

Determination by Gas Chromatography-Mass Spectrometry of [¹⁵N₅]Adenine Incorporation into Endogenous Cytokinins and the Effect of Tissue Age on Cytokinin Biosynthesis in *Datura innoxia* Crown Gall Tissue

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ABSTRACT

In this study gas chromatographic-mass spectrometric techniques have been used to identify and quantify the metabolic incorporation of [¹⁵N₅]adenine into zeatin and its metabolites by 3-week-old *Datura innoxia* Mill, crown gall tissue. In a parallel study the levels of endogenous cytokinins were also determined by the stable isotope dilution technique using deuterium (²H)-labeled internal standards. Incorporation levels of the [¹⁵N₅]adenine after 8 hours of incubation, expressed as a percentage of the endogenous cytokinins, were as follows: zeatin (1.0%), zeatin riboside (1.5%), and zeatin riboside 5'-phosphate (10.2%). These results are consistent with those observed in complementary experiments using [U-¹⁴C]adenine, and support the proposal that the cytokinin biosynthesis occurs primarily at the nucleotide level. The effect of tissue age on cytokinin biosynthesis, determined by [U-¹⁴C]adenine incorporation into cytokinins by tissues at varying growth stages, indicated a steady increase with time reaching maximal synthesis at five weeks following subculture after which the level of ¹⁴C incorporation into cytokinins declined.

Since it was established that crown gall tissues of *Vinca rosea* actively produce free cytokinins (9, 10), a number of endogenous cytokinins have been unambiguously identified and quantified in various crown gall cultures (6, 12, 17, 21-23, 27). Such tissues also release substantial amounts of cytokinins into the culture medium (15). In addition, incorporation of radioactive adenine into cytokinins endogenous to crown gall tissues has been demonstrated (3, 18, 19, 25). In such studies, however, the identifications have been based solely on co-chromatography of the labeled cytokinins produced with authentic compounds, or their derivatives formed by enzymatic or chemical modifications. Furthermore, incorporation of ³H- or ¹⁴C-labeled adenine into cytokinins by crown gall cultures has been measured at a fixed time period, usually 4, 6, or 8 weeks following subculture (details are given in Latham and Palni [7]). Recent work was shown that the level of endogenous cytokinins in crown gall tissues changes substantially following subculture (14, 30) (see also references in Morris [13]).

In this study *Datura* crown gall tissue has been used, first to investigate the level of adenine incorporation into cytokinins 8 h after incubation at different times of tissue growth following subculture, and second to demonstrate unequivocally the incor-

poration of stable isotope labeled adenine into endogenous cytokinins using the GC-MS-MID¹ technique (20).

MATERIALS AND METHODS

Chemicals. Isopentenyladenine (IP), zeatin (Z) and their 9-β-D-ribosides (IPA and ZR, respectively) were obtained from Sigma and the *cis* isomers of Z and ZR were purified from commercial preparations of mixed isomers on silica gel (Merck, PF₂₅₄) TLC plates developed in methanol:chloroform (9:1, v/v; with trace of ammonia). Dihydrozeatin (DZ), dihydrozeatin 9-β-D-riboside (DZR), the *O*-glucopyranosides of cytokinins (ZOG, DZOG, ZROG, and DZROG), 7- and 9-β-D-glucopyranosyl zeatin (Z7G and Z9G), and 5'-monophosphate derivatives of ZR and IPA were synthesized as reported previously (20). The methods for the synthesis of corresponding deuterium-labeled (all were ²H₅, *i.e.* d₅, except [²H₂]DZ, IP, 5'-monoP of IPA, and [²H₆]IPA) cytokinins used as internal standards have also been published (4, 20, 26). [³H]ZR (244 μCi/mmol) was obtained by heating nonradioactive ZR with ³H₂O (24), and [U-¹⁴C]adenine (296 mCi/mmol) was purchased from Amersham. [¹⁵N₅]Adenine was synthesized (20% yield) by slight modification of a published method (11). [¹⁵N]Formamide (Stohler Isotopes, Waltham, MA) was reacted with 2 M excess of redistilled POCl₃ (Ajax) at 120°C for 16 h under anhydrous conditions in a Teflon bomb. The required product [¹⁵N₅]adenine (isotopic purity = 99%) was purified by chromatography on a column of Sephadex LH-20, followed by reversed-phase HPLC on a semipreparative Zorbax C₈ column (elution with 5% aqueous methanol containing 1% acetic acid; UV detection at 265 nm; R_t = 6.0 min). The identity of purified product was confirmed by TLC, UV spectroscopy, MS (electron impact; underivatized, TMS and tBDMS derivatives), GC-MS (TMS and tBDMS derivatives), high resolution MS (molecular ion, M⁺; observed = 140.0397, expected for C₅H₅¹⁵N₅ = 140.0440) and elemental analysis (found, C = 42.61%, H = 3.56% and ¹⁵N = 53.7%; calculated for C = 42.86%, H = 3.57%, ¹⁵N = 53.57%).

