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BIOSYNTHESIS OF N^6-(Δ^2-ISOPENTENYL)ADENOSINE IN THE TRANSFER RIBONUCLEIC ACID OF CULTURED TOBACCO PITH TISSUE

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Abstract—When mevalonic acid-[2-^{14}C] is added to the culture media of tobacco pith tissue (Wisconsin strain 38), a significant amount of radioactive label is incorporated into the transfer ribonucleic acid (tRNA). All of this radioactivity is located in the N^6-(Δ^2-isopentenyl)adenosine component of the tRNA. The addition of N^6-(Δ^2-isopentenyl)adenosine to the media does not appear to depress the degree of incorporation of the radioactive label into the tRNA. A crude enzyme system was extracted from the pith tissue that catalyzes the attachment *in vitro* of the Δ^2-isopentenyl side-chain to preformed tobacco tRNA. This *in vitro* system is analogous to the *in vitro* system for the biosynthesis of N^6-(Δ^2-isopentenyl)adenosine in yeast and rat liver tRNA (Fittler *et al.*, *Biochem. Biophys. Acta* **31**, 517, 1968). The addition of D,L-mevalonic acid (12 μmoles/l) to the culture media containing indoleacetic acid (2 mg/l) appears to partially replace the requirement of the tobacco tissue for a cytokinin. Under these conditions a two- to five-fold increase in yield over control cultures is obtained.

INTRODUCTION

THE EXOGENOUS application of a cytokinin to tobacco pith tissue grown in culture stimulates cell-division and cell-differentiation. This and related observations obtained in other experimental plant systems have led to the proposition that the experimentally produced cytokinin phenomenon is indicative of a hormone function in growing plants that involves regulation of normal growth and development. In spite of considerable research on the cytokinin effect,[1–3] the basic questions: what is the regulator hormone(s) produced in the plant tissue and what is its mechanism of action, remain unanswered.

Before a meaningful attempt can be made to answer these questions it will be necessary to characterize the compound(s) synthesized in tissue that plays the regulatory role. Compounds have been identified in plant tissue that exhibit cytokinin activity in the test systems, but there is no evidence that any of these naturally occurring compounds is involved in a regulatory function in growing plants. The most potent of these compounds (tobacco pith culture assay system) is N^6-(Δ^2-isopentenyl)adenosine, and its hydroxylated derivatives.[4–7] The immediate question that arises is whether these particular compounds are natural

* Abbreviations used: tRNA, transfer RNA; rRNA, ribosomal RNA; DEAE-cellulose, diethylaminoethyl-cellulose anion exchange resin; A_{260} unit, one unit equals 1 ml of a solution with an absorbance of 1·0 at 260 mμ.

[1] J. P. HELGESON, *Science* **161**, 974 (1968).
[2] D. S. LETHAM, *Ann. Rev. Plant Physiol.* **18**, 349 (1967).
[3] J. VAN OVERBEEK, *Science* **152**, 721 (1966).
[4] R. H. HALL, M. J. ROBINS, L. STASIUK and R. THEDFORD, *J. Am. Chem. Soc.* **88**, 2614 (1966).
[5] D. S. LETHAM, J. S. SHANNON and I. R. C. MCDONALD, *Tetrahedron* **23**, 479 (1967).
[6] C. O. MILLER, *Proc. Nat. Acad. Sci. (U.S.)* **54**, 1052 (1965).
[7] R. H. HALL, L. CSONKA, H. DAVID and B. D. MCLENNAN, *Science* **156**, 69 (1967).

cytokinins. If they are produced in the cell for the purpose of exerting a regulatory function, then information about the nature of their biosynthetic pathways might contribute towards a general understanding of this phenomenon.

The parent compound, N^6-(Δ^2-isopentenyl)adenosine, occurs in tRNA[4,8] and evidence shows that its presence is essential to the proper codon–anticodon interaction of those tRNA molecules in which it occurs.[9,10] Its biosynthetic pathway has been studied in bacteria, yeast and mammalian tissue; it has been found that the Δ^2-isopentenyl side-chain is derived from mevalonic acid and is attached to adenosine residues in the tRNA at the macromolecular level.[11–13] In this paper we present evidence that a similar biosynthetic pathway operates in tobacco pith tissue.

