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Mass Spectrometric Analysis of Cytokinins in Plant Tissues[1]

V. IDENTIFICATION OF THE CYTOKININ COMPLEX OF *DATURA INNOXIA* CROWN GALL TISSUE

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ABSTRACT

The cytokinin complex of *Datura innoxia* Mill. crown gall tissue was purified by ion exchange, Sephadex LH-20 chromatography and reversed-phase high performance liquid chromatography. By gas chromatography-mass spectrometry using ^{2}H-labeled compounds, the following cytokinins were identified in the basic fraction eluting from a cation exchange column: zeatin, zeatin riboside, dihydrozeatin, dihydrozeatin riboside, their corresponding *O*-glucosides, 7- and 9-glucosides of zeatin, 9-glucoside of dihydrozeatin, isopentenyladenine, and isopentenyladenosine. Zeatin riboside 5′-monophosphate was the major cytokinin nucleotide in the tissue. In addition, dihydrozeatin riboside and isopentenyladenosine were identified in the nucleotide fraction following enymic degradation.

Recently crown gall tumor tissues have attracted great interest for studies of cytokinin biosynthesis and metabolism. Investigations into the basic mechanisms controlling production and turnover of cytokinins in such tissues are also in progress (17). Since Miller (14) unambiguously established zeatin riboside as the major endogenous cytokinin in crown gall tissue of *Vinca rosea*, a variety of other cytokinins have been identified in several crown gall tissues (2, 8, 15–17, 20–23, 28, 29). Such tissues also release large quantities of cytokinins into the culture medium (18).

Crown gall tumor tissues can be grown on nutrient medium without the addition of phytohormones (cytokinin and auxin) which are essential for growth of corresponding untransformed tissues. The present investigation deals with the isolation and characterization of the cytokinin complex of crown gall tumor tissue of *Datura innoxia* cultured on hormone-free medium. The identifications are based on comparison with synthetic compounds using TLC, Sephadex LH-20 and reversed-phase HPLC, enzymic degradation, and both electron impact and chemical ionization combined GC-MS.

MATERIALS AND METHODS

Chemicals and Plant Material. IP,[3] Z, and their corresponding 9-β-D-ribosides (IPA and ZR, respectively) were obtained commercially (Sigma and Calbiochem). The *cis*-isomers of Z and ZR were purified from preparations of mixed isomers on silica gel (Merck, PF_{254}) TLC plates run in methanol:chloroform (9:1, v/v). DZ, dihydrozeatin-9-β-D-riboside (DZR), the *O*-glucopyranosides of cytokinins (OGZ, OGZR, OGDZ, and OGDZR), 7- and 9-β-D-glucopyranosylzeatin (Z7G and Z9G) and 9-β-D-glucopyranosyldihydrozeatin (DZ9G) were synthesized as reported earlier (3–5, 26). The procedures for the preparation of corresponding deuterium-labeled compounds (all were $^2H_5$, *i.e.* D_5, except [$^2H_2$]DHZ, [$^2H_2$]IP, and [$^2H_6$]IPA) have also been published (6, 25).

Crown gall tumor tissue of *Datura innoxia* Mill. line B6 was grown in 500-ml conical flasks containing 100 ml hormone-free B_5 medium (7) solidified with 0.8% agar. The cultures were maintained at 25°C (±1) in the dark and subcultured every 6–8 weeks. Five-week-old tissue was used in the present study.

Extraction and Purification of Cytokinins. Immediately after harvesting, the tissue was dropped into chloroform:methanol:formic acid:H_2O (5:12:1:2, v/v; 10 ml g^{-1} fresh weight; −20°C; Ref. 1) and left overnight at −20°C. The next day tissue was homogenized in a Waring Blendor and left for a further 8 h at −20°C. After centrifugation (4,000*g*, 30 min) the residue was resuspended in methanol:formic acid:H_2O (6:1:4, v/v; 10 ml g^{-1} fresh weight; −20°C) and left stirring for 12 h at 4°C. Following centrifugation (4,000*g*, 30 min), the residue was discarded and the two supernatants were combined and filtered (Whatman No. 1 paper). The filtrate was evaporated *in vacuo* at 35°C and an aqueous solution was adjusted to pH 3.5 and frozen. After thawing, the suspension was centrifuged (10,000*g*, 1 h) to remove lipid material. The supernatant was passed through a column of insoluble PVP (0.5-ml bed volume g^{-1} tissue) which was eluted with 4 column volumes of H_2O (pH 3.5). The combined effluent was reduced *in vacuo* to about 35 ml, adjusted to pH 3.1, and passed through a column of cellulose phosphate (Whatman P1, floc type; 15-ml bed volume g^{-1} tissue; equilibrated to pH 3.1 in the NH_4^+ form). After washing with 5 column volumes of H_2O (pH 3.1), the column was eluted with 5 column volumes of 2 M NH_4OH. The acid H_2O wash containing cytokinin nucleotides (acidic fraction, Fr A) and the NH_4OH eluate containing cytokinin bases, ribosides, and glucosides (basic fraction, Fr B) were subjected to further analysis as detailed below.