Plant Material. Bacteria-free *Datura innoxia* Mill. line B6

¹ Abbreviations: MID, multiple ion detection; IP, 6-(3-methyl-2-but-nylamino)purine, isopentenyladenine; Z, 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine, zeatin (their corresponding 9-β-D-ribofuranosyl derivatives are IPA and ZR, respectively); dihydro forms of Z and ZR are abbreviated with prefix D; *O*-β-D-glucopyranosyl derivatives and 7- and 9-β-D-glucopyranosyl forms of cytokinins are abbreviated with OG, 7G, and 9G suffixes, respectively; TMS, trimethylsilyl; tBDMS, *tert*-butyldimethylsilyl; Fr., fraction.

crown-gall tumor tissue was cultured in 500-ml conical flasks containing 100 ml hormone-free B₅ medium (5) and 0.8% (w/v) agar. The cultures were maintained in the dark at 26°C and subcultured every 5 weeks. Conical flasks (50 ml) containing 25 ml medium were used for measurement of tissue growth following subculture. Five pieces of inoculum (156 ± 24 mg each) were transferred into each flask and total tissue yield from each flask was recorded at indicated times (weekly intervals). Four flasks were harvested each week to obtain a mean value. The tissue was handled under aseptic conditions, mixed and a portion used in experiments to determine the level of [¹⁴C]adenine incorporation into cytokinins.

Metabolic Studies. Effect of Tissue Age on Cytokinin Biosynthesis Using [¹⁴C]Adenine. To determine the effect of tissue age on cytokinin biosynthesis 2 g tissue were taken at indicated times (1–9 weeks; Table I) under aseptic conditions and incubated in a plastic Petri dish (3.5 cm diameter, 1 cm deep; Sterilin, U.K.), containing 2 ml of hormone-free B₅ medium supplemented with 5 μCi of [¹⁴C]adenine. After 8 h incubation on a shaker (80 rpm) in the dark at 26°C, the tissue was removed by filtration, washed with 5 ml water, and immediately placed into 15 ml of chilled extraction solvent and stored at –20°C. The remaining incubation medium was combined with washings and made up to 10 ml with ethanol and also stored at –20°C. Small aliquots were withdrawn at various steps to determine radioactivity by liquid scintillation counting using an LKB 1215 Rack-beta II counter (Wallac Oy, Turku, Finland).

All extracts were subjected to cellulose phosphate chromatography and small aliquots of the basic and the nucleotide-derived (alkaline phosphatase treated), butanol-soluble fractions were analyzed by two dimensional TLC on silica gel (PF₂₅₄, 0.25 mm thickness; Merck, FRG) plates, developed first in *n*-butanol:acetic acid:water (12:3:5, v/v), and then in *n*-butanol:14 M ammonia:water (6:1:2, v/v, upper phase). The radioactivity co-chromatographing with adenine, adenosine, zeatin, and zeatin riboside markers (added before TLC) was determined by removing the appropriate silica gel layers from TLC plates and placing them directly into counting vials.

[¹⁵N₅]Adenine Incorporation Studies. Three-week-old *D. innoxia* crown gall tissue (243 g) was aseptically transferred to a conical flask (2 L) containing 400 ml of hormone-free B₅ medium supplemented with isotopically labeled adenine (7.1 μM [¹⁵N₅]adenine and 10 μCi [¹⁴C]adenine). Following incubation for 8 h at 26°C in the dark on a shaker (80 rpm), the tissue was removed by filtration and dropped into 500 ml chilled solvent. The tissue extraction and purification of metabolites was carried out as outlined below (also see Scheme I). Following initial fractionation on a column of Sephadex LH-20 the metabolites were purified by HPLC before final examination by GC-MS-MID.