The investigation of the biosynthesis of N^6-(Δ^2-isopentenyl)adenosine in a test system responsive to exogenously applied cytokinin affords the additional opportunity of investigating whether a relation between the mechanims of biosynthesis and the physiological activity of cytokinin exists. The results of the present work do not shed any definitive light on this possible relation but they do show that exogenously applied N^6-(Δ^2-isopentenyl)-adenosine does not significantly depress the degree of incorporation of the Δ^2-isopentenyl side-chain into the tRNA. In addition, mevalonic acid appears to have a stimulatory effect on the growth of the cultured tobacco pith cells.

RESULTS

Incorporation of Mevalonic Acid-[2-^{14}C] *into the* N^6-(Δ^2-*Isopentenyl*)*Adenosine Residues of tRNA*

Many compounds have been tested for cytokinin activity and this activity seems to be limited to the N^6-(substituted)adenosine derivatives. Therefore, with the thought in mind that the active hormone derived from mevalonic acid might be N^6-(Δ^2-isopentenyl)adenosine, we wished to determine whether the mevalonic acid added to the culture media serves as a source of the Δ^2-isopentenyl group of the N^6-(Δ^2-isopentenyl)adenosine residues of the tRNA.

The tobacco tissue was grown at 25° with DL-mevalonate-[2-^{14}C] (12 μmoles/l; 2 mg/l) in the semi-solid basal media containing indole–acetic acid (2 mg/l). After 35 days of incubation in darkness, tissues were weighed and a small portion of fresh tissue (0·5 g each) was either crushed or dried in an oven at 60° for 24 hr and the radioactivity measured. The amount of radioactivity contained in the fresh tissue after incubation is about 5 per cent of the radioactivity initially added to the media (Table 1). After the tissue was dried, about 27 per cent of the incorporated radioactivity remained in the residue.

RNA was extracted by a phenol–sodium lauryl sulfate procedure. Transfer RNA and rRNA were separated and purified in the manner described in the Experimental section.

The specific activity of the purified tRNA (about 60 cpm/A_{260} unit) is at a level corresponding to the occurrence of about one isoprene residue in every 100 chains of tRNA. Since the

[8] H. G. ZACHAU, D. DÜTTING and H. FELDMANN, *Z. Physiol. Chem.* **347**, 212 (1966).
[9] F. FITTLER and R. H. HALL, *Biochem. Biophys. Res. Commun.* **25**, 441 (1966).
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[11] A. PETERKOFSKY, *Biochemistry* **7**, 472 (1968).
[12] F. FITTLER, L. K. KLINE and R. H. HALL, *Biochemistry* **7**, 940 (1968).
[13] F. FITTLER, L. K. KLINE and R. H. HALL, *Biochem. Biophys. Res. Commun.* **31**, 571 (1968).

dilution factor resulting from endogenously synthesized mevalonic acid is unknown, this value is minimal. The amount of radioactivity found in the ribosomal RNA fraction corresponds to about 3 cpm/A_{260} unit. Since this level of incorporation is not significant, the rRNA was not investigated further.

TABLE 1. INCORPORATION OF RADIOACTIVE LABEL INTO THE tRNA OF TOBACCO TISSUE: CULTURES GROWN IN THE PRESENCE OF [2-^{14}C]-MEVALONIC ACID*

Total wt. of fresh tissue (g)	Total radioactivity in the tissue (cpm × 10^{-6})	% of radioactive label incorporated from the media	Total tRNA extracted (A_{260} units)	Radioactivity in the tRNA (cpm/A_{260} units)
32·5	3·78	4·36	42·3	60
25·3	2·52	5·40	36·6	33
43·7	4·51	5·75	101·4	74
39·8	4·19	4·81	58·7	59

* The tissues were grown in culture containing auxin (2 mg/l) and without cytokinin; the culture contained [2-^{14}C]-mevalonic acid (12·0 μmoles/l; 2·8 mc/mmole). After 35 days of growth, the tRNA was extracted and purified by ion-exchange chromatography on a DEAE-cellulose column.

