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Cytokinin biosynthesis in crown-gall tissue of *Vinca rosea*

The significance of nucleotides

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Abstract. When care was taken to minimise the effects of phosphatase activity during extraction of *Vinca rosea* crown-gall tumour tissue, a large proportion of extractable cytokinin activity was present in the nucleotide fraction. Analysis using ion-exchange chromatography followed by enzymic or chemical degradation and subsequent identification of the biologically active material indicated that this activity was due to zeatin riboside 5′-monophosphate. This was also the major radiolabelled cytokinin formed when this tissue was supplied with [^{14}C]adenine. The incorporation of radioactivity from [^{14}C]adenosine into free cytokinins was also shown, but no incorporation of radioactivity was found when [^{3}H]mevalonic acid lactone was supplied to this tissue under the same conditions. In parallel experiments using normal stem callus tissue of *V. rosea*, no incorporation of [^{14}C]adenine into free cytokinins was observed. The significance of these results is discussed in relation to a possible transfer-RNA-independent pathway of cytokinin biosynthesis, operating primarily at the mononucleotide level.

Key words: Crown gall – Cytokinin biosynthesis – Nucleotide – *Vinca*.

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Abbreviations: DEAE = diethylaminoethyl cellulose; HPLC = high-performance liquid chromatography; TEAB = triethylammonium bicarbonate; TLC = thin-layer chromatography; ZR = zeatin riboside; ZMP = zeatin riboside 5′-monophosphate

Introduction

Cytokinins are potent promoters of cell division and like other principal phytohormones, also elicit a wide spectrum of other physiological responses. Crown-gall tumour tissue of *Vinca rosea* L. is a particularly suitable system for studies of the biosynthesis and metabolism of cytokinins (Horgan et al. 1981). Recent work (for review see Letham and Palni 1983) has indicated the importance of cytokinin nucleotides. A number of cytokinins have been identified unambiguously in *V. rosea* crown-gall tissue (Miller 1974, 1975; Peterson and Miller 1976, 1977; Morris 1977; Scott et al. 1982) and a substantial proportion of these apparently leak out into the medium during culture (Palni and Horgan 1982). However, the significance of cytokinin nucloetides in this tissue is not known.

The nature of endogenous cytokinin nucleotides in crown-gall tumour tissue of *V. rosea* and the role of nucleotides in the biosynthesis of free cytokinins are the subjects of the present investigation.

Material and methods

Plant material

Vinca rosea L. The A6 line of crown-gall tumour tissue (gift of Professor C.O. Miller, Indiana University, Bloomington, USA) was grown in 500-cm^3 conical flasks containing 200 cm^3 hormone-free medium of Miller (1974) solidified with 0.75% agar. The normal stem callus tissue of *V. rosea* was initiated from glasshouse-raised plants and grown on medium containing 2 mg l^{-1} N^6-benzylaminopurine and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (Palni, 1980). The cultures were maintained as described by Palni and Horgan (1983). In the present study 4- to 6-week-old tissue was used.

Chemicals and solvents

All solvents were of analytical grade and glass-distilled before use. Triethylammonium bicarbonate (TEAB) was prepared by

saturating a 2.5 M solution of triethylamine (Sigma Chemical Co., St. Louis, MO, USA) with carbon dioxide.

[U-^{14}C]Adenine (10.58 GBq mmol^{-1}), [U-^{14}C]adenosine (21 GBq mmol^{-1}), [8-^{14}C]adenosine 5′-monophosphate (2.26 GBq mmol^{-1}) and DL-[2-^{3}H]mevalonic acid lactone (14.13 GBq mmol^{-1}) were obtained from the Radiochemical Centre, Amersham, Bucks., UK.

Adenine; adenosine; AMP; ADP; ATP; tubercidin (7-Deaza adenosine); isopentenyl adenine; isopentenyl adenosine were obtained from Sigma. The following cytokinins were synthesized by condensing the appropriate side-chain amines with 6-chloropurine or its riboside (Sigma): zeatin (*trans-* and *cis-* isomer); zeatin riboside (ZR, *trans-* and *cis-* isomer); dihydrozeatin; dihydrozeatin riboside; and the O-glucosides of zeatin, zeatin riboside and their corresponding dihydro derivatives. Zeatin riboside 5′-monophosphate (ZMP) was prepared by enzymic phosphorylation of *trans*-ZR according to Giziewiez and Shugar 1978. The product identity was based on its behaviour during thin-layer chromatography (TLC) and enzymic or chemical degradation to ZR or zeatin respectively.

Metabolic studies

Callus tissue was transferred aseptically to a 100-cm^3 conical flask containing liquid medium (10 cm^3 g^{-1} fresh weight (FW) of tissue) and radioactive compound (sterilized by autoclaving for 15 min at 121° C and 1.1 kg cm^{-2}). Incubation flasks were kept on a reciprocating shaker (60 rev min^{-1}) at 25° C. After an appropriate time, the tissue was removed by filtration on a Büchner funnel, washed with 20 cm^3 water and extracted as described below.