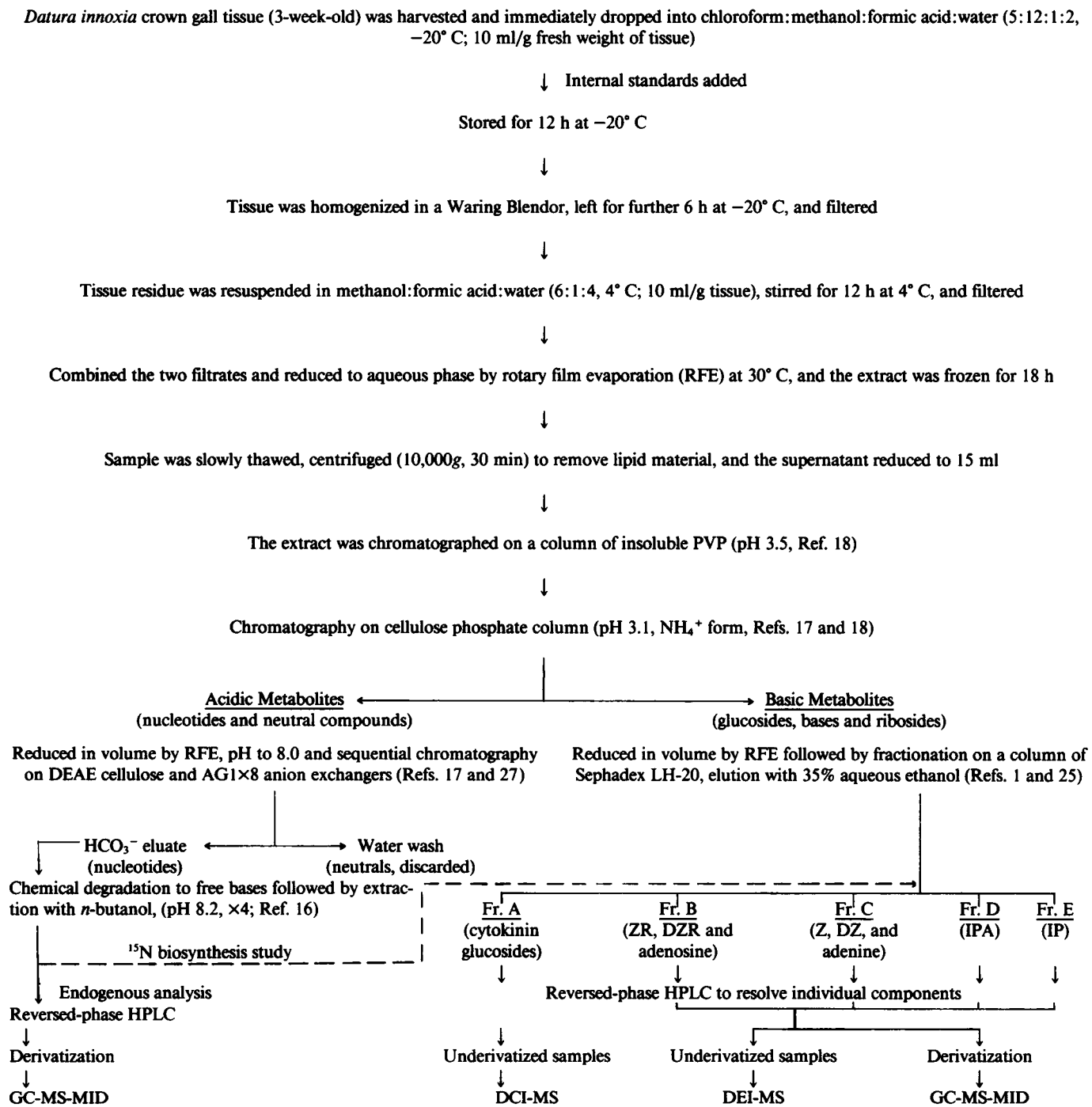
Quantification of Endogenous Compounds. Cytokinin Bases, Ribosides, Glucosides, and Nucleotides. In a parallel study 3-week-old *D. innoxia* crown gall tissue (128 g) was extracted as described below (Scheme I). At the beginning of the extraction known amounts of appropriate deuterium-labeled cytokinin standards (3 μg each of IP, IPA, and its 5'-monoP derivative; 4 μg each of DZR, DZROG, and DZOG; 5 μg of *cis*-ZR; 6 μg each of Z, DZ, ZOG, and ZROG; 10 μg each of ZR and its 5'-monoP derivative; 12 μg each of Z7G and Z9G) were added to permit quantification by GC-MS-MID following final purification of metabolites by HPLC. At the same time a marker amount of [³H]ZR (150,000 dpm) was also added to aid location of the cytokinin fractions eluting from the HPLC column, and to determine losses at various steps during extraction and purification (28). The amount of added [³H]ZR was below the detection level of the quantitation method and hence would not interfere with the measurement of endogenous ZR.

Adenine, Adenosine, and Adenosine 5'-Phosphate. The measurement of endogenous adenine, adenosine, and adenosine 5'-P was also carried out in a separate experiment in which 20 g tissue (3-week-old) was extracted. [¹⁵N₅]Adenine (15 μg) was added as an internal standard prior to tissue homogenization and purification. The basic fraction obtained after cellulose phosphate chromatography was subjected to HPLC and the adenine and adenosine fractions were collected separately. The nucleotide fraction was further purified by anion-exchange chromatography (Scheme I); this and the adenosine fraction were treated with sodium periodate as described below. The determination of adenosine and its 5'-nucleotide(s) in the appropriate fractions was carried out with reference to a known quantity of added [¹⁵N₅]adenine (15 μg each fraction) following their chemical degradation to free base adenine. These fractions were extracted with *n*-butanol (×3) and subjected to HPLC to purify resulting adenine. The final quantification of adenine in the three fractions (purified from the free basic fraction; chemically derived from adenosine, and from adenosine 5'-nucleotide) was carried out by GC-MS-MID. It should, however, be noted that the values for adenosine and adenosine 5'-P (Table III) and the values for the corresponding biosynthesized ¹⁵N₅-compounds (Table IV) could represent considerable underestimates.

Extraction and Purification of Metabolites. The procedures used in this work have been described in detail in the cited references. In brief, tissue extraction was carried out sequentially using solvents known to inactivate phosphatase activity (2, 16, 18) and the extracts were purified as outlined in Scheme I. The nucleotides were treated either enzymatically ([¹⁴C]adenine incorporation work) or chemically ([¹⁵N₅]adenine incorporation studies and endogenous quantification work) as detailed below and then extracted into *n*-butanol (16) for further analysis. *Escherichia coli* alkaline phosphatase (Sigma) was used for the hydrolysis of purine nucleotides to their ribosides according to a published procedure (16). For degradation of 5'-phosphates of adenosine and ZR to their respective bases, the sample was dissolved in a small amount of water containing sodium periodate (10 mg/ml). The mixture was left at 37°C for 16 h, cyclohexylamine (200 μl/ml of reaction mixture) was then added, and this was incubated for a further 3 h (18).

The basic and the nucleotide-derived, butanol-soluble fractions were initially fractionated on a column of Sephadex LH-20 eluting with 35% (v/v) ethanol (1, 25). Various fractions were appropriately pooled (Scheme I) (17) and further purified by HPLC using a semipreparative Zorbax C₈ column. In the endogenous quantification work the nucleotide-derived, butanol-soluble compounds were directly purified by HPLC. Details of HPLC equipment, solvents, and the chromatographic conditions employed in this study were essentially similar to those reported previously (17, 26, 28). The HPLC purified compounds were thoroughly dried, derivatized if required, and subjected to MS and/or GC-MS analysis.

