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Cytokinin Biosynthesis in Plant Tumour Tissues

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Abstract. A range of endogenous cytokinins have been identified in *Datura* crown-gall tissue by GC-MS. Incorporation of [^{3}H]adenine into zeatin riboside, zeatin and its nucleotide(s) is also shown. Metabolism studies using *cis*- and *trans*- isomers of zeatin riboside indicate that interconversion of the two isomers does not occur in this tissue. Data on the identity of major endogenous cytokinins in a genetic tumour line of tobacco is also provided.

Plant tissues normally require the addition of auxin and cytokinin to the basal medium for growth in culture. However, axenic tissues obtained from crown-gall or genetic tumours can be cultured on hormone-free medium. Crown-gall tumours are incited on a range of dicots by *Agrobacterium tumefaciens*, and recent studies indicate that a portion of bacterial plasmid DNA is probably responsible for the synthesis of enzymes involved in hormone production (MORRIS *et al.* 1982, SCHRÖDER *et al.* 1984). A number of endogenous cytokinins have been identified unambiguously in crown-gall tumour (HORGAN *et al.* 1981, PALNI *et al.* 1983a,b). However, the so-called genetic tumours which appear spontaneously on certain interspecific hybrids, particularly in *Nicotiana* spp., arise without any apparent external cause (SMITH 1979). Although phytohormone imbalance is strongly implicated in the induction and maintenance of genetic tumours (BAYER 1982), there is no definitive work on endogenous cytokinins.

In this communication we present data on the endogenous cytokinins in *Datura* crown-gall tissue, report incorporation of [^{3}H]adenine into cytokinins, and examine ZR metabolism in relation to this biosynthesis. In addition, data on major endogenous cytokinins in a genetic tumour line of tobacco is presented, and the possibility of using this system for understanding of cytokinin biochemistry in "normal" plants is discussed.

MATERIALS AND METHODS

Datura innoxia MILL. line B6 crown-gall tumour tissue and tobacco genetic tumour tissues (*Nicotiana glauca* (GRAH.) × *N. langsdorffii* (WEINM.) and *N. suaveolens* (LEHM.) × *N. Langsdorffii*) were grown on hormone-free B_5

(GAMBORG and EVELEIGH 1968) and LINSMAIER and SKOOG (1965) medium, respectively. Soybean callus assay was done according to MILLER (1968). Cytokinin standards were obtained either commercially or synthesized according to published methods; *cis-* and *trans-* [^{3}H]ZR were prepared by heating respective non-radioactive compounds with ^3H_2O (for refs. see PALNI *et al.* 1984). Extraction and purification of endogenous cytokinins (PALNI *et al.* 1983a), and analysis of metabolites in [^{3}H]ZR feeding studies (PALNI *et al.* 1984) were done as in cited references. Cytokinin biosynthesis experiments were performed essentially as reported (PALNI *et al.* 1983b). In every case the extract was initially subjected to cellulose-phosphate chromatography yielding a basic fraction (containing bases, ribosides, glucosides and alanine conjugates) and an acidic/neutral fraction which would include nucleotides. High performance liquid chromatography (HPLC) conditions and equipment used have been described (SUMMONS *et al.* 1980). Per-trimethylsilyl (TMSi) derivatives were prepared according to PALNI *et al.* (1983a). tertiary-Butyl-dimethylsilyl (^{t}BuDMSi) derivatives were prepared by the method of HOCART *et al.* (1985). The sample was heated in 10 µl of pyridine (containing 0.5 µg dimethylamino pyridine as catalyst) and 10 µl of N-methyl-N-ter. butyl-dimethylsilyl trifluoroacetamide (MTBSTFA, containing 1 % t-BuDMCS, Regis Chem. Co.) at 90 °C for 10 min. Both electron impact (E.I.) and chemical ionization (C.I.) gas chromatography-mass spectrometry (GC-MS) were performed on a Finnigan 4500 instrument which was interfaced to a Nova 4 computer, and selected-ion-monitoring (S.I.M.) was done using Incos data system.

