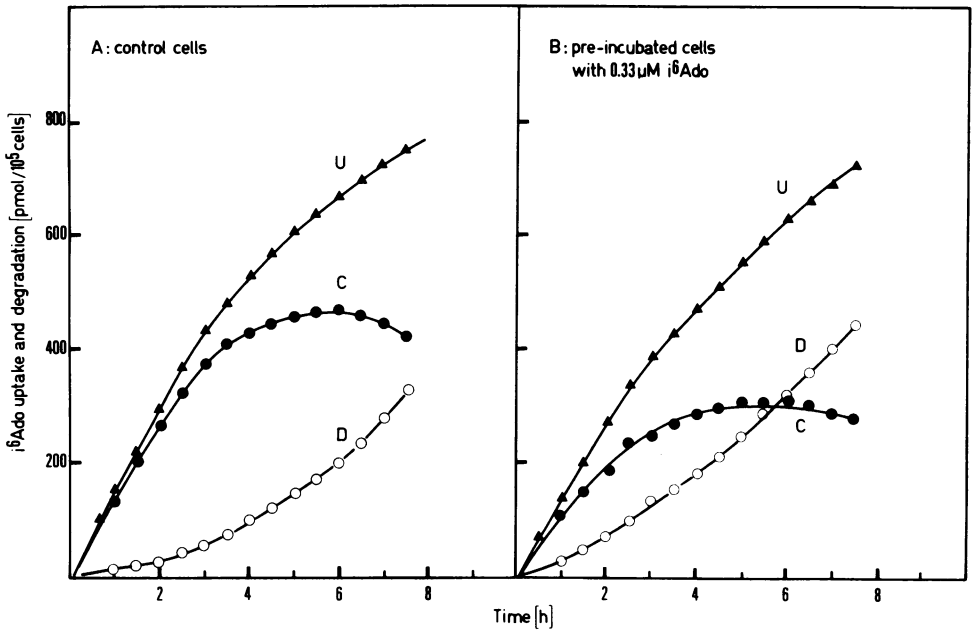

FIG. 4. Time course of N^6-(Δ^2-isopentenyl)adenosine uptake (U) and degradation (D) in tobacco cells. [8-^{14}C]i^{6}Ado was supplied at a concentration of 1 μM to two 100-ml suspensions of tobacco cells (cytokinin-autonomous cells—10^5 cells/ml). (A) control cell suspension; (B) cell suspension incubated previously for 3 h with 0.33 μM i^{6}Ado. Curve C corresponds to i^{6}Ado metabolites which retained the Δ^2-isopentenyl chain: i^{6}ATP, i^{6}ADP, i^{6}AMP and i^{6}Ade.

Table II. *Relation between i^{6}Ado Degradative Activity and i^{6}Ado Metabolism in Autonomous Tobacco Cells*

Influence of duration of incubation or pre-incubation with benzyladenine (experiment described in Fig. 5).

i^{6}Ado Metabolism	Control Cell Suspension		Cell Suspension Preincubated with 0.3 μM Benzyladenine	
	3.5 h	8 h	3.5 h	8 h
Direct analysis:				
i^{6}Ado metabolites	389	509	272	306
Rate of degradation (pmol/10^5 cells·h)	16	49	52	63
Perchloric acid fixation and complete analysis:				
i^{6}Ado metabolites	373	509	249	295
i^{6}ADP + i^{6}ATP	215	217	112	112
i^{6}AMP	153	286	135	179
i^{6}Ado	2	ϵ[a]	ϵ	ϵ
i^{6}Ade	2	6	2	4

[a] Near the limit of detection which was about 0.5 pmol/10^5 cells.

increases. The relation is less than linearly proportional (Table I) which indicates existence of a saturation phenomenon which we interpret in terms of enzyme-substrate concentration relationship. Second, the rate increases severalfold after a 3- to 4-h delay, or when tobacco cells have been previously exposed to either i^{6}Ado or benzyladenine. This stimulation appears to be independent of the absolute level of cellular i^{6}Ado metabolites (Fig. 2).

