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Conversion of N^6-(Δ^2-Isopentenyl)adenosine to Adenosine by Enzyme Activity in Tobacco Tissue

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ABSTRACT

By using [8-^{14}C]-N^6-(Δ^2-isopentenyl)adenosine as a substrate, we detected the presence of enzyme activity in a crude extract of tobacco tissue that converts this cytokinin into adenosine. The extract also contains strong hydrolase activity that converts adenosine to adenine.

N^6-(Δ^2-Isopentenyl)adenosine and its derivatives N^6-(Δ^2-isopentenyl)-2-methylthioadenosine, N^6-(4-hydroxy-3-methylbut-2-*trans*-enyl)-adenosine, and N^6-(4-hydroxy-3-methylbut-2-enyl)-2-methylthioadenosine occur in plant tissue as components of tRNA (2, 3, 8). The parent compound and its derivatives exhibit potent cytokinin activity, for example the stimulation of cell division and cell differentiation in cultured tobacco cells (see review, ref. 6).

The nature of the physiological activity has led to the suggestion that N^6-(Δ^2-isopentenyl)adenosine and/or its derivatives are involved as hormones in the maintenance of normal growth and development of plant tissue. In order to understand the mechanism of action of these compounds, it would be helpful to know what happens when such compounds enter the cell.

We have been studying the fate of N^6-(Δ^2-isopentenyl)adenosine in cultured tobacco cells. Chen *et al.* (4) reported that when N^6-(Δ^2-isopentenyl)adenosine was incubated with a crude extract of normal tobacco pith cells (*Nicotiana tabacum* var. Wisconsin 38), it was metabolized. As part of a program to study the metabolism of N^6-(Δ^2-isopentenyl)adenosine in tobacco tissue, we have now identified an enzyme activity that could be involved in the *in vivo* degradation of this nucleoside. This activity catalyzes degradation of N^6-(Δ^2-isopentenyl)adenosine to adenosine and/or adenine.

MATERIALS AND METHODS

Tobacco tissue, a cytokinin-requiring strain (*Nicotiana tabacum* var. Wisconsin 38), and an autonomous variant that requires neither cytokinin nor auxin were obtained from Dr. J. E. Fox, University of Kansas. We are indebted to Dr. W. Dyson for maintaining and providing these strains.

Adenosine and [8-^{14}C]adenosine (specific radioactivity 55 mc/mmole) were obtained from Schwarz BioResearch Inc. 1-Bromo-3-methylbut-2-ene was obtained from Columbia Organic Chemicals and was freshly distilled prior to the reaction (boiling point 76–78 C at 85 mm). Dimethylformamide (Baker Analyzed Reagent) was dried by refluxing over calcium hydride for 18 hr and freshly distilled (boiling point 151–152 C). Sephadex LH-20 was obtained from Pharmacia Ltd. N^6-(Δ^2-Isopentenyl)adenosine was obtained from Starcks Associates Inc., Buffalo, New York. Polyvinyl pyrrolidone powder (Polyclar AT) was obtained from Irwin Dyestuffs Ltd.

Chromatography. Whatman No. 1 paper was used. Solvents: A: ethanol-0.1 M ammonium borate, pH 9.0 (1:9, v/v); B: isopropanol-concentrated ammonium hydroxide-water (7:1:2, v/v); C: 1-butanol-concentrated ammonium hydroxide-water (86:5:14, v/v). Merck Silica Gel H was used for preparative thin layer chromatography. Plates were developed in a 15% (v/v) methanol in methylene chloride mixture.

Detection of Radioactivity. For the assays of enzyme activity 3.8-cm wide strips from the developed paper chromatograms were cut out and scanned in a Nuclear Chicago Actigraph III instrument. The areas on the chart paper corresponding to the peaks of radioactivity were cut out and weighed. The calculation of the relative amounts of radioactive compounds was based on the weight of the paper cut-outs.

For accurate measurement of radioactivity in the synthetic procedures a liquid scintillation counter was used.