Identification of [^{14}C]-*labeled* N^6-(Δ^2-*Isopentenyl)Adenosine in the tRNA*

The method of characterization is based on the procedures of Fittler *et al.*[12] and Robins *et al.*[14] The tRNA (20–50 OD_{260} units) was hydrolyzed to its constituent 2′(3′)-nucleotides by treatment with 0·3 N potassium hydroxide for 24 hr at 37°. The solution was neutralized with perchloric acid and potassium perchlorate was removed by centrifugation (Solution A). One-half of the solution was treated with bacterial alkaline phosphatase for 16 hr at pH 9·2 and 37° in order to produce the constituent nucleosides (Solution B).

The nucleotide preparation (Solution A) was subjected to paper electrophoresis at pH 10·5 (0·1 M sodium carbonate) for 3 hr (17 V/cm). An authentic sample of N^6-(Δ^2-isopentenyl)adenosine 2′(3′)-phosphate was electrophoresed at the same time. The tRNA hydrolysate showed one radioactive spot that migrated coincidently with the authentic marker (+15·0 cm). The radioactive nucleotide was dephosphorylated with bacterial alkaline phosphatase and this material was mixed with authentic N^6-(Δ^2-isopentenyl)adenosine and the mixture was chromatographed in solvent system I. The radioactive material migrated coincidently with the authentic nucleoside.

The nucleoside-containing hydrolysate (solution B) (1800 cpm) was mixed with six OD_{270} units of authentic N^6-(Δ^2-isopentenyl)adenosine and the mixture was chromatographed on a partition column of Celite-545 according to the method of Robins *et al.*[14] All the radioactivity was eluted in the fraction containing N^6-(Δ^2-isopentenyl)adenosine. The isolated nucleoside was chromatographed on paper in several solvent systems. The radioactive spots and the u.v. absorbing spots coincided in every system (Table 2). This sample was also electrophoresed at pH 2·7 for 2 hr (28 V/cm). Both the isolated nucleoside and an authentic sample of N^6-(Δ^2-isopentenyl)adenosine migrated 13·1 cm (−).

N^6-(Δ^2-Isopentenyl)adenosine undergoes a unique reaction in the presence of hydrochloric acid;[4, 14] therefore, as final confirmation of the identity of the radioactive nucleoside,

[14] M. J. ROBINS, R. H. HALL and R. THEDFORD, *Biochemistry* **6**, 1837 (1967).

a sample was mixed with 3·0 A_{270} OD units of N^6-(Δ^2-isopentenyl)adenosine and this mixture was hydrolyzed with 200 μl of 1 N HCl in a sealed tube at 100° for 15 min. The reaction was chromatographed on Whatman No. 1 paper for 19 hr in solvent system 1. The two radioactive spots migrated coincidently with the two major hydrolytic products, N^6-(3-hydroxy-3-methylbutyl)adenine and 3*H*-7,8,9-trihydropyrimido[2,1-i]purine. The spot corresponding to N^6-(3-hydroxy-3-methylbutyl)adenine was eluted and rechromatographed on Whatman No. 1 paper in solvent systems 2 and 4. In both systems the radioactive spot and that of N^6-(3-hydroxy-3-methylbutyl)adenine migrated together.

The data show that the radioactive label of the mevalonic acid is incorporated into the tRNA, and further, that this radioactivity is located exclusively in the N^6-(Δ^2-isopentenyl)-adenosine residues. This result indicates that the tRNA of the tobacco pith tissue contains N^6-(Δ^2-isopentenyl)adenosine and that its biosynthetic pathway is analogous to that established for the pathway in *Lactobacillus acidophilus*, yeast and mammalian tissue.[11–13]

