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CYTOKININ BIOSYNTHESIS IN CROWN GALL TISSUE OF *VINCA ROSEA*: METABOLISM OF ISOPENTENYLADENINE

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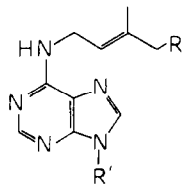
Key Word Index—*Vinca rosea*; Apocyanaceae; crown gall tissue; cytokinin biosynthesis; isopentenyladenine metabolism.

Abstract—When isopentenyl[8-^{14}C]adenine was incubated with crown gall tumour tissue of *Vinca rosea*, it was stereospecifically hydroxylated to *trans*-zeatin and its derivatives, which are the endogenous free cytokinins in this tissue. Adenine, adenosine and adenine nucleotides were the major degradation products.

INTRODUCTION

The metabolism of 6-(3-methyl-2-butenylamino)purine, commonly known as isopentenyladenine (IP, **1**) and its riboside (IPA, **2**) has been studied in several plant systems. In general they undergo one or more of three basic reactions: cleavage of the isopentenyl side chain [1–6], ring substitution [2, 7–9] and *trans*-hydroxylation of the terminal methyl group in the side chain [10, 11]. The first reaction leads to a complete inactivation of the cytokinin molecule, the second results in a slight lowering of activity and the third results in an increase in the cytokinin activity [12] and is known to occur in only a few systems. These reactions are probably very important in controlling endogenous cytokinin levels. Metabolism of isopentenyladenine, to 6-(4-hydroxy-3-methyl-2-butenylamino)purine or zeatin (Z, **3**), in the culture medium of *Rhizopogon roseolus* [10] and of isopentenyladenosine, to 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine or zeatin riboside (ZR, **4**), by *Zea mays* endosperm and *R. roseolus* [11] are examples of the hydroxylation reaction. This conversion may also occur in the phloem exudate of a detached inflorescence stalk of *Yucca* [8].



	R	R′
1	H	H
2	H	β-D-ribosyl
3	OH	H
4	OH	β-D-ribosyl
5	*O*-β-D-glucosyl	H
6	*O*-β-D-glucosyl	β-D-ribosyl

In our earlier study of cytokinin biosynthesis in crown gall tumour tissue of *Vinca rosea*, a number of cytokinins endogenous to the tissue were labelled when [^{14}C] adenine was supplied [13]. These included *trans*-zeatin, *trans*-zeatin riboside, their side chain *O*-glucosides (ZOG, **5** and ZROG, **6**) and ribosyl-*trans*-zeatin monophosphate (ZMP). Although isopentenyladenosine has been detected in minute amounts in this tissue [14], incorporation of label from adenine into isopentenyladenine, its riboside or ribotide was never detected [13].

Work on cytokinin biosynthesis from other *in vivo* [15, 16] and *in vitro* [17–19] systems would suggest IP type compounds to be the first cytokinins synthesized. If this was the case in *V. rosea* crown gall tumour tissue the hydroxylation step leading to their further conversion to Z type cytokinins from adenine must be accomplished very rapidly. The aim of this project was to see if IP could be a precursor of the Z type cytokinins and to ascertain whether or not it could be stereospecifically hydroxylated to *trans*-Z and/or its derivatives.

RESULTS AND DISCUSSION

Table 1 shows the results of a time-course study in which *V. rosea* crown gall tumour tissue (5 weeks after subculture) was incubated with [8-^{14}C]IP for 1, 3 and 8 hr. The radioactivity was rapidly taken-up by the tissue and following 8 hr incubation less than 10% of supplied label remained in the medium. At all times most of the radioactivity extracting into Bieleski solvents (see Experimental) was present in adenine nucleotides and cytokinin nucleotides (compounds that yielded corresponding ribosides after treatment with alkaline phosphatase). The conversion of IP to Z has been demonstrated in the culture medium of *R. roseolus* [10]. The data presented in Table 1 clearly show that crown gall tissue of *V. rosea* is also capable of carrying out this conversion. As early as 1 hr after the start of the incub-