Preparation of [8-^{14}C]-N^6-(Δ^2-Isopentenyl)adenosine. Two syntheses of N^6-(Δ^2-isopentenyl)adenosine have been reported. In the first route, Δ^2-isopentenylamine is condensed with 6-chloro-(9-β-D-ribofuranosyl)purine (12). In the second, adenosine is alkylated with Δ^2-isopentenylbromide (5, 9, 12). For preparation of a radioactively labeled compound, the latter approach offers greater versatility because of the commercial availability of different forms of labeled adenosine.

We synthesized [8-^{14}C]-N^6-(Δ^2-isopentenyl)adenosine, following the route of Martin and Reese (9). The small scale of the reaction presented difficulties because of potential side reactions; further, the product is highly labile and consequently must be purified and stored in a manner different from that used for larger scale synthesis of an unlabeled sample.

An aqueous solution of [8-^{14}C]-adenosine (100 μc; 0.4854 mg; 1.82 μmole; in 10 ml) was evaporated to dryness *in vacuo* at 35 C. The residue was dried from absolute ethanol (2 × 2 ml) and placed in a desiccator over phosphorus pentoxide for 16 hr. Adenosine (4.85 mg, 18.2 μmoles) was dried and added to the radioactive material. A suspension of adenosine (5.335 mg, 20 μmoles), Δ^2-isopentenyl bromide (5.2 mg, 4 μl; 35 μmoles) in dimethylformamide (70 μl) was stirred at room temperature. After 24 hr, aqueous dimethylamine (70 μl) and methanol (100 μl) were added, and the reaction mixture was stirred for a further 16 hr. The reaction mixture was then evaporated to dryness *in vacuo;* the residue re-evaporated with 95% ethanol (2 × 200 μl) and finally with water (2 × 200 μl). The solid residue was triturated with 95% ethanol (2 × 2 ml)

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and ethyl acetate (2 × 2 ml), and the combined organic extracts were purified by preparative thin layer chromatography (20 × 20 cm plates). The silica gel band (R_F: 0.5) containing the product was transferred into a small container with a porous bottom and this was placed on top of a Sephadex LH-20 column (1) (2.5 × 35 cm). The product was washed onto the column with 35 ml of ethanol. The container was removed, and the column was eluted with 35% aqueous ethanol at a flow rate of 35 ml per hr. The peak of the fraction containing the product was eluted approximately 275 ml after the start. The fraction containing the product was evaporated to dryness, and the sample was dissolved in 3.0 ml of water. The product co-chromatographed as a single radioactive spot together with the authentic nucleoside in solvent systems B and C on paper and on the thin layer plates. The specific radioactivity was 5.0 mc/mmole. The yield was 47% based on the [8-^{14}C]-adenosine.

The identity of the radioactive material was confirmed by subjecting it to chemical degradation. Robins *et al.* (12) showed that mild acid hydrolysis converts N^6-(Δ^2-isopentenyl)adenosine to two products, N^6-(3-hydroxy-3-methylbutyl)adenine and 3*H*-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]-purine. A sample of the radioative product was mixed with the authentic nucleoside and the mixture was hydrolyzed for 15 min in 1 N hydrochloric acid. The reaction mixture was chromatographed on paper in solvent systems B and C. Two radioative spots migrating coincidentally with the above two degradation products were obtained in each chromatogram.

To rule out the possibility that the small scale of the procedure could have produced an anomalous product, the synthesis was also carried out on the same scale using unlabeled precursors. The mass spectrum of the obtained product was identical with that of authentic N^6-(Δ^2-isopentenyl)adenosine (12).

Preparation of Enzyme Extract. Freshly harvested tobacco tissue (500 g, autonomous or cytokinin-requiring) was frozen in liquid nitrogen. The cells were broken by grinding them in the presence of superfine washed glass beads in a mortar prechilled in liquid nitrogen. The cells were maintained in a frozen condition by continual addition of liquid nitrogen during the grinding procedure. The frozen flour was poured into 2 liters of a buffer solution consisting of 0.1 M sodium phosphate, 0.1 M sodium ascorbate, ammonium sulfate (15% saturation), pH 6.8, and 150 gm of polyvinyl pyrrolidone. The mixture was stirred with a heavy duty stirrer at room temperature until the suspension reached 4 C. All subsequent operations were performed at 4 C.