Mass Spectrometric Measurements. Trimethylsilyl (TMS) (28) and *tert*-butyldimethylsilyl (tBDMS) (19) derivatives of purified compounds and standards were prepared as in the cited references. Electron impact (EI, 70 eV) GC-MS was carried out on a VG Micromass 7070F instrument interfaced to an Inco Nova 3 data system using a packed GC column (3% OV-101 on Gas chrom Q 100-120, 1.5 m × 2.0 mm) under the following conditions: He carrier gas, 25 ml/min; jet separator, line and ion source temperatures, 280, 280, and 250°C, respectively; column temperature programming (ZR, 250°C [for 2 min] to 280°C; Z, 200°C [for 2 min] to 300°C; adenosine, 200–300°C and adenine, 150–300°C; all at 10°C/min). MID was carried out by computer controlled voltage switching between the ions given in Tables III and IV using an ion sampling time of 0.2 s and a mass window of ± 0.5 mass unit. For mass spectrometric measurement of



SCHEME I. The procedure used in the extraction and purification of samples for quantification of endogenous compounds by MS. An essentially similar method was used to investigate the metabolic incorporation of [^{15}N]₃adenine.

cytokinin glucosides, the individual components of Fr. A (Scheme I), obtained after Sephadex LH-20 chromatography, were purified by HPLC. The six glucosides listed in Table III were examined underderivatized by desorption chemical ionization (DCI) mass spectrometry, and the protonated molecular ion (MH^+) pair was monitored by MID and used for quantification. The DCI spectra of cytokinin glucoside standards (ammonia as a reagent gas, 1 torr) show prominent MH^+ ions (base peak) with only a few fragment ions, and therefore offer enhanced sensitivity for quantification by MID (details to be published elsewhere). A desorption electron impact (DEI) mass spectrum of the underderivatized ZR fraction was also obtained by scanning over a selected

mass range (m/z 300–380), and the molecular ion pair was used for quantification. For analysis by DCI or DEI the sample was coated onto a thin platinum wire attached to the probe tip, and sample desorption was achieved by rapid heating of the wire. The measurement of endogenous compounds (Table III) and [^{15}N]₃adenine incorporation levels (Table IV) were carried out by summing over the ion peak profiles to derive integrated ion intensity values.

RESULTS AND DISCUSSION

Effect of Tissue Age on Cytokinin Biosynthesis. The growth curve for *D. innoxia* crown gall tissue following subculture onto

fresh medium is shown in Figure 1. Tissue growth has been measured in terms of increase in fresh weight. After 1 week of subculture the tissue started to grow rapidly and a sharp increase in growth occurred between weeks 4 and 5; maximum fresh weight was achieved around week 6, after which little increase in tissue fresh weight occurred. Tissue browning started 7 to 8 weeks after subculture and by week 9 the tissue was beginning to dry and appeared uniformly brown on the outside.

Tables I and II show the results of a study of [U-¹⁴C]adenine uptake at varying growth stages, and its incorporation into cytokinins by *D. innoxia* crown gall tissue after a fixed (8 h) incubation period. The length of incubation period used in this investigation has previously been shown to result in maximum incorporation of radiolabeled adenine into cytokinins in a de-

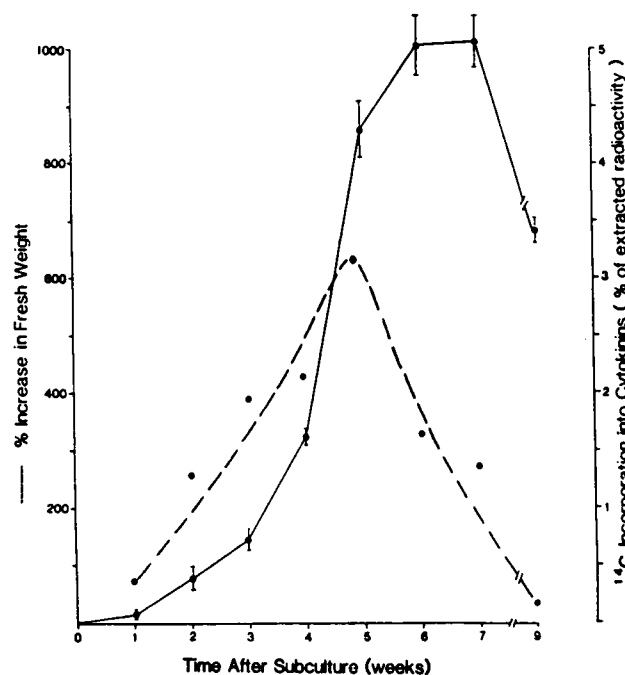


FIG. 1. The growth of *D. innoxia* crown gall tissue and cytokinin biosynthesis over a 9-week-period following subculture. Each point in the growth curve was derived from an average of total tissue yield from four flasks (five callus pieces in each flask). Vertical bars indicate standard deviation values. Cytokinin biosynthesis was determined as described in the text and each point represents sum total [U-¹⁴C]adenine incorporation into zeatin, zeatin riboside, and zeatin riboside 5'-P (values were taken from Table II).