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Table 1. Metabolism of IP by *V. rosea* crown gall tissue

Time (hr)	Uptake	Z	ZR	Z Ntd	Ade	Ado	Ade Ntd	IP	IPA	IP Ntd
1	61.73	0.40	0.03	0.54	1.84	1.18	6.29	6.07	0.36	6.60
3	83.25	0.47	0.14	0.97	1.74	2.07	9.31	1.50	—	18.88
8	93.97	0.39	0.53	3.14	1.97	3.25	17.81	—	—	6.22

10 g ft. wt of tissue was supplied with 10 μg(4×10^5 dpm) of [8-^{14}C]IP for 1,3 and 8 hr. Following incubation the tissue was extracted and the analysis of metabolites was done as described in the text. The radioactivity is expressed as a percentage of dpm added initially to the incubation medium. Ntd: Nucleotides, for other abbreviations see text.

ation radioactivity was detectable in Z, ZR and Z phosphate(s). While the total radioactivity in Z type cytokinins increased with time, the label in IP type compounds was found to decrease substantially. After only 8 hr of incubation only 6% of supplied radioactivity was present as IP nucleotide(s), while IP and its riboside were not detectable. Analysis of *n*-butanol soluble materials (following 8 hr incubation) on a column of Sephadex LH-20 revealed that *ca* 1.25% of the radioactivity administered was present in the elution volume of cytokinin glucosides (ZOG and ZROG, fraction A1). However, no radioactivity was detected in this region after 1 and 3 hr of incubation. Following β-glucosidase treatment of fraction A1 (see Experimental) radioactivity cochromatographed with Z and ZR on 2D-TLC.

Enzymatic hydrolysis of the nucleotide fraction (3 and 8 hr after the start of incubation) followed by Sephadex LH-20 chromatography showed that some radioactivity eluted in fractions 9–13 (inosine elutes in this region). After 2D-TLC of this fraction the major radioactive spot cochromatographed with inosine. This was probably derived from the hydrolysis of phosphate ester(s) of inosine. Similarly, free inosine was found in the base and riboside fraction (fraction A, see Experimental).

The identity of metabolites, shown in Table 1, was based on cochromatography on a column of Sephadex LH-20 (eluted with solvent F) followed by 2D-TLC on Si gel (solvents A and B). The identity of these compounds was also confirmed by cochromatography with authentic standards on TLC plates developed in either solvent system D or E. These two solvent systems clearly separate *cis*- and *trans*-isomers of Z and ZR [21] and the radioactivity always comigrated with the *trans*-isomers.

A similar pattern of metabolism was observed after 1, 3 and 8 hr of incubation when 5 μg (*ca* 2.0×10^5 dpm) of [8-^{14}C]IP was fed to 3.5-week-old tissue (10 g). The uptake of label by the tissue was 59, 83 and 95% after 1, 3 and 8 hr of incubation, respectively.

In a later study, HPLC was used for further examination of the various metabolites. In these experiments, 7-week-old tissue (10 g fr. wt) was supplied with [8-^{14}C]IP (15.0 μg; *ca* 6.2×10^5 dpm) for 3 hr (uptake 86%). The tissue extraction was done as before except that the butanol partition step was replaced by cation exchange chromatography. Preliminary fractionation of fraction A and alkaline phosphatase treated fraction B was achieved on a column of Sephadex LH-20 and further analysis of appropriately combined fractions was carried out by reversed-phase HPLC. Various metabolites (Ade, Ado, Z, ZR, IP and IPA) are well separated by this technique. A typical HPLC analysis of fraction A2 (elution volume of ZR and Ado) from the column of Sephadex LH-20 is shown in Fig. 1. This system clearly resolves *cis*- and *trans*-isomers of Z and ZR. The radioactivity was always found to be associated with the *trans*-isomers only. Dihydrozeatin (DHZ) and dihydrozeatin riboside (DHZR) coelute with *cis*-Z and *cis*-ZR, respectively, under the conditions of HPLC analysis used. Neither the dihydro derivatives nor the *cis*-isomers of Z and ZR were formed as metabolites of IP.