Consequently, only rates of i^{6}Ado degradation measured during the first 3 h of incubation can be considered as representative of the intrinsic cytokinin degradative activity of tobacco cells because the higher rates of i^{6}Ado degradation observed after longer incubation times with [8-^{14}C]i^{6}Ado result from the incubation conditions. Within these limits, our results indicate that cytokinin degradative activity is high in cytokinin-autonomous tobacco cells. For example, when i^{6}Ado is added in the culture medium at the concentration of 0.2 μM, the half life of the resulting cellular i^{6}Ado metabolites, measured after 2 h 30 min of incubation, was about 2 h 40 min. This corresponds to approximately 100 μg i^{6}Ado degraded/h·kg (fresh weight) of cells. Under the same conditions, half lives of cellular i^{6}Ado were about 5 and 9 h, respectively, when i^{6}Ado was supplied at concentrations of 0.5 and 1 μM (Table I). In the later case, this corresponds to about 180 μg i^{6}Ado degraded/h·kg of cells (fresh weight). These values may at first seem very high in comparison to the amounts of endogenous cytokinins reported for cytokinin-autonomous plant tissues (2), but they are physiologically significant because i^{6}Ado is required at a concentration of 0.3–0.5 μM to support optimal growth of cytokinin-requiring tobacco cell suspensions (Laloue, unpublished results).

The initial aim of this study was a comparison of cytokinin degradative activities in cytokinin-requiring and in cytokinin-autonomous tobacco cells. We found that in 5-day-old cultures (in midexponential phase), i^{6}Ado degradation rates were higher in the dependent cell than in the autonomous cells (Table III). This would be expected from the fact that in late exponentially growing cell suspensions, preincubation with BA resulted in increased i^{6}Ado degradative activity, and the dependent cells were cultured continually in the presence of BA. Thus, cytokinin-requiring tobacco cells cannot be compared to cytokinin-autonomous cells with respect to their cytokinin degradative activities because cytokinin degradative activity is stimulated in the cytokinin-requiring cells by the cytokinin added to the culture medium.

After a 3- to 4-h delay, i^{6}Ado degradative activity increases severalfold in tobacco cells exposed to i^{6}Ado or benzyladenine, without any apparent relationship to the total amount of cellular i^{6}Ado metabolites. We considered here the intracellular i^{6}Ado and its metabolites as an unique entity and it is possible that in tobacco cells, different degrading enzymes with different constants are specific for i^{6}Ado, i^{6}Ade, or i^{6}Ado nucleotides. Consequently, any modification in the metabolic distribution of these [8-^{14}C]i^{6}Ado metabolites would change the apparent rates of i^{6}Ado degradation.