Extraction and purification of cytokinins-metabolites

Immediately after harvest, the callus pieces were dropped into chloroform:methanol:formic acid:water (5:12:1:2, by vol.; 10 cm^3 g^{-1} FW; −20° C; Bieleski 1964) and left overnight at −20° C. The tissue was removed by filtration and homogenized using a Waring blendor (or Virtis when less than 30 g tissue was used) in methanol:formic acid:water (6:1:4, by vol.; 10 cm^3 g^{-1} FW; −20° C). After standing overnight at −20° C, the residue was filtered off and washed with a small quantity of 80% methanol. The filtrates were combined and dried by rotary-film evaporation (RFE)[1]. At this stage, known amounts of internal standards were added for recovery estimations. The residue was dissolved in distilled water (2 cm^3 g^{-1} FW of tissue) and centrifuged at 15,000 *g* for 20 min. The supernatant was collected, its pH adjusted to 3.5 with acetic acid, and passed through a column of PVP (insoluble polyvinylpyrrolidone, 1 cm^3 bed vol. g^{-1} FW). The column was washed with five column volumes of water (pH 3.5) and the washings were reduced to approx. 20 cm^3 by RFE. At this point, either procedure (1) or (2) or a combination of both was followed. In procedure (1), the pH of the aqueous extract was adjusted to 8.2 with 1 M NaOH and the extract was partitioned with water-saturated butan-1-ol (6 × equal vol.). The butanol fractions were pooled and dried by RFE (henceforth called Fraction A), and analysed for cytokinin bases, ribosides and glucosides. The aqueous fraction (henceforth called Fraction B) containing cytokinin nucleotides was subjected to enzymic or chemical degradation, either directly or following chromatography on a column of diethylaminoethyl (DEAE)-cellulose (DE-32 microgranular, Whatman Ltd., Maidstone, Kent, UK) before further analysis. In procedure (2), the pH of the aqueous extract was adjusted to 3.1 and loaded on to a cation-exchange column of cellulose phosphate (P1 floc type, Whatman Ltd.; 15 cm^3 bed vol. g^{-1} tissue, equilibrated to pH 3.1 in the NH_4^+ form). The column was washed with water (three column volumes at pH 3.1, followed by one column volume at pH 7.0) and then eluted with five column volumes of 2 M NH_4OH. The acidic wash and the ammonia eluate from the column are equivalent to Fraction B and Fraction A, respectively, of the butanol-partition step.

In some experiments the nucleotides were extracted by an alternative method essentially as reported by Brown (1962). The extract was finally chromatographed on a column of DEAE-cellulose. The nucleotides were eluted with 0.25 M TEAB buffer (pH 8.0) and the eluate was freed of bicarbonate by azeotropic distillation with metahnol. This fraction was analysed in the same way as Fraction B, described above.

Enzymic and chemical degradation of compounds

Cytokinin O-glucosides and nucleotides were hydrolysed by β-glucosidase and alkaline phosphatase respectively, as reported previously (Stuchbury et al. 1979; Palni and Horgan 1983). For degradation of nucleoside 5′-phosphates to bases, the sample was dissolved in 0.5 cm^3 water containing 5 mg sodium periodate. The reaction mixture was kept at 37° C for 12 h, cyclohexylamine (100 µl, Hopkins and Williams Ltd., Chadwell Heath, Essex, UK) was then added and this was incubated for a further 3 h. In all cases the reaction mixture was finally dried, taken up in water and extracted with butan-1-ol (4 × equal vol.) at pH 8.2 for further analysis.

Chromatographic methods

Thin-layer chromatography was performed on 20-cm square plates with a 0.25 or 0.5 mm thick layer of PF_{254} silica gel (Merck, Darmstadt, FRG). The anion-exchange layers (0.25 mm thick) for separation of nucleotides were prepared from commercial DEAE-cellulose (Serva Feinbiochemica, Heidelberg, FRG) containing 0.7% green fluorescent indicator (Woelm Pharma, Eschwege, FRG) or from unmodified cellulose layers impregnated with polyethyleneimine (Poly, 50% solution in water; Eastman Kodak Co., Rochester, N.Y., USA). Polyethyleneimine plates were developed in distilled water, air dried and stored in dark at 4° C before use. The solvents used were (by vol.): A. Butan-1-ol:acetic acid:water (12:3:5); B. Butan-1-ol:14 M NH_4OH:water (6:1:2, upper phase); C. Chloroform:methanol (9:1); D. Chloroform:acetic acid (4:1); E. 0.03 M HCl containing potassium chloride (0.03 M); F. Sodium formate-formic acid buffers (0.25, 2.0 and 4.0 M); G. Butan-2-ol:25% NH_4OH (4:1).

Initial fractionation was done on a column of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) eluted with 35% ethanol (Stuchbury et al. 1979). Suitable aliquots were withdrawn from each fraction for the determination of cytokinin activity or measurement of radioactivity. In biosynthetic experiments in which sufficient internal standards had been added, a UV trace ($\lambda = 254$ nm) of the eluate allowed detection of compounds of interest. The standards eluted as shown in Fig. 1 to yield five fractions (Fr.), LH-20/Fr. 1 to LH-20/Fr. 5.

The high-performance liquid chromatography (HPLC) equipment and chromatographic conditions used have been described by Horgan and Kramers (1979). The column and solvent combinations used were as follows:

(A) Reversed-phase HPLC

Hypersil SAS. Isocratic elution with water:TEAB:Acetonitrile (AcN) (100:1:5, by vol.) followed by bulk elution of cytokinins with methanol – System 1.

1 All rotary-film evaporations were carried out at 30° C

Hypersil ODS. Three-segment linear gradient, water (pH7 with TEAB) to 10% AcN over 10 min, 10 to 20% AcN over 15 min and 20 to 30% AcN over 5 min – System 2 or; two-segment linear gradient, 5% AcN (in water adjusted to pH7 with TEAB) to 20% AcN over 20 min and 20 to 45% AcN over 15 min – System 3.

(B) Adsorption HPLC

Hypersil. Isocratic elution with ammonia-saturated chloroform:methanol:water (1,050:50:3, by vol.) – System 4. This adsorbent-solvent combination was found particularly useful for the final analysis of cytokinins after their bulk elution from reversed-phase Hypersil SAS by methanol (System 1), in studies of cytokinin biosynthesis.

(C) Normal-phase partition HPLC

Partisil 10 PAC. Isocratic elution with AcN:H_2O:formic acid (100:10:0.1, by vol.) – System 5, or; chloroform:methanol:acetic acid (9:5:5, by vol.) – System 6.