The radioactivity remaining in the medium following incubation was also analysed. After initial purification on a cellulose phosphate column, further analysis was carried out on a column of Sephadex LH-20 followed by HPLC. IP, IPA, *trans*-ZR, Ade, Ado and *trans*-Z (in decreasing amounts) were identified. A similar leakage of endogenous cytokinins into the culture medium of *V. rosea* crown gall tissue [22] and of radiolabelled cytokinins [13], produced as metabolites of externally applied [^{14}C]Ade, has been reported.

Externally applied IP appears to undergo rapid metabolic conversion to phosphate esters in *V. rosea* crown gall tumour tissue. These might include the mono-, di- and triphosphate derivatives as in cell suspensions of tobacco and *Acer* [2, 9]. The early rise of radioactivity in the nucleotide fraction would suggest a direct conversion of IP to its nucleotide(s). Adenine phosphoribosyl transferase, which has been partially purified from this tissue [23], or a related cytokinin specific transferase may bring about this conversion.

The observed metabolism of IP to Z and its derivatives in crown gall tissue of *V. rosea* is stereospecific. A similar conversion of IPA to ZR and its derivatives has been reported in *Zea mays* and *Rhizopogon roseolus* [11]. After only 8 hr of incubation the label in *trans*-zeatin derivatives is nearly equal to that remaining in IP derivatives. However, it is not clear whether the *trans*-hydroxylation takes place at the base, riboside or ribotide level, or at all the three levels.

In vivo breakdown of externally applied IP, Z or their derivatives is very well documented in the literature (e.g. see refs. [24] and refs. therein). This reaction involves cleavage of the isopentenyl side chain resulting in the formation of adenine and/or its derivatives. An enzyme, 'cytokinin oxidase', catalyses this reaction [3, 4, 25]. After 8 hr of incubation the radioactivity in Ade and its derivatives was approximately four times that remaining in IP derivatives. When labelled Z was supplied to *V. rosea* crown gall tissue a similar breakdown, but to a lesser extent, was observed [unpublished results]. This is in agreement with the finding of Whitty and Hall [4] that although Z and ZR were degraded by cytokinin oxidase,