TABLE 2. PAPER CHROMATOGRAPHY

Compound	Solvent system (R_f values)					
	1	2	3	4	5	6
Mevalonic acid	0·16	0·07	0·88	0·60	0·81	0·75
N^6-(Δ^2-Isopentenyl)adenosine	0·82	0·80	0·77	0·86	0·75	0·91
N^6-(Δ^2-Isopentenyl)adenosine 2′(3′)-phosphate	0·04	0·08	0·88	0·54	0·77	0·80
Adenosine 2′(3′)-phosphate	0·01	0·02	0·83	0·16	0·43	0·46
Ribosylzeatin	0·64	0·43	0·80	0·77	0·62	0·82
Zeatin	0·74	0·62	0·63	0·77	0·73	0·80
N^6-(3-hydroxy-3-methylbutyl)-adenine	0·73	0·56	0·59	0·79	—	—
3H-7,7-Dimethyl-7,8,9-trihydropyrimido [2,1-i]purine	0·49	0·07	0·59	0·61	—	—

(1) nBuOH–H_2O–conc. NH_4OH (86:14:5); (2) ethyl acetate–1-propanol–water (4:1:2); (3) EtOH–0·1 M ammonium borate (pH 9) (1:9); (4) 2-PrOH–H_2O–conc. NH_4OH (7:2:1); (5) *t*-BuOH–formate–H_2O (20:5:8); (6) 1-PrOH–conc. NH_4OH–H_2O (55:10:35).

Growth of Pith Tissue in Presence of N^6-(Δ^2-*Isopentenyl*)*Adenosine and* [2^{14}C]-*Mevalonic Acid*

Tobacco pith tissue was grown 35 days on media containing DL-mevalonic acid[2-^{14}C] (12 μmoles/l) and N^6-(Δ^2-isopentenyl)adenosine (1·5 μmoles/l) (40 flasks). The average yield of fresh tissue was 5·1 g/flask. Tissue from all the flasks was combined and the tRNA extracted and purified. This sample of tRNA contained 28·5 cpm/OD_{260} unit; all the radioactivity was located in the N^6-(Δ^2-isopentenyl)adenosine residues. Although the specific activity of this tRNA sample is about one-half of that of tissue grown in the absence of N^6-(Δ^2isopentenyl)adenosine (Table 1) no significance can be attached to this result since the yield of tissue is about twice as much. The data suggest that if exogenously applied N^6-(Δ^2-isopentenyl)adenosine has an effect on the biosynthesis of N^6-(Δ^2-isopentenyl)-adenosine residues in tRNA the effect is more subtle than can be demonstrated by this experimental approach.

Biosynthesis of N^6-(Δ^2*Isopentenyl*)*Adenosine in Tobacco Pith tRNA* in vitro

Treatment of tRNA with potassium permanganate under mild conditions cleaves off the Δ^2-isopentenyl side-chain leaving adenosine residues in place of the N^6-(Δ^2-isopentenyl)-

adenosine residues.[13, 14] The permanganate-treated tRNA serves as a substrate for the reattachment of the Δ^2-isopentenyl group *in vitro*.[13] The presence of an enzyme system in tobacco pith tissue that catalyzes the attachment of the Δ^2-isopentenyl side-chain derived from mevalonic acid to homologous tRNA was confirmed using this experimental approach.

Permanganate-treated tRNA was incubated for 2 hr with a crude cell-free extract (see Experimental section) and DL-mevalonic acid-[2-^{14}C]. The results listed in Table 3 show that the tobacco tissue contains an enzyme that catalyzes the biosynthesis of N^6-(Δ^2-isopentenyl)-adenosine in homologous tRNA. This shows that the pathway established *in vitro* for the biosynthesis of N^6-(Δ^2-isopentenyl)adenosine in the tRNA of yeast and rat liver[13] also exists in the plant tissue. Kline *et al.*[15] have shown that in the yeast system the enzyme specifically catalyzes the attachment of the Δ^2-isopentenyl group to the adenosine residue adjacent to the 3′ end of the anticodon.