Radioactivity measurements

A radiochromatogram spark chamber (Birchover Instruments Ltd., Letchworth, UK) was used for rapid location of radioactive areas on thin-layer plates. Conventional autoradiography was also done using Kodak Kodirex X-ray (no-screen) films. Liquid scintillation counting was done using either an LS-200B (Beckman Instruments, Fullerton, Calif., USA) or a PW4540 (Phillips, Eindhoven, The Netherlands) instrument, as reported by Stuchbury et al. (1979).

Ultraviolet spectroscopy

Ultraviolet spectra were recorded on an SP 800 spectrophotometer (Pye Unicam Ltd., Cambridge, UK). The optical density (Δ 542–620) determinations for *Amaranthus* bioassay were done on a Pye Unicam SP 1800 spectrophotometer.

Bioassays

Cytokinin activity was detected using soybean cotyledonary callus (Miller 1968) and *Amaranthus* betacyanin (Biddington and Thomas 1973) assays.

Results

Endogenous cytokinins

Although the special extraction procedures of Bieleski (1964) and-or Brown (1962) were used at subzero temperatures to inactivate phosphatases, this probably did not prevent cytokinin nucleotide breakdown completely; even with the Bieleski extraction method 13.7% [^{14}C]AMP was degraded to adenosine and adenine.

Figure 1 shows the results of *Amaranthus* betacyanin assay of the basic fraction containing cytokinin bases, ribosides and glucosides, and the nucleotide-derived, butanol-soluble compounds from an extract of 3.3 g FW of *V. rosea* crown-gall tis-

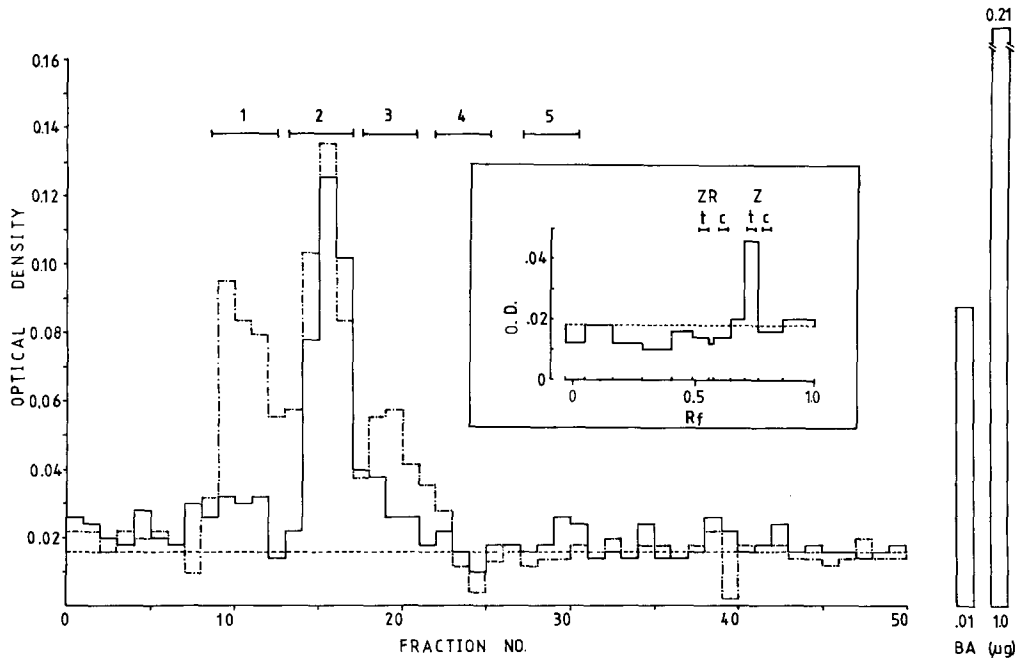

Fig. 1. *Amaranthus* betacyanin bioassay profile following Sephadex LH-20 chromatography of the endogenous cytokinins of *V. rosea* crown-gall tissue (3.3 g). Nucleotide-derived, butanol-soluble cytokinins (——); cytokinin base, riboside and glucoside fraction (-·-·-); control (-----); *BA*, N^6-benzylamino purine. Known standards eluted in the following regions: *1*, cytokinin glucosides; *2*, adenosine, ZR and dihydrozeatin riboside; *3*, adenine, zeatin and dihydrozeatin; *4*, isopentenyl adenosine; *5*, isopentenyl adenine. *Inset: Amaranthus* betacyanin bioassay following TLC of a small portion of chemically degraded mononucleotide fraction. *Z*, Zeatin; *t*, *c*, *trans-* and *cis*-isomers, respectively

Table 1. Uptake and incorporation of radioactivity into cytokinins in *Vinca rosea* crown-gall tissue. Isolation and analysis of metabolites was performed 8 h after the start of incubation. The results are expressed as a percentage of supplied radioactivity

Expt. No.[a]	Compounds added to incubation medium	Uptake of radioactivity	Incorporation into cytokinins		
			Zeatin	Zeatin riboside	Zeatin nucleotide (s)
1	[^{14}C]Adenine	95.1	0.032	0.073	0.900
2	[^{14}C]Adenine and 10 mM AMP	91.3	0.008	0.058	0.127
3	[^{14}C]Adenine and 1 μM tubercidin	90.7	0.018	0.055	0.184
4	[^{14}C]Adenosine	66.6	–	0.108	1.072
5	[^{14}C]Adenine	96.2	0.057	0.159	1.135

[a] In experiments 1–3 final analysis was by HPLC, and experiments 4 and 5 by two-dimensional TLC. The data in experiment 5 are based on the results of Stuchbury et al. (1979) and have been included for comparison

sue following Sephadex LH-20 chromatography. In the basic fraction (Fraction A) there are three clear peaks of biological activity. Peaks 1, 2 and 3 correspond to the elution volumes of cytokinin glucosides, ZR-dihydrozeatin riboside, and zeatin-dihydrozeatin respectively. The active components of these peaks have been identified previously (Miller 1974, 1975; Morris 1977; Scott and Horgan 1980; Scott et al. 1982).