Fr A. This fraction was evaporated to dryness, taken up in 20 ml H_2O, adjusted to pH 8.0 with NH_4OH, and loaded onto a DEAE-cellulose column (Whatman DE1, coarse fibrous, 10-ml bed volume g^{-1} tissue, equilibrated to pH 8.0 in the HCO_3^- form). The column was washed with 5 column volumes of H_2O (pH 8.0) and then eluted with 5 column volumes of NH_4HCO_3 solution (10%, w/v). The eluate was evaporated *in vacuo*, freed of bicarbonate by repeated addition and evaporation of methanol, and treated with *Escherichia coli* alkaline phosphatase as reported earlier (19). The hydrolyzed material was dissolved in 25 ml H_2O, adjusted to pH 8.2 with 1 N NaOH, and extracted with H_2O-

[1] This is Part V of a series; see Ref. 28 for Part IV.

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[3] Abbreviations: IP, isopentenyladenine; Z, zeatin (their corresponding 9-β-D-ribosides are IPA and ZR, respectively); BSTFA: *N,O*-bis(trimethylsilyl)trifluoroacetamide; CI, chemical ionization; EI, electron impact; TMS, trimethylsilyl. *O*-Glucopyranosyl derivatives and dihydro forms are abbreviated with OG and D prefixes; 7- and 9-β-D-glucopyranosyl forms are shown with 7G and 9G suffixes.