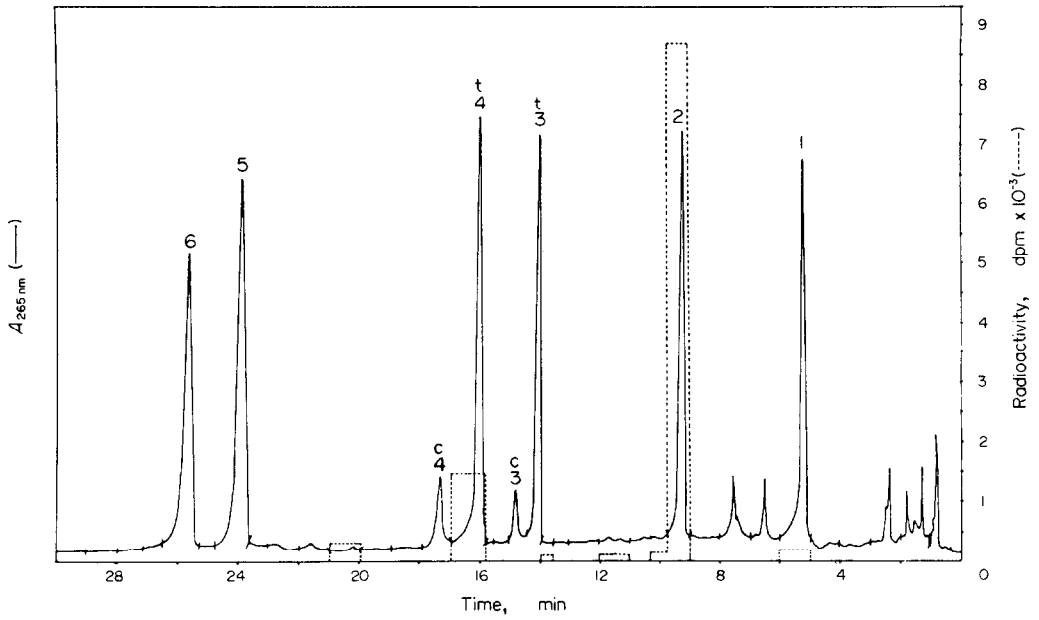

Fig. 1. *trans*-Hydroxylation of isopentenyl[8-^{14}C]adenine by *V. rosea* crown gall tissue. Analysis of a portion of fraction A2 (elution volume of Ado and ZR on Sephadex LH-20 column) by reversed-phase HPLC. Standards (1–6) were mixed with the sample prior to injection. Column: Hypersil ODS (150 × 4.5 mm); flow rate: 2 ml/min; mobile phase: three segment linear gradient, water (pH 7 with triethylammonium bicarbonate) to 10% acetonitrile over 10 min, from 10 to 20% acetonitrile over 15 min, and from 20 to 30% acetonitrile over 5 min. (1) Ade; (2) Ado; (3) Z; (4) ZR; (5) IP; (6) IPA. c and t, Represent *cis*- and *trans*-isomers, respectively. Fractions were collected as marked on the UV trace for radioactivity measurements. Radioactivity is not shown in fractions containing less than 100 dpm.

IP and its derivatives were the most efficiently utilized substrates.

As little as 3 hr after the start of the incubation, the presence of polar metabolites, such as inosine and its phosphate ester(s), were detectable. Cytokinins do not appear to be good substrates for adenosine deaminases in plants [26], hence further breakdown of IPA to inosine possibly occurs after the cleavage of the side chain, i.e. via adenosine.

In contrast to tobacco cells [2, 7], the metabolism of IP to its 7-glucoside was not detected in crown gall tissue of *V. rosea*. Although in tobacco cells some degradation of externally applied IP and IPA to Ade compounds occurred, their hydroxylation to Z compounds was minimal, thus possibly allowing further metabolism to 7-glucoside in significant amounts [2]. Glucosylation may render some protection to the N^6-isopentenyl side chain from cleavage by cytokinin oxidase [24, 27]. Side chain *O*-glucosides of Z and ZR which begin to appear 8 hr after the start of incubation may simply represent their further metabolites.

Taya *et al.* [17] using a cell-free system from *Dictyostelium discoideum*, have shown that 5′-adenosine monophosphate (AMP) is the acceptor of the Δ^2-isopentenyl side chain in the biosynthesis of isopentenyladenosine monophosphate (IPMP). A similar conversion, resulting in the formation of IPMP, has also been demonstrated using a partially purified enzyme preparation from cytokinin-autonomous tobacco tissue [18]. Once formed, IPMP or its derivatives could be further metabolized to Z type cytokinins as shown in this study. Chen *et al.* [14], using a method to detect very low levels of IPA, identified it as an endogenous cytokinin in *V. rosea* crown gall tissue. However, in all our investigations biological activity in fractions possibly containing IP (or its derivatives) could not be observed [13]. Perhaps, because of the speed of hydroxylation reaction, steady state concentrations of IP and its derivatives may be very low. This may also be the reason why IP type compounds could not be detected in our biosynthetic work. In contrast, IPA has also been identified as an endogenous compound in cytokinin-autonomous tobacco tissue [28] but very little hydroxylation of the isopentenyl side chain was reported in this tissue [2]. This may account for the detection of considerable radioactivity in IP, in addition to Z and ZR, 5 hr after cytokinin-autonomous tobacco callus tissue was incubated with [^{14}C]Ade [16].

Several attempts were made to demonstrate the presence of label in IP and/or its derivatives by 'cold trapping'. However, attempts to cold trap radioactivity in IP type compounds, by the addition of different amounts of unlabelled IP to the tissue at various times during the incubation with [^{14}C]Ade, were unsuccessful (results not shown here). The addition of unlabelled IP to the system resulted in a marked increase of adenine catabolism and conversion to more polar compounds, e.g. inosine was noticed. This highlights the problems of investigations into the pathways of plant hormone biosynthesis, as only small amounts of added hormone can totally upset metabolism of the compound of interest and channel it in other directions. A several-fold increase in the degradation of [8-^{14}C]IPA in tobacco cells was observed when the cells had been exposed to the same or other cytokinins [6].