RESULTS

Datura Crown-Gall Tumour Tissue

Endogenous cytokinins: Results of soybean callus bioassay following Sephadex LH-20 column chromatography of tissue extract are shown in Fig. 1. This analysis yielded five active fractions in the basic fraction, and two active fractions in the alkaline phosphatase treated acidic fraction. The active fractions were pooled as marked in Fig. 1 and appropriate deuterium labelled compounds were added as "carriers" to facilitate purification and as internal standards to obtain reliable identification and quantification. After extensive HPLC purification putative cytokinin peaks were derivatised and subjected to E.I. and/or C.I. GC-MS analysis. As a result a total of following 16 cytokinins were identified unambiguously, and quantified in tissue extract (PALNI *et al.* 1983a). Basic Fraction: Fr1 — Z7G, Z9G, DZ9G and O-glucosides of Z, DZ, ZR, DZR; Fr2 — ZR, DZR; Fr3 — Z, DZ: Fr4 — IPA; Fr5 — IP. Acidic Fraction: Fr2 — nucleotides of Z and DZ; Fr4 — IP nucleotide.

Cytokinin biosynthesis: 10 g tissue was incubated for 20 h in 50 ml B_5 medium containing [^{3}H]adenine (1 µCi ml^{-1}; 23.6 µCi mmol^{-1}, Amersham). Following cellulose phosphate chromatography of the tissue extract, both the basic and acidic (after hydrolysis with alkaline phosphatase) fractions were fractionated on a Sephadex LH-20 column. Further analysis of appropriately

Abbreviations used: IP = isopentenyladenine; Z = zeatin (their corresponding 9-β-D-ribosides are IPA and ZR, respectively). 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. Ado = adenosine; Ade = adenine.

combined fractions confirmed ^3H incorporation into Z, ZR and Z nucleotide(s) in the ratio of 1 : 5 : 8. The total ^3H incorporation into cytokinins was less than 0.8 % of ^3H supplied initially.

Fig. 2 illustrates rigorous characterization of [^3H]ZR isolated from the tissue. After Sephadex LH-20 column chromatography of the basic fraction

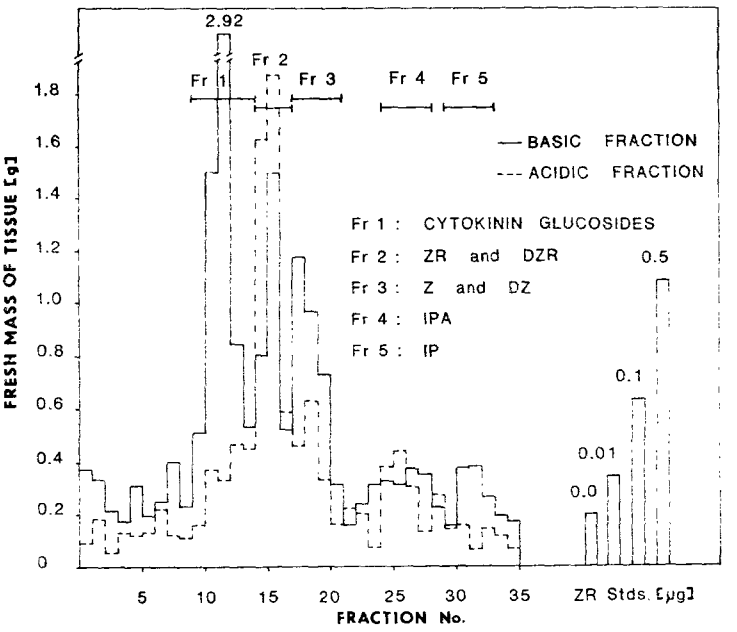

Fig. 1. Soybean callus bioassay profile following Sephadex LH-20 column fractionation of the basic and alkaline phosphatase treated acidic fraction from an extract of 10 g *Datura innoxia* crown-gall tissue. The bars (Fr1—Fr5) indicate the elution volume of cytokinin standards.