The suspension was filtered through Miracloth (Chicopee Mills Inc., N.Y.) (or several layers of cheesecloth), and the filtrate was centrifuged at 10,000*g* for 25 min. Solid ammonium sulfate was added to the supernatant until 60% saturation was reached. The pH was maintained at 6.8 to 7.0 by addition of a few drops of concentrated ammonium hydroxide. The solution was gently agitated during the saturation procedure and then left for 15 min. The precipitate was collected by centrifugation for 20 min at 10,000*g* and redissolved in 70 ml of 50 mM sodium phosphate buffer, pH 6.8. The turbid opalescent solution was dialyzed overnight against 5 liters of 50 mM sodium phosphate buffer, pH 6.8, and the dialysate was clarified by centrifugation at 10,000*g* for 10 min. The protein concentration of this solution was 2.9 mg/ml. This solution represented the stock solution of enzyme activity. It could be stored at −20 C for several weeks without loss of activity.

Concentration of this solution by various means did not result in any substantial improvement in specific activity of the enzyme.

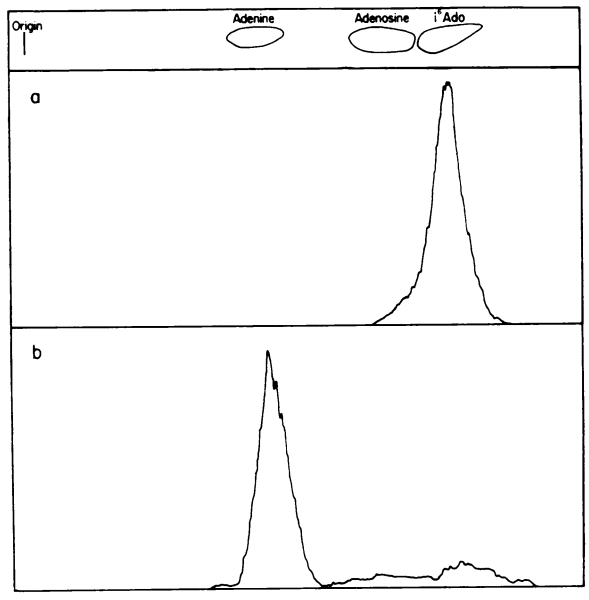

FIG. 1. Radioactive profiles of developed chromatograms (system A) of a stock sample of [8-^{14}C]-N^6-(Δ^2-isopentenyl)adenosine. a: After incubation for 18 hr at 37 C with heat-inactivated enzyme extract of the Wisconsin-38 strain of tobacco tissue. The profile of the untreated stock sample of [8-^{14}C]-N^6-(Δ^2-isopentenyl)adenosine is identical with this profile. b: After incubation for 18 hr at 37 C with enzyme extract from the Wisconsin-38 strain of tissue (see section 1 of "Results"). The paper strips were counted at 120 cm/hr. The top of the chart represents 1500 cpm. Counting efficiency was about 8%.

RESULTS

1. **Conversion of N^6-(Δ^2-Isopentenyl)adenosine into Adenosine and Adenine.** A sample of [8-^{14}C]-N^6-(Δ^2-isopentenyl)-adenosine, 20 μl of a 227 μM aqueous solution, was added to 200 μl of stock enzyme solution obtained from the Wisconsin-38 strain. This solution was incubated for 18 hr at 37 C. In the control sample, the substrate was incubated with 200 μl of the enzyme solution that had been previously heated for 2 min at 100 C. After 18 hr, 0.5 ml of cold ethanol was added to each reaction. The mixture was chilled to 0 C for 10 min and the precipitated protein was removed by centrifugation. The supernatant was concentrated *in vacuo* and the concentrated solution was applied to chromatographic paper. Standard markers of adenine, adenosine and N^6-(Δ^2-isopentenyl)-adenosine were applied to the same strip of paper. The paper was developed in system A. The radioactive profiles of the developed chromatogram are shown in Figure 1. The reaction is about 70% complete (see data in Table I) at the end of 10 hr, but the reaction was left 18 hr to achieve completion.