The analysis of nucleotides (Fraction B) following enzymic degradation showed a major peak of biological activity in the elution volume of ZR and dihydrozeatin riboside; slight breakdown to free base was also frequently observed (Fig. 1). Thin-layer chromatography on silica-gel plates (solvent C or D) and HPLC (System 2) analysis followed by bioassay showed that the activity in the main peak was caused by *trans*-ZR. To investigate further the nature of the cytokinin nucleotides, parallel tissue extractions were done using the methods of Bieleski (1964) and Brown (1962). The nucleotides were fractionated into mono-, di- and triphosphates on a DEAE-cellulose column (see biosynthesis section for details) and treated individually with alkaline phosphatase. The resulting ribonucleosides were chromatographed on Whatman 3MM paper (developed descendingly in solvent G for 30 cm). The chromatograms were divided into ten zones and subjected to the soybean callus or *Amaranthus* betacyanin bioassay (results not presented). The majority of biological activity was present in compounds derived from the monophosphate peak and had the chromatographic mobility of ZR. Thus further analysis was limited to the monophosphate peak only. Analysis by HPLC (System 2) of a portion of alkaline-phosphatase-treated monophosphate fraction followed by *Amaranthus* bioassay revealed a peak of activity in the elution volume of *trans*-ZR. The remainder of the mononucleotide peak was degraded chemically and a portion analysed by TLC followed by bioassay. The only peak of biological activity had the R_f of *trans*-zeatin (see inset, Fig. 1), confirming that the nucleotide was in fact a 5′-monophosphate. Further evidence for the identity of ribosyl zeatin 5′-monophosphate has now been obtained by comparison with authentic standard using ion-pair HPLC and gas chromatography-mass spectrometry (Scott et al. 1982).

Cytokinin biosynthesis

a) Crown-gall tumour tissue

(i) Adenine as the precursor. The results obtained were essentially similar to those reported earlier (Stuchbury et al. 1979). Analysis carried out in the present study using high-resolution techniques provided further confirmation of incorporation of label into various cytokinins.

[^{14}C]Adenine (740 kBq) was supplied to 20 g tissue. The tissue was extracted by the method of Bieleski (1964) 8 h following the start of incubation (uptake 95.13%[2]). Cellulose-phosphate chromatography of the extract yielded Fraction A (containing cytokinin bases, ribosides and glucosides) and Fraction B (containing cytokinin nucleotides) which were analysed as follows:

Fraction A (7.55%). This was extracted with butan-1-ol, followed by fractionation on a column of Sephadex LH-20. Appropriate fractions (Fr.) were combined, and dried by rotary-film evaporation for further analysis.

The radioactivity eluting in LH-20/Fr. 1 was minimal (see Stuchbury et al. 1979) and no radioactivity eluted in LH-20/Fr. 4 and Fr. 5, hence further analysis of these fractions was not attempted. Small portions of LH-20/Fr. 2 and Fr. 3 were analysed using HPLC Systems 5 and 4 respectively. In both cases approx. 90% or more of the radioactivity eluted either with adenosine or adenine, and only a small fraction with *trans*-ZR (Fr. 2) or *trans*-zeatin (Fr. 3) as shown in Table 1.

2 Unless mentioned otherwise the radioactivity in various fractions is represented as percentage of supplied radioactivity

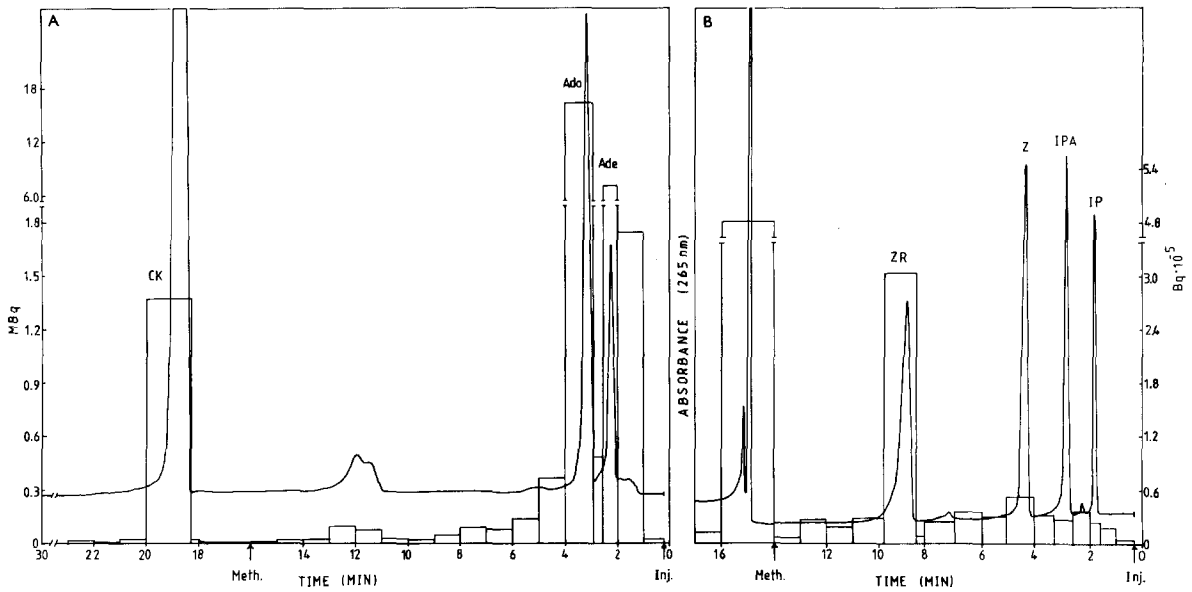

Fig. 2. **A** Bulk HPLC (System 1) of a small portion of LH-20/Fr. 2 of nucleotide-derived materials from [^{14}C]adenine feed to *V. rosea* crown-gall tissue. The bulk of radioactive compounds including adenosine (*Ado*) elute within the first 5 min and the remaining radioactive materials including cytokinins (*CK*) were then eluted with methanol (*Meth.*). **B** Further fractionation of a portion of cytokinin peak (CK) by HPLC (System 4). *Inj.*, Point of sample injection; *Z*, zeatin; *IPA*, isopentenyl adenosine; *IP*, isopentenyl adenine; *Ade*, adenine