tailed quantitative and qualitative time-course study (25). The uptake of supplied radioactivity was rapid in keeping with previous results which showed that 38 and 77% of supplied radioactivity was taken up after 20 min and 1 h incubation, respectively, and that adenine uptake was essentially quantitative ($\geq 90\%$) after 3 h (25). In the present investigation, after 8 h about 90 to 95% of ¹⁴C initially added to the incubation medium was taken up by the tissue, except in the case of old, browning tissue (9 weeks after subculture) where about 60% of the supplied radioactivity still remained in the medium following incubation (Table I). This reduced uptake of adenine by 'aged' tissue probably relates to its greatly reduced metabolic state. Nevertheless, reasonably uniform values obtained between weeks 1 to 7 would suggest that uptake of adenine is not influenced to any extent by the rate of tissue growth. In all cases approximately 60% of the radioactivity taken up by the tissue could be extracted using the procedure outlined in "Materials and Methods," except in the case of 3-week-old tissue where only 41.7% was extracted (Table I). A large portion of the 'unextracted' radioactivity is likely to be incorporated into insoluble materials, e.g. nucleic acids. The maximum incorporation of supplied [U-¹⁴C]adenine into the insoluble material by 3-week-old tissues may reflect maximum cell division activity and related nucleic acid synthesis at about this time after subculture. The period of maximum cell division activity would be expected to be followed by a period of rapid tissue growth, largely due to cell enlargement, as is reflected by the increase in fresh weight (Fig. 1).

It can be seen from Table I that up to 7 weeks after subculture only a small amount of extracted radioactivity (about 20%) was associated with the basic metabolites, while the bulk of extracted ¹⁴C (44–51%) was incorporated into the nucleotide-derived, butanol-soluble materials. This situation was reversed for extracts of 9-week-old tissue, where 70% of extracted radioactivity was in the basic metabolites mainly due to greatly reduced metabolism of adenine. The incorporation of [U-¹⁴C]adenine into Z, ZR, and ZR 5'-P by different aged tissues is shown in Table II. It should be noted that at all times the ¹⁴C incorporation into ZR 5'-phosphate (which yielded ZR following enzymatic hydrolysis) was considerably higher than in ZR and Z. Similar results have been obtained previously with this and *V. rosea* crown gall tissue (18, 19, 25). The [U-¹⁴C]adenine incorporation into total cytokinins by *D. innoxia* crown gall tissue increased steadily with time following subculture and maximum incorporation occurred at 5 weeks, after which the level of incorporation declined sharply (Fig. 1). The analysis and identification of metabolites presented in Table II was based principally on two-dimensional TLC. Further analysis by other chromatographic techniques, e.g. HPLC, was not considered necessary because

Table I. Uptake and Recovery of ¹⁴C in Extracts of *D. innoxia* Crown Gall Tissue 8 h after Incubation with [U-¹⁴C]Adenine

Time after Subculture	Uptake of Adenine	Extracted Radioactivity	Radioactivity after Cellulose Phosphate Chromatography		Nucleotide-Derived, Butanol-Soluble Radioactivity
			Basic fraction	Acidic fraction	
weeks	% of ¹⁴ C supplied	% of ¹⁴ C taken up	% of ¹⁴ C extracted		
1	95.5	57.1	15.2	84.5	48.2
2	93.5	67.4	11.7	86.0	48.1
3	91.3	41.7	17.2	82.5	51.2
4	92.2	57.8	16.4	80.0	43.8
5	95.0	62.6	17.4	78.5	46.9
6	95.7	64.4	20.2	76.6	45.0
7	90.4	61.5	16.4	78.5	49.5
9	42.0	64.7	69.9	27.3	19.5

Table II. Metabolism [^{14}C]Adenine by *D. innoxia* Crown Gall Tissue

The tissue (2 g) was incubated with 5 μCi adenine for 8 h at various times (1–9 weeks) after subculture. Values are the mean of three determinations.

Time after Subculture	Radioactivity in Adenine and Other Metabolites					
	Zeatin	Zeatin riboside	Zeatin riboside 5'-P	Adenine	Adenosine	Adenosine 5'-P
weeks	% of extracted radioactivity					
1	0.03 \pm 0.01	0.15 \pm 0.04	0.30 \pm 0.03	2.97 \pm 0.28	3.31 \pm 0.27	38.82 \pm 2.07
2	0.10 \pm 0.01	0.10 \pm 0.02	1.02 \pm 0.09	1.76 \pm 0.22	1.32 \pm 0.22	38.92 \pm 3.07
3	0.20 \pm 0.02	0.26 \pm 0.08	1.43 \pm 0.15	3.92 \pm 0.41	1.37 \pm 0.12	37.09 \pm 3.11
4	0.06 \pm 0.01	0.28 \pm 0.09	1.71 \pm 0.48	3.20 \pm 0.06	1.14 \pm 0.05	39.63 \pm 1.57
5	0.17 \pm 0.01	0.42 \pm 0.04	2.55 \pm 0.64	2.76 \pm 0.47	3.00 \pm 0.37	42.47 \pm 4.28
6	0.07 \pm 0.01	0.27 \pm 0.03	1.26 \pm 0.13	3.34 \pm 0.28	4.21 \pm 0.11	39.23 \pm 1.60
7	0.14 \pm 0.01	0.16 \pm 0.04	1.13 \pm 0.04	3.21 \pm 0.19	4.34 \pm 0.23	40.73 \pm 0.74
9	0.06 \pm 0.02	0.07 \pm 0.02	0.11 \pm 0.03	40.19 \pm 3.57	4.41 \pm 0.30	14.48 \pm 0.77

rigorous analysis of adenine metabolites, particularly cytokinins (Z, ZR, and ZR 5'-P), in crown gall tissues of *D. innoxia* (19) and *V. rosea* (18, 25) has previously been reported. Furthermore the metabolic incorporation of adenine into Z, ZR, and ZR 5'-P in *D. innoxia* crown gall tissue has been unequivocally verified using mass spectrometric techniques (see below).