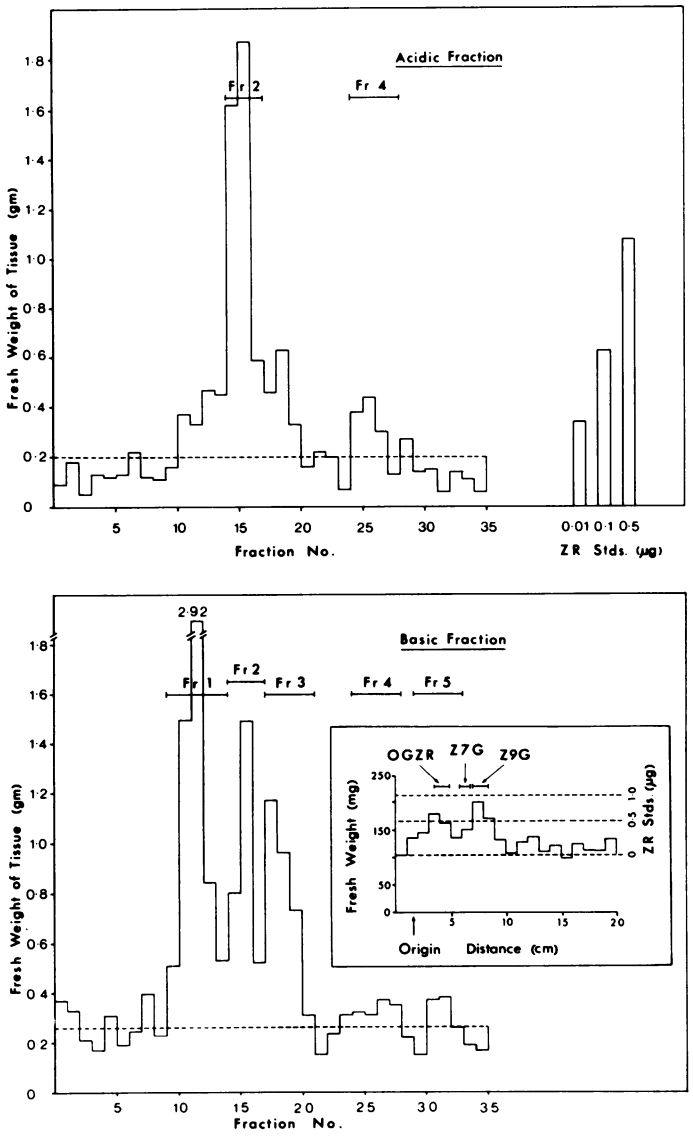

FIG. 1. Soybean callus bioassay profile following Sephadex LH-20 chromatography of the basic fraction and alkaline phosphatase-treated acidic fraction from an extract of 10 g *D. innoxia* crown gall tumor tissue. The basic and acidic fractions refer to Fr B and Fr A, respectively, obtained after ion exchange chromatography on a column of cellulose phosphate. Dotted lines: control. Cytokinin standards eluted in the following regions: Fr 1, cytokinin glucosides; Fr 2, ZR and DZR; Fr 3, Z and DZ; Fr 4, IPA; Fr 5, IP. Inset: radish cotyledonary assay of one-tenth of Fr 1 (basic fraction) after chromatography on a cellulose thin layer plate (see text for details). The bars indicate the position of cytokinin standards (OGZR, Z7G, and Z9G).

saturated butan-1-ol (4 × 25 ml). The butanol extract was evaporated to dryness, dissolved in 2.5 ml 35% (v/v) ethanol, and fractionated on a column of Sephadex LH-20 (80 × 2.5 cm) eluting with the same solvent at a descending flow rate of 30 ml h^{-1} (details in Ref. 24). Thirty-five, 1-h fractions were collected and a small aliquot from each was taken for soybean callus assay (13). Two peaks of biological activity were obtained. Appropriate fractions were combined to yield Fr 2 and Fr 4 (see Fig. 1, acidic fraction) and known amounts of appropriate ^{2}H-labeled standards added. Fr 2 and 4 were subsequently further purified by HPLC before GC-MS analysis.

Fr B. After evaporation of the NH_3, this fraction was made up to 40 ml with H_2O, adjusted to pH 8.2 and extracted with butanol (6 × 40 ml), and fractionated on a Sephadex LH-20 column as above. Following soybean callus assay, five peaks of cytokinin activity were obtained. Appropriate fractions were combined to yield Fr 1 to 5 (see Fig. 1, basic fraction) and further analyzed. One-tenth of Fr 1 was applied as a 10-cm streak on a cellulose plate (0.5 mm in thickness, Serva) developed once in butan-1-ol:14 N NH_4OH:H_2O (6:1:2, v/v; upper phase). One-cm wide zones were scraped off the plate, put in Petri dishes (5.5 cm) containing 1.5 ml of phosphate buffer (pH 5.7), and left for 6 h with occasional shaking. One filter disc (Whatman No. 1) was put in each Petri dish and cytokinin activity was determined by the radish cotyledon assay (9) according to the cited reference. The results of the assay are shown in Figure 1 (inset). The remainder of Fr 1 was treated with sweet almond β-glucosidase as in (19), and then extracted with butanol. Known quantities of ^{2}H-labeled standards (Z7G, Z9G, Z, DZ, ZR, and DZR) were added to the residue obtained by evaporation of the butanol extracts and this was then purified by HPLC prior to GC-MS analysis.

Other fractions containing biological activity (Fr 2–5) were also subjected to HPLC purification after adding known quantities of appropriate ^{2}H-labeled standards.

HPLC. The details of equipment and chromatographic conditions used have been described elsewhere (26). All the columns were eluted at 3 ml min^{-1} and UV-absorbing compounds were monitored at 254 and/or 280 nm. The solvents used were binary mixtures of methanol and water containing 0.2 N acetic acid, the proportion of methanol being expressed as a percentage by volume. All columns and packings were obtained from Waters Associates and the column/solvent combinations used were as follows: (a) C_8 radial compression column (8 × 100 mm) eluted with a linear gradient of 5 to 50% methanol (System A) or, 5 to 60% methanol (System B) in 30 min, and 15 to 60% methanol in 20 min (System C); (b) μBondapak C_{18} column (7.8 mm × 30 cm) for isocratic elution with 20% (System D) or 32% (System E) methanol.