The remainder of LH-20/Fr. 2 and Fr. 3 were analysed by two-dimensional TLC on silica-gel plates developed first in solvent A, and then in solvent B, as in our earlier study (Stuchbury et al. 1979). The radioactive zones co-chromatographing with adenine, adenosine, zeatin and ZR were scraped off and eluted separately with 80% ethanol (1 cm^3 × 5). The radiochemical purity of these TLC eluates was determined by HPLC (System 4) as follows: adenine (81%), adenosine (93%), *trans*-zeatin (37%) and *trans*-ZR (88%). Radioactivity did not co-elute on HPLC with either *cis*- isomers of zeatin and ZR, or their dihydro derivatives (dihydrozeatin and dihydrozeatin riboside). The identity of *trans*-zeatin and ZR was also confirmed by co-chromatography on silica-gel TLC plates developed in either solvent C or D.

Fraction B (42%). The sample was divided into two equal parts for further analysis. One half of the sample was treated with alkaline phosphatase followed by butanol partition and the butanol-soluble material (33%) was subjected to Sephadex LH-20 chromatography. The appropriate fractions were combined, dried by rotary-film evaporation and further examined as follows: LH-20/Fr. 1: 53% of total radioactivity eluting from the column was located in this fraction (elution volume of cytokinin glucosides). However, when this fraction was treated with β-glucosidase and rechromatographed on the same column the elution volume of radioactivity remained essentially unaltered. While no further attempt was made to characterize the radioactivity, a large proportion of it could be inosine, based on its behaviour on Sephadex LH-20 column and two-dimensional TLC (Palni and Horgan, 1983). LH-20/Fr. 2: this was subjected to analytical HPLC (System 1). Most of the radioactivity (in common purines including adenosine) eluted within the first 5 min and reached background level by approx. 15 min. At this point, the column was eluted with methanol and the remaining radioactive compounds (including cytokinins) eluted as a bulk fraction, approx. 5% of the radioactivity initially loaded onto the HPLC column eluting in this "cytokinin peak" (CK, see Fig. 2A). When a portion of the "cytokinin peak" was further analysed by HPLC (System 4), only 26% of the radioactivity co-eluted with ZR (Fig. 2B). LH-20/Fr. 3: the analysis was carried out exactly as for Fr. 2. Less than 5% of the radioactivity present in the "cytokinin peak" co-eluted with zeatin (derived from complete hydrolysis of zeatin nucleotide to the free base). The radioactivity co-eluting with ZR and zeatin was combined to represent incorporation into zeatin nucleotide as shown in Table 1. LH-20/Fr. 4 and Fr. 5: no radioactivity was present in these fractions.

The other half of Fraction B was taken up in 2 cm^3 of TEAB (0.02 M, pH 9.0) and unlabelled standards (AMP, ADP and ATP, 100 μg each) were added. The sample was loaded onto a column

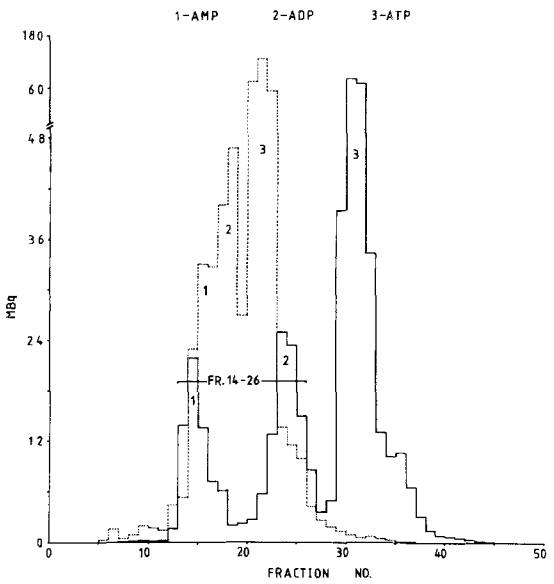

Fig. 3. Fractionation of [^{14}C]adenine-derived radioactivity in the nucleotide fraction using a DEAE-cellulose column (40 cm long, 1 cm diameter) eluted with a linear gradient of TEAB buffer (pH 9.0). (-----); Half of the nucleotide fraction was applied to the column and eluted with 0.02 to 0.4 M buffer. (——); Fractions 14–26 from the above analysis were combined and rechromatographed on the same column using a shallower gradient (0.02 to 0.3 M). *1*, *2* and *3* indicate elution volumes of AMP, ADP and ATP, respectively

of DEAE-cellulose (40 cm long, 1 cm diameter) and eluted with a linearly increasing gradient of TEAB. Of the radioactivity initially present in Fraction B, 93% eluted in fractions 14–16 (Fig. 3). These fractions were combined, dried and rechromatographed (after exhaustive washing of the column with starting buffer) on the same column with a shallower gradient. Now the standards AMP, ADP and ATP eluted discretely (Fig. 3). These fractions were appropriately pooled and dried by rotary-film evaporation. Henceforth these fractions (1–3, Fig. 3) will be referred to as mono-, di- and triphosphate fractions, respectively. The ratio of radioactivity in these fractions was 1:2.3:4.5.