The crude extract from the autonomous variant of tobacco tissue was assayed for the presence of the enzyme activity under identical conditions. The radioactive profile of the developed chromatogram was identical with that shown in Figure 1b.

These results show that the plant extract contains at least two enzyme activities that catalyze cleavage of the Δ^2-isopentenyl side-chain and the *N*-glycosylic bond.

2. **Incubation of [8-^{14}C]-N^6-(Δ^2-Isopentenyl)adenosine in the Presence of Excess Unlabeled Adenosine.** [8-^{14}C]-N^6-(Δ^2-Isopentenyl)adenosine was incubated for 10 hr at 37 C with the enzyme extract from the Wisconsin-38 strain of tissue in the presence of about a 300-fold excess of unlabeled adenosine in order to dilute the endogenous ^{14}C-adenosine. The results are shown in Table I. The amount of adenine formed under these conditions is reduced from 69 to 25%. These results are equiv-

Table I. *Effect of Excess Adenosine on Enzymic Cleavage of N^6-(Δ^2-Isopentenyl)adenosine*

Each incubation mixture consisted of 10 μl of stock [8-^{14}C]-N^6-(Δ^2-isopentenyl)adenosine (227 μM), 200 μl of the stock enzyme solution obtained from the Wisconsin-38 strain of tobacco tissue and the additions. In experiment 2 the enzyme solution had been heated for 2 min at 100 C prior to incubation.

The samples were incubated for 10 hr at 37 C. The solutions were diluted with 1 ml of cold ethanol, and the precipitated protein was removed by centrifugation. The solutions were concentrated *in vacuo*, and the concentrate was chromatographed in system A. The amount of radioactivity was calculated as described in "Materials and Methods."

Experiment no.	Additions			Products		
	50 mM phosphate, pH 6.8	Adenosine	i^{6}Ado[1]	i^{6}Ado	Adenosine	Adenine
	μl	2 mg/ml	2 mg/ml	%		
1	100	0	0	31	0	69
2 (control)	100	0	0	92	7	1
3		100 μl	0	32	43	25
4		0	100 μl	93	0	7

[1] i^{6}Ado: N^6-(Δ^2-isopentenyl)adenosine.

ocal but suggest that the conversion of N^6-(Δ^2-isopentenyl)-adenosine to adenine may proceed via either the nucleoside (*i.e.*, conversion to adenosine) or via the free base (*i.e.*, conversion first to N^6-(Δ^2-isopentenyl)adenine).

3. **Identification of the End Product of Reaction as Adenine.** In order to confirm the identity of the final product of conversion as adenine, the radioactive spot corresponding to adenine in the developed chromatogram was cut out and the radioactive material was rechromatographed in systems B and C. In each case a single radioactive spot migrated coincidentally with the adenine marker. The results are tabulated in Table II.

4. **Demonstration of Adenosine Hydrolase Activity in the Enzyme Extract.** A stock solution of radioactive adenosine was prepared as follows: 300 μl of [8-^{14}C]-adenosine (100 μc/10 ml, 55 mc/mmole) 400 μl of an aqueous solution of unlabeled adenosine (1 mg/ml), water was added to make a total volume of 1.2 ml. The enzyme incubation mixture consisted of 30 μl of the stock adenosine solution and 50 μl of the stock enzyme extract from the Wisconsin-38 strain of tobacco tissue. A control sample consisted of the same amount of substrate and 50 μl of stock enzyme solution that had been heated for 2 min at 100 C. The reaction mixtures were incubated for 15 min at 37 C. The reaction was stopped by addition of an equal volume of cold ethanol. The centrifuged solutions were concentrated *in vacuo*, and the samples were applied to Whatman No. 1 paper, which was developed in system A. The results are shown in Figure 2.