TABLE 3. *In vitro* FORMATION OF N^6-(Δ^2-ISOPENTENYL)ADENOSINE FROM [2-^{14}C]-MEVALONIC ACID

tRNA	A_{260} units of tRNA substrate	Radioactivity (cpm) RNA	Radioactivity (cpm) N^6-(Δ^2-isopentenyl)adenosine*
Tobacco—MnO_4-treated + enzyme prep.	72	1512	1008
Tobacco—MnO_4-treated + boiled enzyme prep.	65	56	—
Tobacco—untreated + enzyme prep.	68	367	—
Yeast—MnO_4-treated + enzyme prep.	115	138	—
Yeast—untreated + enzyme prep.	105	288	—

* Identified in each sample using the methods described in the *in vivo* system.

Incubation and Assay: Tobacco enzyme preparation was used for both of the tobacco and yeast tRNA incubation. 2 ml of the enzyme preparation was added to a solution of tRNA, 14 mg of Na_2 ATP (adjusted to pH 7·0), 0·001 M $MgCl_2$, 0·01 M mercaptoethanol and [2-^{14}Cl-D,L-mevalonic acid ($1{\cdot}62 \times 10^6$ cpm) in 2 ml of water (pH adjusted to 7·0 with 1 N NaOH). The mixture was incubated at 37° for 2 hr and the reaction was stopped by the addition of 4 ml of 88% phenol. The re-isolation of the RNA, and the characterization of Δ^2-isopentenyladenosine was performed as described in the methods. Replicate experiments with a separate sample of permanganate-treated t-RNA gave similar results.

In the tobacco tissue system, about 70 per cent of the incorporated radioactivity in the tRNA was identified as N^6-(Δ^2-isopentenyl)adenosine. The remaining 30 per cent of the radioactivity was located in a second product that was not identified.

The crude tobacco tissue enzyme system does not catalyze a significant amount of incorporation of radioactive label into heterologous tRNA (yeast). The enzyme system appears to incorporate a significant amount of radioactivity into untreated tobacco tRNA. The amount of radioactivity incorporated, however, was not sufficient to enable a definitive identification of the product to be made.

Effect of Mevalonic Acid on the Growth of Tobacco Pith Tissue Culture

A series of 125-ml Erlenmeyer flasks containing the basal media and mevalonic acid at varying concentrations was prepared. Ten replicates were used for each concentration of mevalonate. Each flask was inoculated with three separate pieces of tobacco tissue and the

[15] L. K. KLINE, F. FITTLER and R. H. HALL, manuscript in preparation.

flasks were incubated for 35 days at 25° in the dark. The results are summarized in Fig. 1. There is some variability in total yields within each concentration series but this can be ascribed to differences in environmental conditions and treatment of stock cultures.

The results are not due to a carry-over of cytokinin in the stock tissue because these data show a definite dose-response relationship. The favorable influence of mevalonic acid on growth at these concentrations is also evident from the physical appearance of the tissues. At concentrations between 9·0 and 18·0 μmoles/l a translucent, friable tissue, whitish in color, is obtained (Fig. 2). This morphologic appearance is similar to that obtained when the tissue is grown in the presence of low concentrations of cytokinin (3×10^{-3} to 9×10^{-3} μM kinetin).[16] Those tissues grown on the basal medium without added mevalonic acid turn brown and early necrosis is noticeable.

As a control, N^6-furfuryladenine was added at a concentration of 0·5 mg/l to one series of replicates. The fresh yields of tissue were two to five times that obtained for mevalonic acid at the optimum concentration (12 μmoles/l).

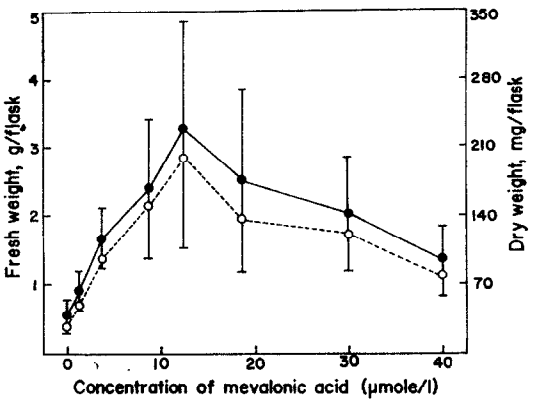

FIG. 1. THE EFFECT OF MEVALONIC ACID CONCENTRATION ON THE YIELD OF TOBACCO CALLOUS TISSUE. THE BASAL MEDIA CONTAINED 2·0 mg/l OF INDOLEACETIC ACID. GROWTH PERIOD WAS 35 DAYS. Ten replicate cultures were grown at each concentration. The open circles represent fresh weight, the closed circles dry weight. The indicated range for each concentration is for the wet weight values.