GC-MS. per-TMS derivatives of HPLC-purified and thoroughly dried (by azeotropic evaporation of methylene dichloride) cytokinins were prepared by heating the sample in a mixture of pyridine:BSTFA (1:1, v/v) at 90°C for 10 min. Electron impact mass spectra of TMS derivatives were obtained with a DuPont 21-491B instrument (source temperature, 200°C; line and separator temperature, 300°C) interfaced to a VG2025 data system (VG Analytical, Altrincham, Cheshire, U.K.). The samples were introduced via a gas chromatograph (2 m × 2 mm i.d., 3% OV-101 on 100-120 mesh Gas-chrom Q, with He flow of 12 ml/min) temperature programmed from 230 to 300°C for Z, DZ, ZR, and DZR or, 250 to 300°C for glucosides, at 10°C min^{-1}. The low resolution mass spectra were recorded (full scans from 800 to 200 atomic mass units) at 70 ev and a scan speed of 2 s/mass decade.

Electron impact GC-MS was also performed on a Finnigan 4500 instrument (GC and other conditions as above). Chemical-ionization spectra were determined on the Finnigan machine using methane as reagent gas at 1 torr. The samples were introduced either via a GC or, where indicated, on the direct inlet probe using a Vespel extension so that the sample was in direct contact with the reagent gas plasma.

RESULTS

Cytokinins in the Basic Fraction. The basic fraction eluted from cellulose phosphate was further purified by chromatography on Sephadex LH-20 to yield five active fractions, Fr 1–5.

Fr 1. Cytokinin glucosides are known to elute in this fraction and considerable biological activity eluted in this region (Fig. 1). A portion of this fraction was run on TLC under conditions where 7- and 9-glucosides of Z are clearly resolved from each other and from all four *O*-glucosides. The resulting chromatogram zones were bioassayed using the radish cotyledon test in which OGZ

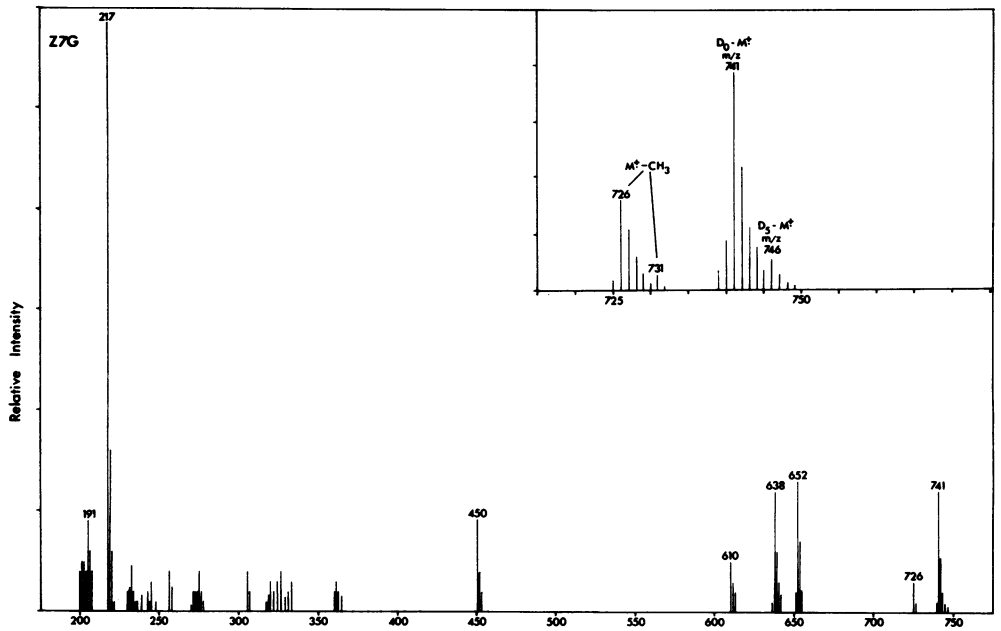

FIG. 2. Electron impact mass spectrum of pentatrimethylsilyl 7-β-D-glucopyranosylzeatin isolated from *D. innoxia* crown gall tumor tissue taken during combined GC-MS. Inset: enlargement (× 10) of the molecular ion region of the spectrum showing ions at *m/z* 741 and 726 derived from endogenous compound (D_0) in addition to corresponding ions at *m/z* 746 and 731 arising from penta-^{2}H-labeled internal standard (D_5).