The results of this study suggest that IP type cytokinins are likely intermediates in the synthesis of Z type cyto-

kinins in *V. rosea* crown gall tissue. However, the possibility that Z type cytokinins may be formed directly from adenine or its derivatives cannot be ruled out.

EXPERIMENTAL

Plant material and chemicals. The *V. rosea* L. A6 line of crown gall tumour tissue (the generous gift of Professor C. O. Miller) was grown in 500 ml conical flasks containing 200 ml hormone-free medium of Miller [29] solidified with 0.75% agar. The cultures were maintained under diffused light (6 lm/ft^2) at 25° (± 1) and were subcultured every 6–8 weeks. For metabolic studies 3–7-week-old tissue was used.

All the cytokinin standards were synthesized by RH. The solvents used were of analytical grade and were redistilled before use.

Synthesis of N^6-(Δ^2-*isopentenyl*) [8-^{14}*C*]*adenine* (3.6 *mCi/mmol*). [8-^{14}C]IP was prepared by the condensation of 3-methyl-2-butenylamine with 6-chloro[8-^{14}C]purine (Amersham) according to the method of Hall and Robins [30]. The recrystallized product (once from H_2O and once from EtOH–H_2O, 1:25) was purified by TLC on Si gel (1 mm, pre-washed for 72 hr in 90% EtOH) developed in solvent C. Following autoradiography the radioactive zone (R_f 0.54–0.62) cochromatographing with the IP marker was eluted and stored in 80% EtOH at 0°. The purity of labelled IP was checked on TLC (Si gel) in solvents A–E and in later expts by reversed-phase HPLC on a column of Hypersil ODS just before use.

Metabolic studies: purification and analysis of metabolites. 10 g tissue was transferred aseptically to a 100 ml conical flask containing 10 ml liquid medium and [8-^{14}C]IP (filter sterilized). Flasks were incubated on a reciprocating shaker (60 rpm) at 25° in the dark and after the appropriate time (1, 3 and 8 hr) tissue was removed by filtration, washed with 10 ml H_2O and immediately dropped into 100 ml cocktail 1 ($CHCl_3$–MeOH–HCO_2H–H_2O, 5:12:1:2; −20°; ref. [20]). The next day, the tissue was removed by filtration and homogenized in 100 ml cocktail 2 (MeOH–HCO_2H–H_2O, 6:1:4; −20°) in a Virtis blendor (half speed, 2 × 30 sec). After standing overnight at −20° the residue was filtered off and the two filtrates combined. At this point 100 μg each (in 80% aq. EtOH, of which the concn was determined by UV *A*) of the int. standards Z, ZR, IP, IPA, adenine (Ade) and adenosine (Ado) were added to the extract. The combined filtrates were evaporated to dryness by rotary film evaporation (RFE) at 30° and the residue taken-up in 20 ml H_2O (pH to 8.2 with 1N NaOH) and filtered. The filtrate was partitioned with H_2O-satd *n*-BuOH (6 × 20 ml). The materials extracting into *n*-BuOH (fraction A) and those remaining in the aq. phase (fraction B) were examined as described below. In some expts the partition step was replaced by CC on a cation exchanger (cellulose phosphate, Whatman P1 floc type, 100 ml, equilibrated to pH 3.1 in the NH_4^+ form) as in ref. [22]. The H_2O wash and the NH_4OH eluate from the column were equivalent to fractions B and A, respectively, of the BuOH partition step.