Each fraction was freed of bicarbonate by azeotropic distillation with methanol and analysed in three ways. (i) One quarter of each fraction was hydrolysed with alkaline phosphatase. The resulting ribosides were subjected to two-dimensional TLC on silica-gel plates developed in solvent A followed by solvent B. Radioactive adenosine was the main compound present in all three cases; an unknown minor radioactive spot with R_f lower than adenosine in both dimensions was also located, and probably represents a more polar metabolite of adenine. Fractions between mono- and diphosphate peaks and between di- and triphosphate peaks (Fig. 3) were also analysed. Radioactive ZR was found only in the hydrolysed monophosphate fraction. (ii) One quarter of each of the mono-, di- and triphosphate fractions was also analysed directly by TLC on polyethyleneimine-cellulose plates developed stepwise in 0.25 M, 2.0 M and 4.0 M buffer (solvent F). The major radioactive spots from the mono-, di- and triphosphate fractions co-chromatographed with AMP, ADP and ATP respectively. However, a radioactive spot running slightly ahead of AMP was located only in the monophosphate fraction and had the expected chromatographic behaviour of ZMP. This was later shown to co-chromatograph with standard ZMP on a DEAE-cellulose layer developed in solvent E. (iii) The remainder of the mono-, di- and triphosphate fractions was hydrolysed enzymatically to ribosides, and the bulk of radioactivity in adenosine and other common adenine metabolites was separated from the labelled putative "cytokinin peak" using HPLC (System 1), e.g., as shown in Fig. 2A. Further HPLC (System 4) of "cytokinin peaks" showed that radioactive *trans*-ZR was found only in the hydrolysed monophosphate fraction.

In a separate experiment, the analysis of metabolites was performed by reversed-phase HPLC, following initial chromatography on Sephadex LH-20. This is in contrast to work presented above where adsorption HPLC was employed for the final analysis. The identity of labelled cytokinins present in the basic eluate (Fraction A from the cellulose-phosphate column) was confirmed by HPLC (System 3) analysis as *trans*-ZR and *trans*-Z.

Nucleotides derived from cellulose-phosphate Fraction B were further fractionated by DEAE-cellulose column chromatography into mono-, di- and triphosphate fractions. These fractions were degraded with alkaline phosphatase or chemically (see Material and methods) and the products were subsequently analysed using HPLC System 2. Following enzymic hydrolysis, radioactive *trans*-ZR was found only in the monophosphate fraction (Fig. 4A). When a portion of monophosphate fraction was analysed subsequent to chemical degradation, a peak of radioactivity was detected at the elution volume of *trans*-zeatin (Fig. 4B).

(ii) Mevalonic acid as the precursor. In preliminary experiments, 740 kBq of DL-[2-^{3}H]mevalonic acid lactone was fed to 10 g tissue. After 8 h of incubation, tissue was extracted and further analysis was carried out as described by Stuchbury et al. (1979). Two-dimensional TLC of Sephadex LH-20 frac-

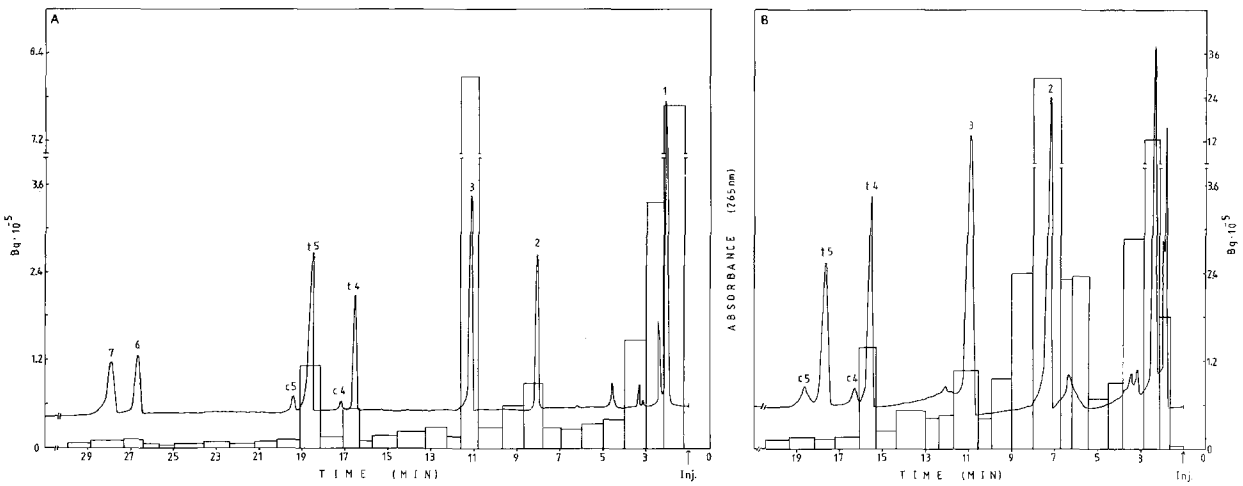

Fig. 4A, B. Reversed-phase HPLC (System 2) of nucleotide-derived radioactive compounds. The nucleotides were fractionated into mono-, di- and trinucleotides as per Fig. 3, and a portion of the mononucleotide peak analysed after **A** enzymic hydrolysis, or **B** chemical degradation. *Inj.*, point of sample injection; *1*, AMP; *2*, adenine; *3*, adenosine; *4*, zeatin; *5*, ZR; *6*, isopentenyl adenine and *7*, isopentenyl adenosine; *t*, *c*, *trans*- and *cis*- isomers, respectively

tions did not show any evidence of incorporation into cytokinins. In identical experiments, when unlabelled adenine (2.36 μg) was also supplied to the tissue together with [^{3}H]mevalonic acid lactone, no incorporation into free cytokinins was noticed.