Quantification of Endogenous Cytokinins, Adenine, Adenosine, and Its Nucleotide. The endogenous cytokinins in *D. innoxia* crown gall tissue were unambiguously identified, and their levels determined in 3-week-old tissue by GC-MS-MID, using deuterium-labeled cytokinins as internal standards (Fig. 2, A–C, and Table III). The purification procedures used resulted in some cases in essentially pure individual cytokinins, and therefore in these instances both GC-MS and direct probe techniques could be applied. For example, identical results were obtained where both methods were used to quantify endogenous ZR (Fig. 2, A and B). Table III also shows the derivatives used and ions monitored during the analysis of individual compounds (bases and ribosides) by GC-MS-MID.

The glucosyl conjugates of cytokinins (both the *O*- and *N*-glucosides) were quantified without prior derivatization as intact molecules by DCI-MS (Table III) to avoid the difficulties associated with the analysis of these compounds by GC-MS of derivatives (20). Although the nucleotide derivatives of cytokinins and adenosine were analyzed after their chemical degradation to corresponding bases (Tables III and IV), the use of DCI-MS for analysis of intact cytokinin nucleotides from *D. innoxia* has previously been reported (27).

In addition to the cytokinins listed in Table III, DZ9G and 5'-P of DZR have been previously identified from *D. innoxia* crown gall tissue (17). However, the quantification of these two cytokinins was not attempted in the present study due to the lack of relevant deuterium-labeled standards. The *cis* isomer of ZR was not detected as a free cytokinin in this tissue in agreement with the earlier study cited above. Although the results of these two studies agree qualitatively, the levels of endogenous cytokinins determined in this study (Table III), can not be compared directly with the previous report (17) on two accounts. Firstly the tissue extracted in the earlier study was older (5 weeks after subculture), and secondly the deuterium-labeled standards were added well after the initial purification and fractionation steps in that study.

The endogenous levels of adenine, adenosine, and adenosine 5'-P were also determined by GC-MS-MID (Table III). In all the three cases [$^{15}\text{N}_5$]adenine was used as internal standard (see "Materials and Methods"). This was necessary in the absence of [$^{15}\text{N}_5$]adenosine and its 5'-phosphate standards. The values quoted for these two compounds in Table III, and the values for the corresponding biosynthesized [$^{15}\text{N}_5$]compounds in Table IV would therefore certainly be considerable underestimates.

Measurement of [$^{15}\text{N}_5$]Adenine Incorporation into Cytokinins.

In a parallel study [$^{15}\text{N}_5$]adenine, together with a small amount of [^{14}C]adenine, was supplied to 3-week-old *D. innoxia* crown gall tissue. The tissue extraction was performed 8h after incubation. The uptake of adenine was 95%. Preliminary fractionation of the extract on a column of Sephadex LH-20 following ion-exchange chromatography (Scheme 1), indicated (by scintillation counting) adenine incorporation into the free base (Fr. C; adenine, Z, DZ), riboside (Fr. B; adenosine, ZR, DZR) and nucleotide (Fr. C; 5'-phosphate derivatives of adenosine, ZR, and DZR), which were analyzed as their respective bases following chemical degradation) fractions, but no incorporation into cytokinin glucosides (Fr. A) or isopentenyladenine type (Frs. D and E) cytokinins (results not shown). These three fractions (Frs. B and C from basic metabolites and Fr. C derived from acidic metabolites) were further purified by HPLC. HPLC and TLC (silica gel; chloroform-methanol, 9:1) analyses indicated that there was no incorporation of [^{14}C] into either the *cis* isomer of Z, its riboside and nucleotide or the corresponding dihydrozeatin derivatives. Individual compounds following HPLC purification were subjected to MS analysis (Fig. 2, D–F) using methods given in Table III. The incorporation levels of the [$^{15}\text{N}_5$] label as a percentage of the endogenous ($^{15}\text{N}_0$) compound(s) (from Table III) can be seen in Table IV. The identity of compounds derived from the incorporation of [$^{15}\text{N}_5$]adenine (Table IV) is based on several important criteria, namely co-chromatography with the corresponding endogenous ($^{15}\text{N}_0$) compounds right through to the final purification of samples by HPLC before GC-MS analysis, and co-elution from the GC column (Fig. 2, D–F) at the correct retention times. For selectivity, high mass ions (M^+ and $\text{M}^+-\text{CH}_3\cdot$) were chosen for MID analysis, and the identifications were further strengthened by comparable ion intensity ratios obtained for these ions ($\text{M}^+-\text{CH}_3\cdot$ and M^+) for the endogenous ($^{15}\text{N}_0$) and the biosynthesized [$^{15}\text{N}_5$]compounds (Table IV).