Fraction A. This fraction (combined BuOH layers or NH_4OH eluate) containing purine bases, ribosides and glucosides was evaporated to dryness by RFE at 30°. The residue was taken-up in 1.5 ml 35% aq. EtOH and fractionated on a column (85 × 2.5 cm) of Sephadex LH-20 (bead size 25–100 μM, Pharmacia, continually eluted with solvent F; flow rate 30 ml/hr) as reported earlier [13]. 50 1-hr fractions were collected and an aliquot from each was counted in a Beckman LS-200 scintillation counter using 10 ml scintillant (toluene–Triton X-100, 2:1; 4 g PPO/1.5 l.). A UV trace ($A_{254\,nm}$) of column eluate permitted detection of the added int. standards which eluted as follows. Fractions 9–13 (fraction A1): cytokinin glucosides (int. standards not added) known to elute in these fractions; fractions 14–16 (fraction A2): Ado and ZR; fractions 18–20 (fraction A3): Ade and Z; fractions 23–26 (fraction A4): IPA; fractions 29–32 (fraction A5): IP.

These fractions were appropriately combined as indicated above and evaporated to dryness by RFE at 30°. Suitable aliquots of fractions A2–A5 together with 20 μg of each of the six standards which they lacked were applied to analytical Si gel TLC plates. The plates were developed in the first dimension with solvent A and in the second with solvent B. The plates were viewed under short-wavelength UV for detection of standards and the radioactive spots were located by a radiochromatogram spark chamber (Birchover Instruments, U.K.). Areas of the plate corresponding to the appropriate standards were scraped off, eluted with 80% aq. EtOH (4 × 1 ml) and made up to a final vol. of 5 ml. An aliquot was used for counting radioactivity, and a UV spectrum of the eluted material was recorded to measure the recovery of the added int. standards. The radioactivity was then corrected for purification losses. The radioactivity cochromatographing on 2D-TLC plates with Ade, Ado, Z, ZR, IP and IPA was also coincidental with the appropriate standards on Si gel plates developed in solvent D or E.

Fraction A1 corresponding to the known elution vol. of cytokinin glucosides ZOG and ZROG was evaporated to dryness and treated with β-glucosidase (from sweet almonds, suspension in 3.2 M NH_4SO_4, Boehringer; 10 μl enzyme in 250 μl 0.1 M NaOAc buffer, pH 5.4, 15 hr at 37°). The mixture was heated to 60° to terminate the enzyme reaction and evaporated to dryness under a stream of N_2. 20 μg each of the six standards was added to the hydrolysed sample which was then directly analysed by 2D-analytical TLC as described for fractions A2–A5.

Fraction B. Materials not extracted into BuOH from aq. soln at pH 8.2 or washed from the cellulose phosphate column with H_2O included purine nucleotides. The sample was evaporated to dryness by RFE at 30°, dissolved in 5 ml ethanolamine–HCl (0.03 M containing 10 mM $MgCl_2$, pH 9.5) and alkaline phosphatase (25 μl, calf intestinal mucosa, Sigma) was added. After incubation for 20 hr at 37°, 100 μg each of the six standards (Z, ZR, IP, IPA, Ade and Ado) were added and the soln was evaporated to dryness. All the standards and ribosides released by enzymatic hydrolysis were purified either by extraction with *n*-BuOH or cation exchange chromatography as described earlier. Further analysis of appropriate fractions by 2D-TLC following chromatography on a column of Sephadex LH-20, measurement of radioactivity and its correction for losses was achieved as for fraction A.

It should be noted that the recovery of glucosides and nucleotides could not be measured accurately because of the non-availability of standards and, thus, the reported values are certainly underestimates of the true radioactivity present in these compounds.

Chromatographic procedures. TLC: air-dried Kieselgel PF_{254} (Merck). The solvents used were as follows: (A) *n*-BuOH–HOAc–H_2O (12:3:5); (B) *n*-BuOH–1 4 N NH_4OH–H_2O (6:1:2, upper phase); (C) *sec*-BuOH–25% NH_4OH (4:1); (D) $CHCl_3$–MeOH (9:1); (E) $CHCl_3$–HOAc (4:1); (F) aq. EtOH (35%, degassed before use).

HPLC: Pye LC 3X system (Pye–Unicam, Cambridge); reversed-phase Hypersil ODS column (150 × 4.5 mm i.d.) with gradient-elution; sample injections were made via an Altex 905–42 syringe loading sample injector fitted with a 100 μl loop. Other details were as in ref. [31].

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