and OGZR are highly active, Z9G, OGDZ, and OGDZR are moderately active, and Z7G is very weakly active (11). A prominent peak of biological activity was found at the R_F of Z9G (Fig. 1 inset), the other peak corresponding to the R_F of OGZ and OGZR (*cf.* Ref. 12). The *N*-glucosides of Z are resistant to β-glucosidase treatment whereas OGZ, OGDZ, OGZR, and OGDZR are all hydrolyzed to their corresponding aglycones. The remainder of Fr 1 was subjected to HPLC (System B) following β-glucosidase hydrolysis and butanol extraction, and UV peaks corresponding to the elution volumes of Z9G (R_t 16.85), Z7G (R_t 23.03), ZR (R_t 24.45), DZR (R_t 26.28), Z (R_t 33.35), and DZ (R_t 36.05) were collected separately. In addition, a large UV peak at R_t 18.95 was observed. All these peaks were individually rechromatographed on HPLC (System D for *N*-glucosides and System A for others) and further analyzed by GC-MS as TMS derivatives.

The TMS derivative of putative Z9G eluted as a sharp GC peak at the correct R_t for Z9G and yielded a EI-mass spectrum characteristic of penta-TMS 9-β-D-glucopyranosylzeatin (26). The ions in the molecular ion region, *m/z* 741 ($M^{\cdot+}$) and 726 ($M^{\cdot+} - CH_3^{\cdot}$) were approximately 10 times more intense than the corresponding ions (746 and 731) arising from the penta-^{2}H-labeled analog. The other prominent ions were seen at *m/z* 651, 639, 638, 450, 361, 360, 276, 271, 257, 218, 217, 204, 202, and 201.

The TMS derivative of the compound with R_t 18.95 on HPLC eluted as a sharp GC peak just before penta-TMS-Z9G. The EI-mass spectrum showed a prominent molecular ion at *m/z* 743 and an ion at 728 (loss of $CH_3^{\cdot}$) indicative of a glucosyl dihydrozeatin moiety. The remaining spectrum resembled closely that of penta-TMS-9-β-D-glucopyranosylzeatin except for the expected mass shifts and absence of fragmentation specifically directed by the double bond in the side chain of Z-type compounds. The TMS derivative of this compound co-eluted with penta-TMS-DZ9G on GC and the EI-mass spectrum was in complete accord with the published spectrum of 9-β-D-glucopyranosyl dihydrozeatin (26). Since the ^{2}H-labeled analog of DZ9G was not available, a known quantity of [$^2H_5$]Z9G was mixed with the sample before GC-MS. Although the two compounds fractionally separate on GC, an approximate quantification was achieved against *m/z* 746 and 731 arising from penta-TMS-[$^2H_5$]Z9G.

Putative Z7G after HPLC purification was also converted to its TMS derivative for further confirmation by GC-MS. Z7G elutes later than Z9G on GC and as a characteristic broad peak. The EI-mass spectrum of this compound (Fig. 2) was in complete agreement with the spectrum of authentic penta-TMS-7-β-D-glucopyranosylzeatin. The molecular ion region (see inset of Fig. 2) shows ions at *m/z* 746 ($M^{\cdot+}$) and 731 ($M^{\cdot+} - CH_3^{\cdot}$) derived from $^2H_5$-labeled internal standard (D_5) in addition to corresponding ions at 741 and 726 arising from endogenous compound (D_0). The CI-mass spectrum of the penta-TMS derivative of this compound exhibited an intense peak at *m/z* 742 (and 747) attributable to the protonated molecular ion (MH^+) and the correct M-15 peaks.

The *O*-glucosides of cytokinins were hydrolyzed to the corresponding aglycones and purified by HPLC before further analysis. This was necessary as the intact molecules would be degraded under the GC conditions employed. The HPLC peaks corresponding to the elution volumes of Z, DZ, and their ribosides were individually analyzed as their TMS derivatives by GC-MS. These compounds had the correct R_t on GC and their identity was established by comparison of their complete EI-mass spectra with those of relevant synthetic standards. The molecular ion regions of their spectra are shown in Figure 3, B and D. The molecular ions ($M^{\cdot+}$) and ions resulting from the loss of a $CH_3^{\cdot}$ radical are clearly indicated for the endogenous (D_0) and the corresponding deuterium-labeled internal standards (all D_5 except D_2 in the case of DZ which had only two ^{2}H atoms).