There is the possibility that the mevalonic acid samples contain trace quantities of some cytokinin-active substance that is responsible for the observed stimulatory effect. However, if we assume that mevalonic acid partially replaces the cytokinin requirement of the pith tissue, then the question may be raised as to its mode of action. Mevalonic acid serves as a source of the five-carbon building blocks of the isoprenoid constituents of plant tissue in addition to being a source of the Δ^2-isopentenyl side-chain of the N^6-(Δ^2-isopentenyl)adenosine residues in tRNA. Consequently, the synthesis of a number of isoprenoid products, including known plant hormones, may be stimulated by the exogenously supplied mevalonic acid and one of these products may have a growth stimulatory effect.

Effect of Mevalonic Acid on the Rate of Leaf Senescence

The results shown in Table 4 indicate that mevalonic acid does not replace a cytokinin as a retarder of the senescence process. This is probably due to the fact that there is little bio-

[16] F. SKOOG, H. Q. HAMZI, A. M. SZWEYKOWSKA, N. J. LEONARD, K. L. CARRAWAY, T. FUJII, J. P. HELGESON and R. N. LEOPPKY, *Phytochem.* **6**, 1169 (1967).

synthetic activity in the excised leaf and, therefore, the active cytokinin hormone is not synthesized from mevalonic acid. This result supports the inference that mevalonic acid *per se* is not an active compound but that in the tobacco tissue bioassay system where synthetic processes are occurring, mevalonic acid is converted into the active hormone.

TABLE 4. CHLOROPHYLL RETENTION OF SECTIONED BARLEY LEAVES FLOATED ON THE TEST SOLUTION FOR 4 DAYS IN THE DARK

Compound†	Chlorophyll retention (A_{665} unit/four sections compound of leaves)* at various concentrations of the test			
	4 mg/l	8 mg/l	12 mg/l	16 mg/l
Mevalonic acid	0·83	0·78	0·88	0·72
N^6-Furfuryladenine	4·29	—	3·16	—
N^6-(Δ-Isopentenyl)adenosine	1·87	—	2·41	—

* Average of three duplicates.
† The chlorophyll content of four leaf sections floated on water for 4 days in darkness is 0·91 A_{665} units.

DISCUSSION

With respect to the biosynthesis of N^6-(Δ^2-isopentenyl)adenosine, these results show that the compound is synthesized in the tobacco pith tissue by attachment of a Δ^2-isopentenyl group derived from mevalonic acid to preformed tRNA. This mode of biosynthesis is consistent with those of other modified constituents of nucleic acids. The methylated components, for example, are synthesized after formation of the macromolecule by attachment of methyl groups derived from *S*-adenosylmethionine.[17] There is no evidence that any of the modified nucleosides is synthesized by incorporation of a completed mononucleotide into the RNA chain, although this possibility cannot be discounted.

The data also demonstrate that the biosynthetic mechanism for the N^6-(Δ^2-isopentenyl)-adenosine residues in the tobacco pith tissue tRNA appears to be functioning satisfactorily. Further, the exogenous addition of N^6-(Δ^2-isopentenyl)adenosine to the tissue culture system appears to have no effect on this biosynthetic process. Therefore, is there any connection between the physiological activity of N^6-(Δ^2-isopentenyl)adenosine and its presence in the tRNA? Assuming that there is a connection, the following model might be advanced.