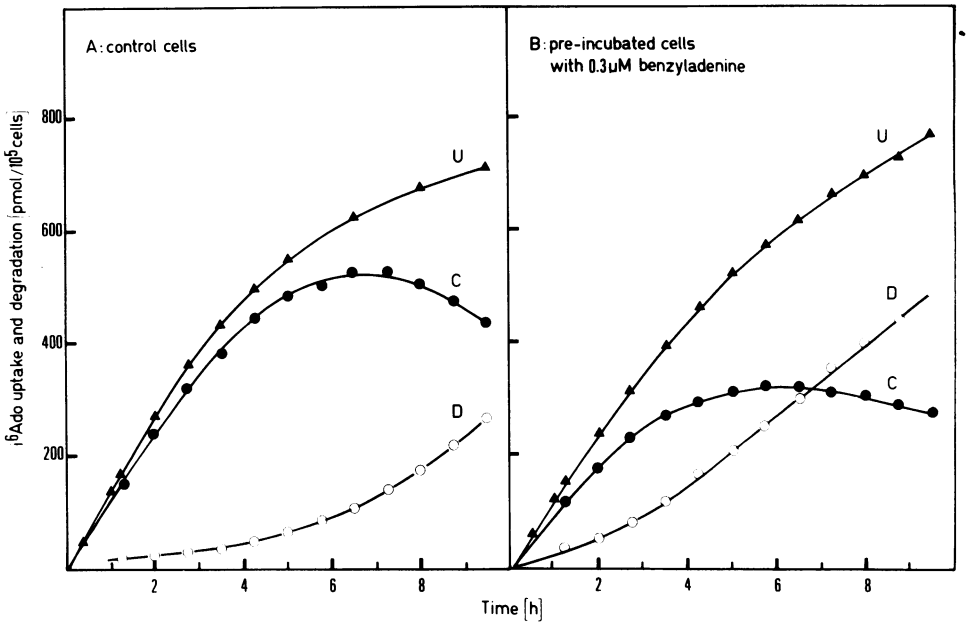

FIG. 5. Time course of N^6-(Δ^2-isopentenyl)adenosine uptake (U) and degradation (D) in tobacco cells. [8-^{14}C]i^{6}Ado was supplied at a concentration of 1 µM to two 100-ml suspensions of tobacco cells (cytokinin-autonomous cells, 10^5 cells/ml). (A) control cell suspension; (B) cell suspension incubated previously for 3 h with 0.3 µM BA. Curve C corresponds to i^{6}Ado metabolites which retained the Δ^2-isopentenyl chain: i^{6}ATP, i^{6}ADP, i^{6}AMP and i^{6}Ade. After 3.5 h and 8 h of incubation, respectively, cells corresponding to 20 ml of both cell suspensions were fixed with $HClO_4$ for detailed analysis of i^{6}Ado metabolites (see Table II).

Table III. *Rates of i^{6}Ado Degradation in Cytokinin-requiring Tobacco Cells*

[8-^{14}C]i^{6}Ado was supplied to a 5-day-old suspension of cytokinin-requiring tobacco cells (cell line 19, 0.85 × 10^5 cells/ml) at the concentration of 1 µM. Rates of i^{6}Ado degradation and quantities of cellular cytokinins measured after 2.5 and 7 h of incubation, respectively, are reported in order to allow a comparison with the data obtained in the case of cytokinin-autonomous cells (Table I).

Incubation Time	Intracellular Quantities of Cytokinin	Rate of Degradation
h	*pmol/10^5 cells*	*pmol/10^5 cells·h*
2.5	230	40
7	260	65

This hypothesis was evaluated by studying i^{6}Ado metabolism and degradation in tobacco cells previously supplied with 0.3 µM BA. The rate of i^{6}Ado degradation was higher in cells preincubated with 0.3 µM BA than in control cells (Fig. 5). Indeed, after 3 h–30 min of incubation, i^{6}Ado was degraded at rates of 16 and 43 pmol/h·10^5 cells in control cells and in BA treated cells, respectively. The various i^{6}Ado metabolites were in lesser amounts in BA pretreated cells than in control cells, with the exception of i^{6}Ade which was in equal amounts (Table II). Furthermore, in control cells, no evident relationship could be observed between the levels of i^{6}Ado metabolites after 3 h–30 min and 8 h of incubation and the corresponding rates of i^{6}Ado degradation which were of 16 and 49 pmol/h·10^5 cells, respectively.

Hence, these results do not support the hypothesis that higher rates of Δ^2-isopentenyl chain cleavage from i^{6}Ado or i^{6}Ado metabolites correspond to higher levels of specifically degradable substrates. As an alternative hypothesis based mainly on the increased degradative activity observed after 3–4 h to exposure to i^{6}Ado or BA, we hypothesize that it is due to increased levels of the degradative enzyme(s).

Whitty and Hall (14) reported that BA was not degraded by a cytokinin oxidase purified from corn kernels and that, moreover, it inhibited degradation of i^{6}Ade. In the autonomous tobacco cells, ^{14}C-labeled adenylic products were detected when [8-^{14}C]BA was supplied in the culture medium, indicating that BA is degraded to a small extent, presumably via the same degradative pathway. The rate of BA degradation was estimated to be about 0.3 pmol/h·10^5 cells over a 24-h period when 0.3 µM [8-^{14}C]BA was added aseptically to a cell suspension. BA appears to be degraded about 50 times less rapidly than i^{6}Ado under comparable intracellular quantities of cytokinin metabolites (Table I). Under these conditions, it would be expected that benzyladenine or its metabolites would inhibit removal of the isopentenyl chain from i^{6}Ado or its degradable metabolites. If such effects occur however, they may not be observed because of the fact that BA simultaneously stimulates the cytokinin degradative activities (Fig. 5).

In summary, results presented in this paper establish that cytokinin degradative activity is high in tobacco cells grown as cell suspensions. This activity plays an important role in controlling cytokinin levels in these cells. This conclusion is supported further by the fact that this cytokinin-degradative activity, whatever the exact mechanism, is under the control of cytokinins themselves.

Acknowledgments—The authors wish to thank Professor J. Guern for his constant support during this investigation and Professor R. H. Hall for reviewing the manuscript.

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