Fr 2. A strong peak of biological activity eluted in this region (elution volume of ZR and DZR, Fig. 1). Following HPLC analysis (System A) of Fr 2, a major peak at R_t of ZR and a minor peak at R_t of DZR were obtained. These peaks were collected individually and further purified by a second HPLC step (System D) and the identity of ZR and DZR was confirmed by GC-MS as their tetra-TMS derivatives. The complete EI-mass spectra were recorded which were in total agreement with the spectra of synthetic compounds. The molecular ion regions of the spectra showing ions for the endogenous (D_0) and corresponding penta-^{2}H-labeled internal standards (D_5) are shown in Figure 3C.

Fr 3. Significant biological activity eluted in this fraction corresponding to the elution volume of Z and DZ (Fig. 1). Following further purification by HPLC (System A followed by System D), Z and DZ were identified as di-TMS derivatives by GC-MS.

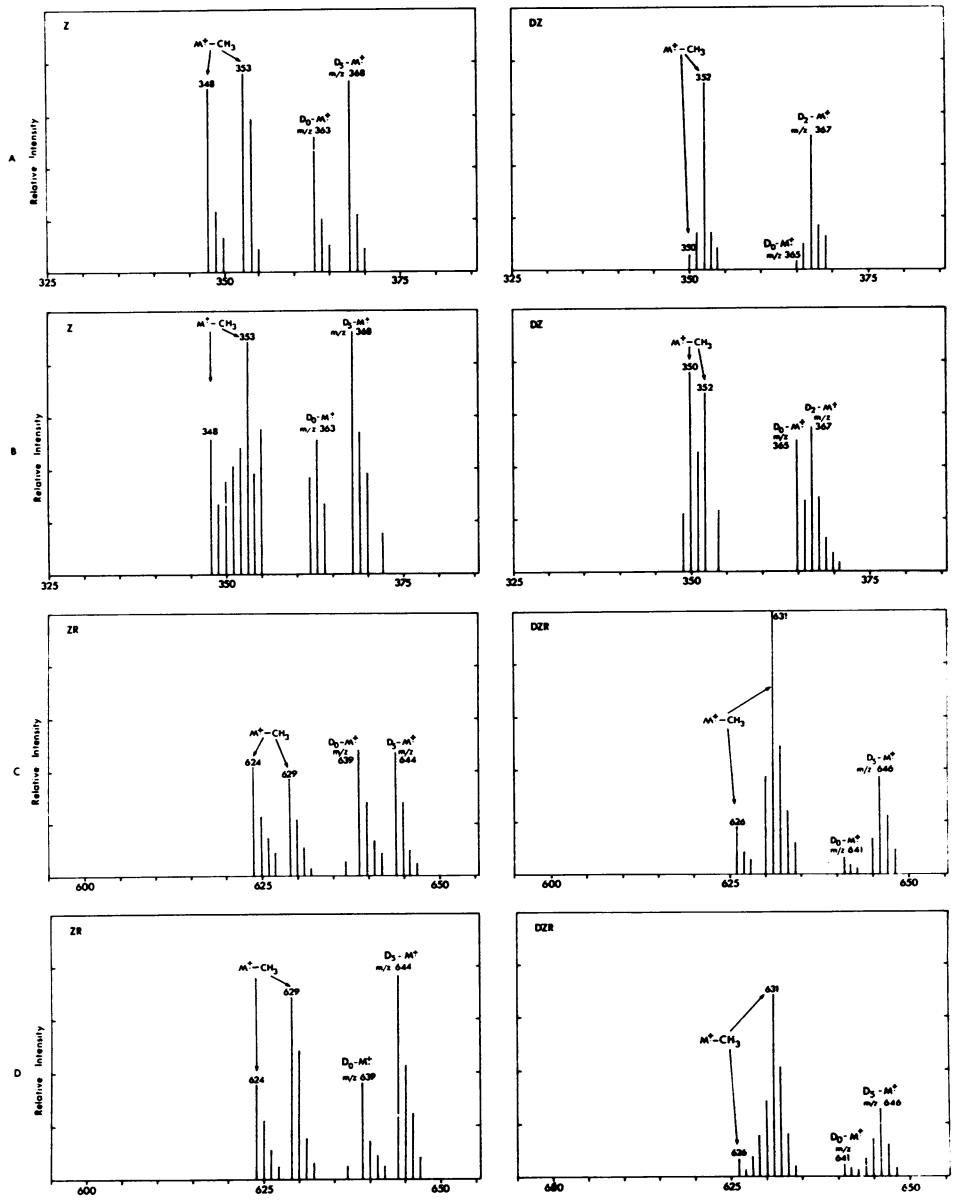

FIG. 3. Molecular ion regions of the electron impact mass spectra of trimethylsilyl derivatives of cytokinins purified from *D. innoxia* crown gall tumor tissue. The complete spectra were recorded; selected regions presented here show ions derived from endogenous cytokinins (D_0) and ^{2}H-labeled internal standards (D_5, except D_2 in the case of DZ). A, Z and DZ from Fr 3; B, Z and DZ from Fr 1 following treatment with β-glucosidase; C, ZR and DZR from Fr 2; D, ZR and DZR from Fr 1 following treatment with β-glucosidase. Fr 1-3 (see Fig. 1) were obtained following Sephadex LH-20 chromatography of the basic fraction eluting from a cellulose phosphate column.

Their full EI-mass spectra were identical to those of authentic compounds. The molecular ion regions of spectra for Z and DZ are shown in Figure 3A.

Fr 4 and 5. The elution volumes of IPA and IP correspond to Fr 4 and 5, respectively. These fractions exhibited only weak cytokinin activity (Fig. 1) and were purified individually by HPLC (System C followed by System E) before analysis by chemical ionization GC-MS. Their full spectra were in complete accord with those of appropriate synthetic compounds. The ions derived from endogenous compounds were very weak in comparison to the ions derived from corresponding ^{2}H-labeled compounds.