Failure to detect incorporation of mevalonic acid into free cytokinins in above experiments may be partly due to the use of less sensitive techniques. In a later study employing HPLC, the incubations with [^{3}H]mevalonic acid lactone were carried out with or without 5 μg unlabelled adenine for 3 or 8 h. After extraction, further analysis of materials partitioning into butanol was carried out using HPLC System 1, followed by further analysis on HPLC System 4 or two-dimensional TLC on silica-gel plates. None of the samples showed incorporation into zeatin, ZR, isopentenyl adenine or isopentenyl adenosine, except one sample (8 h incubation without adenine) where a very small amount of radioactivity co-eluted with ZR during HPLC analysis. Further characterisation could not be attempted, hence the identity of this compound is uncertain.

(iii) Other experiments. The uptake and incorporation of [^{14}C]adenosine (185 kBq, 10 g tissue in 10 cm^3 medium, 8 h incubation) was also studied in *V. rosea* crown-gall tissue. The extraction and analysis of metabolites was performed essentially as reported by Stuchbury et al. (1979). The results are presented in Table 1.

Table 1 also lists results of [^{14}C]adenine incorporation (185 kBq, 10 g tissue in 10 cm^3 medium, 8 h incubation) into cytokinins in the presence of AMP and tubercidin. Preliminary experiments were done to determine the concentration of AMP (10 mM) and tubercidin (1 μM) at which maximum inhibition of adenine incorporation into cytokinins occurred. In the case of tubercidin it was found necessary to preincubate tissue with it for approx. 20 min before adding [^{14}C]adenine.

b) Normal stem callus tissue

Adenine as the precursor. Adenine (185 kBq) was supplied to 10 g tissue in 10 cm^3 liquid medium either with (+H Expt.) or without (–H Expt.) the usual concentration of phytohormones. The tissue was extracted 8 h after the start of incubation and the uptake of label was similar (approx. 94%) in both cases. The extract was purified as usual and the profile of butanol-soluble radioactivity (+H Expt.) eluting from a column of Sephadex LH-20 is shown in Fig. 5A.

With one exception, two-dimensional TLC on silica-gel plates of appropriately combined Sephadex LH-20 fractions followed by auto-radiography or exposure in the spark chamber failed to provide evidence of incorporation into cytokinins. Analysis of LH-20/Fr. 2 from butanol-soluble materials in the +H Expt. revealed a radioactive spot with chromatographic behaviour similar to ZR (mobility in solvent B was fractionally slower). This spot was eluted (80% ethanol, 1 cm^3 ×4) but further examination by HPLC System 6 showed the radioactivity was easily separated from ZR (Fig. 5B).

In a later experiment (740 kBq adenine, 10 g tissue, 8 h incubation), following initial fractiona-

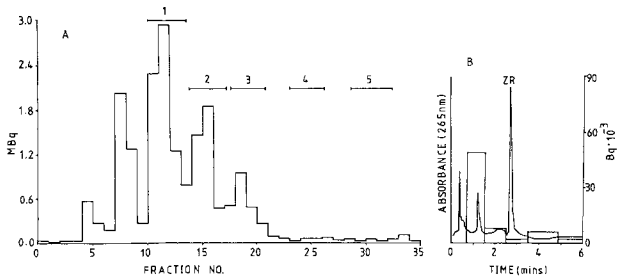

Fig. 5. **A** Profile of the butanol-soluble radioactivity eluting from a column of Sephadex LH-20 from [^{14}C]adenine fed to *V. rosea* normal tissue. Standards (1–5) elute as described in the legend to Fig. 1. **B** Fraction 2 was further analysed by two-dimensional TLC on a silica-gel plate and the radioactivity co-chromatographing with ZR was eluted and subjected to HPLC analysis (System 6)

tion of the ammonia eluate from the cellulose-phosphate column by Sephadex LH-20, further analysis of appropriate fractions was done using HPLC System 3. No radioactivity eluted with any of the cytokinin standards. Similarly, when the acidic wash from the cellulose-phosphate column was treated with alkaline phosphatase, either directly or following further separation into mono-, di- and triphosphate fractions (ratio of radioactivity, 1:2.3:3.7) and further analysed, no incorporation of radioactivity into cytokinin nucleotides was observed.

Discussion

In earlier work, Miller (1974) identified ZR as the major endogenous cytokinin in *V. rosea* crown-gall tissue. Although the presence of zeatin and a more polar derivative of it, probably nucleotide(s), was also indicated, the apparent high cytokinin activity found mainly in ZR could have resulted from breakdown of corresponding nucleotides during extraction. Our results (Fig. 1) show that there are similar amounts of cytokinin activity present in free ZR and ZR released from the enzymically hydrolysed nucleotide fraction. The cytokinin activity detected in the nucleotide fraction is likely to underestimate the amount present in the tissue, in view of the breakdown of [^{14}C]AMP to adenosine and adenine observed during extraction even with Bieleski (1964) solvents.

Because of problems of purification, work on cytokinin nucleotides per se is almost non-existent. Past studies have employed direct hydrolysis of cytokinin nucleotides to the corresponding ribosides for further study. In this investigation, anion-exchange chromatography on a DEAE-cellulose column, using gradient elution with TEAB has proved very useful. Considerable purification of the nucleotide fraction is also achieved because neutral compounds, e.g. sugars which give this fraction a very viscous consistency, elute in the void volume, and the mono-, di- and triphosphates can then be eluted in discrete fractions. Triethylammonium bicarbonate was found to be more convenient than NH_4HCO_3 or triethylammonium acetate as the eluent, because it could be removed easily by azeotropic distillation with methanol. We also investigated high- and low-voltage electrophoresis to fractionate nucleotides (results not shown). Although these methods gave good separation of standards, they did not work satisfactorily with plant extracts. The majority of biological activity in the nucleotide fraction was present in the hydrolysis products of the mononucleotide peak; very little activity was released from the corresponding di- and trinucleotide peaks. It is unlikely that this resulted from the breakdown of di- and trinucleotides to mononucleotide as identical results were obtained with Bieleski (1964) or perchloric-acid (Brown 1962) extraction methods. We conclude that the main active component of the nucleotide fraction in *V. rosea* crown-gall tissue is *trans*-ZMP. This is based on the identification of ZR and zeatin (*trans*- isomers) following enzymic and chemical degradation respectively, and initial fractionation of nucleotides on DEAE-cellulose. Letham (1973) has used similar methods to establish the identity of zeatin nucleotide in *Zea mays* kernels. These results have been substantiated recently by Scott et al. (1982).