N^6-(Δ^2-isopentenyl)adenosine probably exerts no cytokinin activity as long as it is an integral part of the tRNA molecule, thus release of this tRNA component as the nucleoside or nucleotide through the normal catabolism of tRNA would produce a biologically active compound. The tobacco pith tissue contains enzyme systems that metabolize N^6-(Δ^2-isopentenyl)adenosine.[18, 19] Thus, the rate of release of N^6-(Δ^2-isopentenyl)adenosine from the tRNA and the rate of its metabolism would serve as a balancing mechanism for maintaining the proper level of this compound in the cell. On the basis, if there is a defect in the tobacco pith cells in the production of free N^6-(Δ^2-isopentenyl)adenosine the defect lies in the release of this nucleoside from tRNA or possibly in the balancing mechanism.

[17] E. BOREK and P. R. SRINIVASAN, *Ann. Rev. Biochem.* 35, 275 (1966).
[18] C-M. CHEN, D. M. LOGAN, B. D. MCLENNAN and R. H. HALL, *Plant Physiol.* 43, S-18 (1968).
[19] B. D. MCLENNAN, D. M. LOGAN and R. H. HALL, *Proc. Am. Assoc. Can. Res.* 9, 47 (1968).

Such a model, therefore, makes tRNA an intermediate in the biosynthesis of a cell regulatory factor. The evidence, however, does not exclude the possibility that N^6-(Δ^2-isopentenyl)adenosine can be synthesized by an alternative route and that this route is non-operative in these particular plant cells.

In summary, the results show that the mevalonic acid serves as a source of the Δ^2-isopentenyl side-chain of the N^6-(Δ^2-isopentenyl)adenosine components of the tobacco tissue tRNA and that in tobacco pith tissue that is responsive to an exogenously-added cytokinin the biosynthesis of this nucleoside is functioning normally. There is no evidence that the presence of N^6-(Δ^2-isopentenyl)adenosine in the tobacco tissue tRNA is related to a hormone-like activity. On the other hand, neither do the data negate these possibilities. Finally, there is the observation that mevalonic acid has a weak stimulatory effect in the growth of the pith cells. The relevance of this observation to the cytokinin phenomena remains obscure although the mevalonic acid may somehow be stimulating the synthesis of a cytokinin-active compound.

EXPERIMENTAL

Materials

DL-Mevalonate-[2-^{14}C] (2·8 mc/mmole) was obtained from the New England Nuclear Corp. Unlabeled DL-mevalonic acid (lactone form) was obtained from Sigma Chemical Co. N^6-(Δ^2-isopentenyl)adenosine and its degradation products were prepared in this laboratory by Mr. L. K. Kline. Zeatin, N^6-(*trans*-4-hydroxy-3-methylbut-2-enyl)adenine, and its 9-ribosyl derivative were obtained from Calbiochem. Unlabeled nucleotides and nucleosides used as reference markers were obtained from Sigma Chemical Co. Purified bacterial alkaline phosphatase was purchased from the Worthington Biochemicals Corp., and Celite-545 was obtained from the Johns-Manville Co.

Methods

Tissue culture. The origin of the tobacco tissue (*Nicotiana tobacum*, var. Wisconsin No. 38), its absolute dependence upon a cytokinin for growth *in vitro* and its method of cultivation have been previously reported.[20, 21] The tissue was incubated for 35 days on a semi-solid medium containing a mixture of minerals, vitamins, trace elements, sucrose and indole-3-acetic acid (2 mg/l.)[20, 21] This basal media did not contain a cytokinin. Mevalonic acid-[2-^{14}C] was sterilized by passage through a nalgene filter (0·2 μ plain membrane) and added to the autoclaved media. The unlabeled mevalonate was introduced into the basal media and autoclaved for 10 min at 16 lb/in^2 of pressure. Examination of an autoclaved solution by means of TLC in solvents A and B (see Chromatography) showed that no degradation of mevolanic acid had occurred.

Chlorophyll retention assay. The test of the ability to retard the senescence of excised barley leaves was performed in a manner similar to the methods decribed by Miller.[22] After germination in the dark, the barley was grown at 22° continuous illumination for 9 days. Plants were selected for uniformity and a midsection of each leaf was cut and aged for 24 hr by floating it on H_2O at 22° in darkness. The midsections were then blotted and transferred to a 125 ml flask containing 40 ml of test solution and 200 unit/ml of penicillin G. Four sections were floated in each flask and each sample was tested in triplicate. The leaf sections were incubated at 22° for 72 hr in darkness. The leaf sections from each flask were extracted with 80% EtOH and the chlorophyll content was measured by reading the absorptivity of the extracts at 665 nm.