Cytokinins in the Acidic Fraction. Two clear peaks of biological activity (Fr 2 and 4, Fig. 1) eluted from a column of Sephadex LH-20 during chromatography of the phosphatase-treated acidic fraction. Appropriate fractions were combined and known quantities of ^{2}H-labeled standards were added before further analysis.

Fr 2. Purification of this fraction by HPLC, as indicated above for the basic fraction, produced two UV-absorbing peaks corresponding to the elution volumes of ZR and DZR. The complete EI-mass spectra of their TMS derivatives were recorded by GC-MS and compared with those of authentic compounds. The identifications were also confirmed by direct chemical ionization mass spectrometry.

In a separate experiment, zeatin riboside 5′-monophosphate was identified as the major endogenous cytokinin nucleotide in *D. innoxia* crown gall tumor tissue. It was quantified as an intact molecule using direct CI-MS (28).

Fr 4. The biologically active compound in this peak was purified by HPLC and identified subsequently as isopentenyladenosine by chemical ionization GC-MS. Once again, the ions derived from the hexa-^{2}H-labeled internal standard were very intense compared to the ions derived from the endogenous compound.

Table I lists the various cytokinins identified in *D. innoxia* crown gall tumor tissue. It should, however, be noted that the

Table I. *The Levels of Cytokinins in D. innoxia Crown Gall Tumor Tissue Extract*

Following chromatography on a column of Sephadex LH-20 and soybean callus assay, the biologically active fractions were appropriately combined. At this stage, relevant ^{2}H-labeled internal standards were added to achieve quantification of cytokinins by GC-MS. All estimations are based on the ratios of $M^+ - CH_3$ ions. Values presented for glucosides (Fr 1) are underestimates as the internal standards were added after β-glucosidase treatment.

Sephadex LH-20 Fractions and Cytokinins	Cytokinin Equivalent to 100 g Tissue Extract
	μg/fraction
Basic fraction	
Fr 1: OGZ,[a] OGZR,[a] OGDZ,[a] OGDZR,[a] Z9G, Z7G, and DZ9G	1.92, 2.67, 0.74, 0.4, 25.0, 19.75, and 13.0
Fr 2: ZR and DZR	7.84 and 0.69
Fr 3: Z and DZ	2.70 and 0.36
Fr 4: IPA	0.05
Fr 5: IP	0.11
Acidic fraction[b]	
Fr 2: ZR Ntd and DZR Ntd	4.66 and 0.76
Fr 4: IPA Ntd	0.11

[a] Values are for the derived aglycones.