Among the cytokinins, maximum incorporation levels were found for ZR 5'-P, followed by ZR and Z. This is in accord with the results obtained with [^{14}C]adenine incorporation studies outlined in Table II and previously reported observations (18, 19, 25). Furthermore it is interesting to note that the relative level of adenine incorporation into Z, ZR, and ZR 5'-P parallels the situation observed for corresponding endogenous cytokinins in *D. innoxia* (Table III) and some other crown gall tissues, e.g. *Nicotiana tabacum* (23). The absence of any adenine incorporation into IP, DZ, and *cis* isomer of Z, and their derivatives is in accord with published reports (6, 8, 19, 25). In addition the lack of adenine incorporation into cytokinin glucosides, despite their reasonably high endogenous levels (Table III), would suggest that these compounds are not on the primary metabolic pathway and must be formed after longer incubation periods (25). This is in

QUANTIFICATION

BIOSYNTHESIS

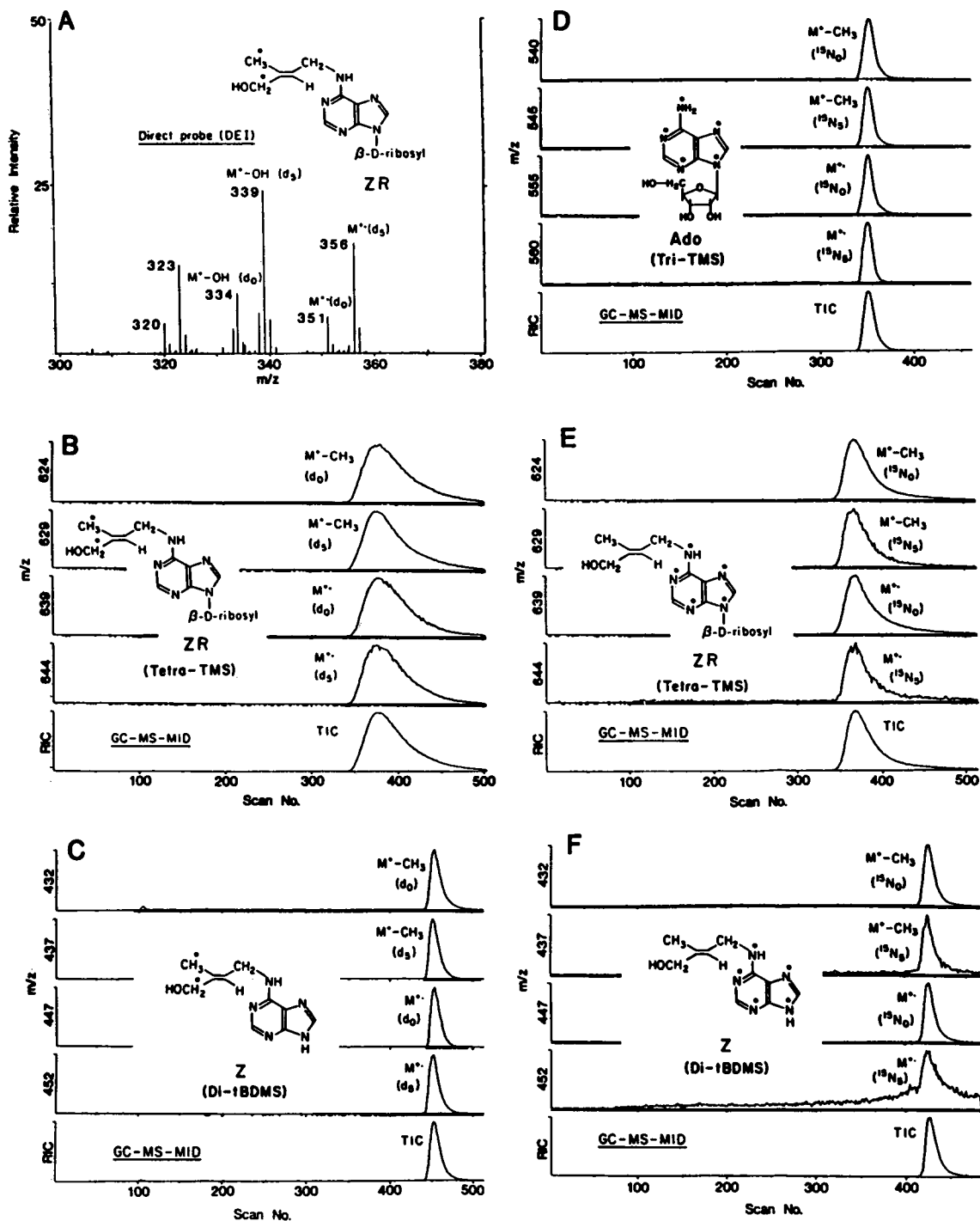


FIG. 2. Representative examples of mass spectrometric quantification of endogenous cytokinins in 3-week-old *D. innoxia* crown gall tissue (A–C) and GC-MS-MID measurement of incorporation of supplied [¹⁵N₅]adenine into cytokinins (D–F). Molecular ion region in the DEI mass spectrum of underivatized ZR (A), and MID tracings obtained during GC-MS of (TMS)₄-ZR (B) and (tBDMS)₂-Z (C) isolated from the tissue. Known quantities of pentadeuterium-labeled (d₅) internal standard were added at the time of extraction to permit quantification. Molecular (M⁺) and fragment ions (M⁺-OH or M⁺-CH₃) derived from the endogenous (d₀) and corresponding internal standards (d₅) are indicated. D–F are MID tracings obtained during GC-MS and indicate [¹⁵N₅]adenine incorporation into adenosine (D), ZR (E), and Z (F) following 8 h incubation. The position of isotopically labeled atoms is indicated with a dot on the chemical structures.