RNA preparations, RNA was extracted from the tissue using a phenol–sodium lauryl sulfate method according to Cherry.[23] The tRNA was purified on a DEAE-cellulose column by means of a linear gradient of 0·1 to 1·2 M NaCl in 0·1 M Tris-HCl (pH 7·5) (total volume, 500 ml). The fraction eluted between 0·45–0·70 M NaCl was collected and dialyzed overnight against distilled water. Transfer RNA prepared in this manner consists of 4–5 S material, as shown by sucrose density gradient centrifugation in a perparative ultracentrifuge. It has a ratio of A280/A260 = 0·48 to 0·50. The tRNA sample accepted 17 $\mu\mu$moles of leucine per O.D.$_{260}$ unit using a crude amino acyl synthetase extract prepared from the tobacco pith tissue. The conditions of the

[20] F. SKOOG and C. O. MILLER, *Symp. Soc. Exptl Biol.* **11**, 118 (1957).
[21] J. E. FOX, *Physiol. Plant.* **16**, 793 (1963).
[22] C. O. MILLER, in *Modern Methods of Plant Analysis* (edited by H. F. LINSKENS and M. V. TRACEY), Vol. 6, p. 194, Springer Verlag, Berlin (1963).
[23] J. H. CHERRY, *Science* **146**, 1066 (1964).

assay were similar to those used to charge yeast tRNA. tRNA was separated by the method described by Barlow and Mathias.[24]

Treatment of tRNA with permanganate. A solution of 200 A_{260} units of tobacco pith tissue tRNA or yeast tRNA in 5 ml H_2O was treated with 0·5 ml or 0·2 ml of 0·1% $KMnO_4$ for 15 min at room temparature. The tRNA was precipitated by adding K acetate (2 mg/ml) and 2·5 volume of 95% cold ethanol and kept for 5 hr at −15°. The tRNA was collected by centrifugation, washed twice with 95% cold EtOH, twice with Et_2O, and redissolved in H_2O.

Preparation of N^6-(Δ^2-*isopentenyl)adenosine biosynthetic enzyme extracts.* All operations were done at 4°. 100 g of fresh normal tobacco pith tissues grown for 35 days on standard culture media containing N^6-(Δ^2-isopentenyl)adenosine as the cytokinin was mixed with 100 ml of cold buffer, 0·05 M K phosphate, 0·005 M $MgCl_2$, 0·02 M mercaptoethanol, pH 7·0. The mixture was homogenized in a Waring blender at maximum speed for three 1-min periods spearated by 2-min cooling intervals, and centrifuged for 40 min at 30,000 × *g*. The supernatant solution was decanted and further concentrated with UM-10 Diaflo* membrane for 4 hr. The concentrated enzyme extract containing 18 mg protein per ml was readjusted to pH 7·0 with 2 N NH_4OH, and was used for incubation immediately.

Measurement of Radioactivity. Samples were counted in a Beckman model LS-200 B scintillation spectrometer. Counting efficiency was about 80 per cent.

Chromatography

Paper chromatography was conducted on Whatman No. 1 paper in the descending fashion. The solvent systems (v/v) are given in Table 2.

TLCs was used for the unlabeled mevalonic acid separations which were carried out on silica gel in the following solvent systems (v/v):

A. n-ProH–conc. NH_4OH (7:3),
B. 2-ProH–conc. NH_4OH–H_2O (6:3:1).

Visualization agent: 0·04% bromocresol purple in ethyl alcohol.

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* Trade name of Amicon Corporation, Lexington, Mass., U.S.A.

[24] J. BARLOW and A. P. MATHIAS, in *Procedures in Nucleic Acid Research* (edited by G. L. CANTONI and D. R. DAVIES), p. 444, Harper and Row, New York, N.Y.