[b] Ntd: nucleotide; measurements were done after hydrolysis to respective ribosides.

internal standards were added well after the initial extraction steps, and therefore, the values reported do not define the true cytokinin levels in the tissue. The extraction losses may differ for individual cytokinins.

DISCUSSION

Almost invariably, past investigations of cytokinins in plant tissue have been attempts to account for the principal components of extracted biological activity in terms of chemical entities. However, such an approach is unlikely to detect potentially important cytokinin metabolites with low cytokinin activity (*e.g.* 7- and 9-glucosides), especially if normal tissue culture bioassays are employed (see "Discussion" in Ref. 11). A desirable alternative type of study would integrate the classical bioassay-based approach with a purely chemical one. The latter would involve mass spectrometric detection methods for all known natural cytokinins using ^{2}H-labeled compounds as "carriers" to facilitate purification and as internal standards to achieve reliable identification and quantification. Such an integrated approach has been adopted for the first time in the present study of *D. innoxia* crown gall tumor tissue. As a result, 16 cytokinins were identified unambiguously which included 7- and 9-glucosides, *O*-glucosides, bases, ribosides, and nucleotides.

Cytokinins with a glucose moiety at positions 7 or 9 of the purine ring (except DZ9G) and compounds in which the sugar moiety is conjugated to the N^6 side chain have been isolated as metabolites of exogenously supplied cytokinins in a variety of plant tissues (10). Although OGZ and OGZR were originally identified as endogenous cytokinins in *V. rosea* crown gall tumor tissue (16) grown on medium containing reduced nitrogen, *O*-glucosides have subsequently been isolated in substantial amounts from tumor tissue cultured on normal medium (23), as also reported in the present study. Indeed, these compounds now appear to be ubiquitous cytokinins (10). However, the *N*-glucosides have been reported to occur in only a few plant tissues; Z9G in *Zea mays* kernels (26) and *V. rosea* crown gall tissue and culture medium (18, 22), and DZ9G and Z7G in *Z. mays* kernels (26) and radish seed (27), respectively. Hence, the identification of all three compounds in one plant tissue now reported is of interest. These compounds are likely to be more widely distributed than is evident at present. They are probably often not detected because of their very low biological activity in conventional callus bioassays and the difficulty of separating them from the *O*-glucosides. However, all the cytokinin glucosides can be readily resolved by HPLC (8) and TLC methods (12). All three *N*-glucosides are present in large amounts in crown gall tissue extracts of *D. innoxia* (Table I).

Using a special detection method, IPA was tentatively identified as a minor cytokinin in *V. rosea* crown gall tumor tissue (2). However, in subsequent studies, IP-type cytokinins have not been isolated from the same or any other tumor tissues (21, 23, 24). We now know that IP is rapidly metabolized to Z-type compounds in tumor tissues (19); hence, the steady state concentrations of IP its riboside and/or its nucleotide may be very low in such tissues. However, the results of this study clearly indicate that these compounds are present, but in barely detectable levels (Table I).

Although the dihydro compounds, namely DZ, DZR, and their *O*-glucosides have not previously been identified from tumor tissues, they were isolated from culture medium on which crown gall tissue of *V. rosea* had been growing (18). DZ, DZR, and their *O*-glucosides are present in *D. innoxia* tumor tissue, but in considerably lower amounts compared to their respective Z-like compounds (Table I). Inasmuch as DZ and DZR cochromatograph with Z and ZR, respectively, on the systems used in previous studies of tumor cytokinins, they were probably not detected in the presence of large excesses of Z and ZR. HPLC can be employed conveniently to separate not only these compounds, but also *cis* and *trans* isomers of Z and ZR; the compounds isolated in this investigation corresponded to the R_t of *trans* isomers only.

For many years, plant tumor tissues have been known to contain abnormally high levels of cytokinin activity. However, the identities of major endogenous cytokinins, including bases, ribosides, nucleotides, and *O*-glucosides, and the possible presence of weakly active conjugates (*e.g.* 7- and 9-glucosides), have been studied rigorously with only two such tissues, namely, *V. rosea* (14–16, 22, 23) and *D. innoxia* (the present study). Predominance of cytokinin glycosides seems to be a feature common to both tumor tissues mentioned. However, both tissues contain a diversity of cytokinin metabolites and it is interesting that *D. innoxia* tumor tissue contains, with the exception of alanine conjugates and *cis*-ZR, all the principal naturally occurring free cytokinins isolated so far.

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