This activity appears to be a hydrolase and not a phosphorylase activity. In one experiment equal aliquots of the same stock enzyme solution were dialyzed exhaustively against 0.1 M phosphate, pH 7.0, 6 mM mercaptoethanol, and 0.1 M tris-HCl, pH 7.3, 6 mM mercaptoethanol, respectively. The ability of the two extracts to catalyze conversion of adenosine to adenine was identical.

DISCUSSION

Metabolism of naturally-occurring cytokinins has been reported previously. Zeatin [N^6-(*trans*-4-hydroxy-3-methylbut-2-enyl)adenosine] is extensively metabolized by excised bean axes to form the riboside and the corresponding dihydrozeatin derivatives (13). There was no evidence of cleavage of the side-chain to form adenine or hypoxanthine. Miura and Miller (10) have shown that N^6-(Δ^2-isopentenyl)adenosine is converted to zeatin by *Rhizopogon roseolus*. This type of enzymic reaction presumably represents conversion of the cytokinin to a more usable or active form. Degradation, on the other hand, represents inactivation.

Enzyme activity that catalyzes the degradation of N^6-(Δ^2-isopentenyl)adenosine is not limited to plant tissue. Hall *et al.* (7) have partially purified from chicken bone marrow an enzyme that catalyzes the conversion of this nucleoside to inosine. This particular enzyme is specific for the nucleoside form. The chicken enzyme system also exhibits considerable specificity with respect to the structure of the side-chain. Ribosyl zeatin [N^6-(*trans*-4-hydroxy-3-methylbut-2-enyl)adenosine], for example, does not serve as a substrate.

The presence of an inactivation mechanism for N^6-(Δ^2-isopentenyl)adenosine in plants has been suggested previously (6,

Table II. *Comparison of Presumed Radioactive Adenine with Standards by Means of Paper Chromatography*

Compound	R_F Value (× 100) in System		
	A	B	C
N^6-(Δ^2-Isopentenyl)adenosine	79	85	86
Inosine	84	34	04
Adenosine	67	55	27
Adenine	41	48	35
Xanthine	64	23	02
Hypoxanthine	66	36	11
Radioactive Spot	41	48	35

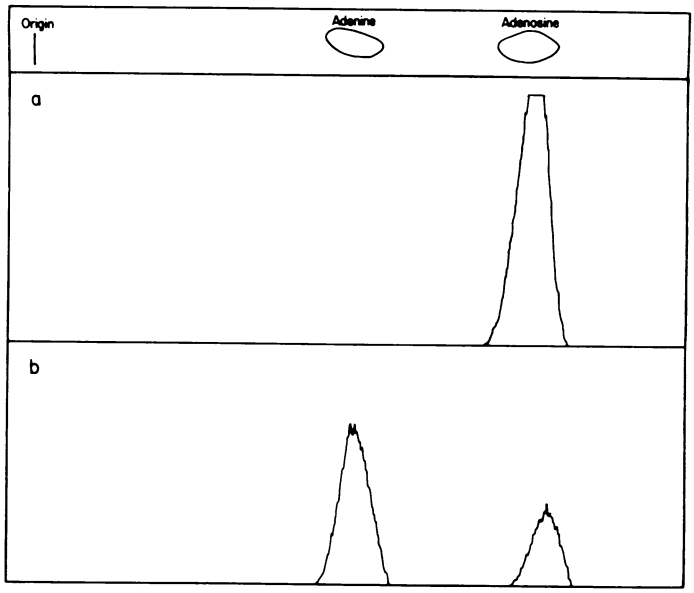

FIG. 2. Radioactive profiles of developed chromatograms (system A) of [8-^{14}C]-adenosine after incubation for 15 min at 37 C with enzyme extract from Wisconsin-38 strain of tobacco tissue (see section 3 of "Results"). a: With heat-inactivated enzyme extract; b: with native extract. The paper strips were counted at 30 cm/hr; the top of the chart represents 5000 cpm. Counting efficiency was about 8%.

11), and the cleavage enzyme reported in this study may be part of such a mechanism.

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