Table III. Levels of Endogenous Cytokinins, Adenine, and Its Common Derivatives in *D. innoxia* Crown Gall Tissue
Three-week-old tissue was used for analysis.

Compounds Analyzed	MS Method Used	Derivatives Used	Ions Monitored by MID ^a			Endogenous level ^b µg/100 g tissue
			M ⁺	M ⁺ -CH ₃ ⁺	MH ⁺	
Z	GC-EIMS	Di-tBDMS	447 (452)	432 (437) ^c		3.0
DZ	GC-EIMS	Di-tBDMS	449 (451)	434 (436) ^c		5.7
ZR	GC-EIMS	Tetra-TMS	639 (644)	624 (629) ^c		3.5
DZR	GC-EIMS	Tetra-TMS	641 (646)	626 (631) ^c		1.9
<i>cis</i> -ZR	GC-EIMS	Tetra-TMS	639 (644)	624 (629) ^c		Not detected
IP	GC-EIMS	Mono-tBDMS	317 (319)	302 (304) ^c		0.15
IPA	GC-EIMS	Tri-TMS	551 (557) ^c	536 (542)		0.18
ZR 5'-P ^d	GC-EIMS	Di-tBDMS	447 (452)	432 (437) ^c		5.5
IPA 5'-P ^d	GC-EIMS	Mono-tBDMS	317 (319)	302 (304) ^c		0.56
ZOG	DCI-MS	Underivatized			381 (386) ^c	3.9
DZOG	DCI-MS	Underivatized			383 (388) ^c	5.7
ZROG	DCI-MS	Underivatized			513 (518) ^c	4.4
DZROG	DCI-MS	Underivatized			515 (520) ^c	3.7
Z7G	DCI-MS	Underivatized			381 (386) ^c	8.8
Z9G	DCI-MS	Underivatized			381 (386) ^c	8.4
Adenine	GC-EIMS	Di-TMS	279 (284)	264 (269) ^c		28.2
Adenosine ^d	GC-EIMS	Di-TMS	279 (284)	264 (269) ^c		39.9 ^e
Adenosine 5'-P ^d	GC-EIMS	Di-TMS	279 (284)	264 (269) ^c		60.6 ^e

^a Ions in parentheses were derived from ²H- or ¹⁵N-labeled internal standards. ^b Determinations were carried out in duplicate to obtain mean values. ^c Quantification was based on this ion pair. ^d Analysis was carried out after chemical degradation to respective bases. ^e Values could be considerable underestimates (see text).

Table IV. Biosynthesis of Cytokinins from [¹⁵N₅]Adenine Incorporation in *D. innoxia* Crown Gall Tissue
Three-week-old tissue was used for analysis.

Compounds Analyzed	Derivatives Used	Ions Monitored by MID ^a		[¹⁵ N ₅]Adenine Incorporation ^{b,c} %	Ion Intensities (M ⁺ -CH ₃ ⁺ / M ⁺)		Biosynthesized [¹⁵ N ₅]-compounds ^d ng/g tissue
		M ⁺	M ⁺ -CH ₃ ⁺		ratio		
					¹⁵ N ₀	¹⁵ N ₅	
Zeatin	Di-tBDMS	447 (452)	432 (437)	1.0	2.6	2.4	0.30
Zeatin riboside	Tetra-TMS	639 (644)	624 (629)	1.5	3.7	3.2	0.525
Zeatin riboside 5'-P ^f	Di-tBDMS	447 (452)	432 (437)	10.2	3.1	3.1	5.61
Adenine	Di-TMS	279 (284)	264 (269)	15.4	6.0	6.3	43.43
Adenosine	Tri-TMS	555 (560)	540 (545)	10.4	27.0	29.0	41.50 ^g
Adenosine 5'-P ^f	Di-TMS	279 (284)	264 (269)	16.0	7.1	6.9	96.96 ^g

^a Ions in parentheses were derived from [¹⁵N₅]adenine incorporation. ^b Determinations were carried out in duplicate to obtain mean values. ^c [¹⁵N₅]Adenine incorporation was measured 8 h following incubation, and the values were expressed as a percentage of the corresponding endogenous ¹⁵N₀ compounds. ^d Calculated from measured endogenous levels (Table III). ^e Percent [¹⁵N₅]adenine incorporation values were based on this ion pair. ^f Analysis of nucleotides was carried out after chemical degradation to respective bases. ^g Values could be considerable underestimates (see text).

agreement with their proposed role as storage forms of cytokinins (7).

A comparison of the amounts of ¹⁵N₅-labeled adenine, adenosine, adenosine 5'-P, and cytokinins formed (Table IV) indicates that the transfer of the isopentenyl side chain to adenine moiety occurs at the nucleotide level, resulting in the production of ZR 5'-P. Subsequent formation of ZR and Z would be expected to result by the action of appropriate enzymes (7). Although no attempt was made to distinguish between the 5'-mono-, di-, or triphosphate derivatives of ZR in this study, it has previously been demonstrated in *V. rosea* crown gall tissue that ZR 5'-monoP was the only cytokinin nucleotide formed under identical experimental conditions (18). Data obtained with partially purified isopentenyl transferase enzyme (13, 29) and the reported occurrence of active hydroxylation systems in crown

gall tissues (16) support this pathway of free cytokinin synthesis in such tissues. In summary, these results further strengthen the proposal that the cytokinin nucleotides play a central role in the biosynthesis of these compounds (18).

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