In our previous study of adenine incorporation into putative cytokinins (Stuchbury et al. 1979), paper and Sephadex LH20 column chromatography, two-dimensional TLC, and in a few cases gas-liquid chromatography were utilised. These results have in general been substantiated in the present study using HPLC as the final analytical step, with one significant difference, in that the levels of incorporation are considerably lower than previously reported (Table 1). This is interpreted to indicate that the putative cytokinins in earlier investigations were not radiochemically pure, and highlights one of the technical problems frequently found in work of this nature.

In this study, all extractions were done 8 h following incubation; this was chosen because maximum radioactivity in cytokinins was found at this time; the radioactivity in zeatin nucleotide(s) was considerably higher than in other zeatin derivatives at all times (Stuchbury et al. 1979). Chromatography on DEAE-cellulose, followed by further chro-

matographic, enzymic and chemical analysis revealed that ZMP was the only labelled cytokinin nucleotide after 8 h. However, the adenine nucleotides included, in increasing amounts, AMP, ADP and ATP. More than 90% of supplied adenine was taken up after 8 h and a substantial proportion of it was found in adenine nucleotides. This indicates initial conversion of adenine into AMP, catalyzed by adenine phosphoribosyl transferase. Such an in-vitro reaction has been reported with a partially purified enzyme from *V. rosea* crown-gall tissue (Omar 1979). It would appear that the attachment of the isoprenoid side chain occurs at the 5′-AMP level, which is in full agreement with published work from cell-free systems from both higher and lower plants (Taya et al. 1978; Chen and Melitz 1979). It is likely that isopentenyl adenosine-5′-monophosphate is formed as an intermediate. However, because of the speed with which it is further stereospecifically hydroxylated resulting in the formation of ZMP, the steady-state concentration of isopentenyl adenosine-5′-monophosphate or its derivatives may be below the limits of detection. A very rapid conversion of isopentenyladenine to zeatin derivatives has been observed in this tissue (Palni and Horgan 1983).

Our data would indicate that biosynthesis of free cytokinins occurs primarily at the mononucleotide level, and subsequent production of ribosides and bases would be expected to take place by the action of specific enzymes (Chen 1981). However, the possibility of side-chain transfer at the base-riboside level cannot be excluded. The uptake of [U-^{14}C]adenosine by *V. rosea* crown-gall tissue was appreciably lower than that of adenine, 8 h following the start of incubation (Table 1). Again there was about 8–10 times more radioactivity in zeatin nucleotides than in ZR, but labelled zeatin was not detected.

Incorporation of labelled adenine into zeatin nucleotide(s) in *V. rosea* crown-gall tissue was decreased by a factor of five after 8 h in the presence of tubercidin (Table 1). It has been reported that tubercidin inhibits the formation of phosphoribosyl pyrophosphate in Ehrlich ascites tumour cells (Henderson and Peterson 1973), thus the synthesis of purine ribotides from the bases may also be reduced. Similarly, AMP was also found to reduce incorporation of adenine into all cytokinins (Table 1).

The lack of adenine incorporation into cytokinins by normal stem callus tissue of *V. rosea* may be the result of various factors. The level of endogenous cytokinin, namely zeatin riboside, in this tissue is extremely low in comparison with its tumourous counterpart (Horgan et al. 1981). Although externally applied adenine is readily taken up by normal callus and metabolised to nucleotides (ratio of AMP, ADP and ATP after 8 h is similar to that observed in crown-gall tissue), further conversion to cytokinins does not occur, possibly because of the absence of enzyme(s) responsible for side-chain transfer. Einset and Skoog (1973) also failed to get incorporation of adenine into cytokinins in cytokinin-dependent tobacco callus, while in the corresponding cytokinin-autonomous strain labelled cytokinins were detected.

Barnes et al. (1980) and Burrows and Fuell (1981) have reported low incorporation of radioactivity from mevalonic acid into free cytokinin. However, in our investigations we failed to get any incorporation (except one case of suspected incorporation). Perhaps there is more than one endogenous pool of mevalonic acid in crown-gall tissue of *V. rosea* and the supplied label does not enter the pool(s) utilized in the biosynthesis of free cytokinins.

The rapid rate of incorporation of adenine (as 5′-AMP) into ZMP indicates a route independent of transfer-RNA (tRNA) turnover. Burrows and Fuell (1981) have also suggested that free cytokinin production in cytokinin-autonomous and crown-gall tissues of tobacco takes place by a route not involving tRNA. If free cytokinin production takes place via tRNA, the conversion of externally applied adenine to its trinucleotide (ATP) would presumably be required for its incorporation into tRNA molecules. Following the limited or complete breakdown of tRNA, corresponding mononucleotides including cytokinin mononucleotides would be released. In this connection, it is interesting to note that after 8 h of incubation when maximum radioactive ZMP is present, radioactivity in ATP is also at least four times greater than in AMP. However, further studies on the rate of turnover of cytokinin moieties in individual tRNA species in this tissue are needed before firm conclusions can be drawn. This is particularly relevant in the light of the findings by Borek et al. (1977) that certain tRNA species can turnover at a much faster rate than the bulk of tRNA in animal tumours.

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