radioactivity corresponding to the elution volume of Ado/ZR/DZR (Fr 2, as in Fig. 1) was combined and subjected to preparative TLC (silica gel; n-BuOH: HOAc : H_2O, 12 : 3 : 5, v/v). The TLC zone corresponding to the R_f of Z/DZ/ZR/DZR was eluted and analysed by HPLC (Fig. 2A); the major peak of radioactivity eluted at the retention time of *trans*-ZR and was shown to cochromatograph with *trans*-ZR in diverse TLC and HPLC systems. This fraction was also chemically degraded (Palni *et al.* 1983b) to Z. Following extraction with n-BuOH about 80 % of ^3H extracting into BuOH was shown to be due to *trans*-Z by HPLC. Half of this was chromatographed directly on a silica gel TLC plate (sol; $CHCl_3$: MeOH, 9 : 1), and the remaining half after converting into ^{t}BuDMSi derivative. Results of TLC analysis are shown in Fig. 2B.

ZR Metabolism: 10 g crown-gall tissue was incubated under aseptic conditions for 6 h in 15 ml B_5 liquid medium containing 3 µM [^3H]*cis*-ZR (172 mCi mmol^{-1}) or [^3H]*trans*-ZR (142 mCi mmol^{-1}). The data on the stability of *cis*- and *trans*- isomers of ZR in *Datura* crown gall tissue, and the distribution of radioactivity in various identified metabolites are presented in Table 1.

TABLE 1

Metabolism of [^{3}H]ZR by *Datura innoxia* crown-gall tissue

Isomer supplied (6H)	Recovery of ^{3}H in metabolites								
							Nucleotide (s)		
	cis-ZR	*trans*-ZR	*cis*-Z	*trans*-Z	Ado	Ade	*cis*-Z	*trans*-Z	Ade
cis-ZR	16.55	ND	2.56	ND	0.95	0.74	1.91	ND	1.41
trans-ZR	ND	12.64	ND	1.33	1.38	0.82	ND	5.66	1.32

Radioactivity is expressed as a % of ^{3}H supplied. ND = Not detected.

Tobacco Genetic Tumour Tissue

The strategy for analysis of endogenous cytokinins was the same as in *Datura* crown-gall tissue. Following soybean callus bioassay of Sephadex LH-20 fractions, the major peak of activity was found in the elution volume of ZR/DZR in both the acidic and the basic fraction (results not shown). Following addition of appropriate ^{2}H standards and further purification by HPLC of active components, the samples were subjected to GC-MS analysis. The cytokinins identified in *N. glauca* × *N. langsdorffii* tissue extract include ZR, small amounts of Z, DZ, and nucleotides of Z and DZ (Fig. 4). Preliminary experiments were also conducted to investigate incorporation of [^{3}H]adenine into cytokinins using this line and a tumour line derived from *N. suaveolens* × *N. langsdorffii*. The tissue was incubated with [^{3}H]adenine in liquid medium for different times (1, 4, 8 and 24 h). The incorporation of ^{3}H (if any) into cytokinins was inconsistent and was insufficient to characterize rigorously.

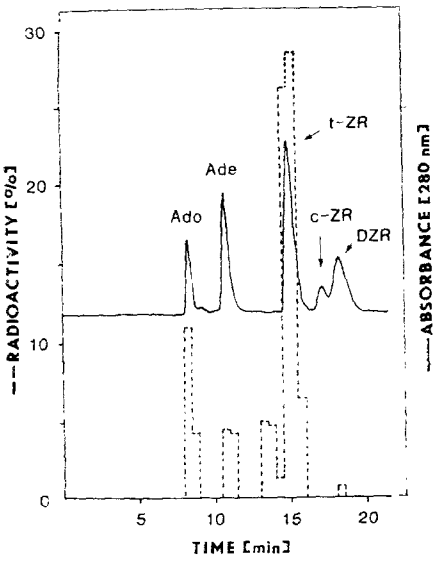

Fig. 2A. HPLC analysis of putative ZR/DZR fraction following preparative TLC (see text for details). HPLC was done on a C_8 Zobax column (250 × 9.4 mm) eluted isocratically with 20 % aqueous methanol containing 1 % HOAc at 4 ml min^{-1}.

DISCUSSION

Tumour induction results in specific biochemical changes including production of elevated levels of auxin and cytokinin (GORDON 1981). We have presented data on the identity of endogenous cytokinins in *Datura* crown-gall tissue and a genetic tumour line of tobacco, both maintained on hormone-free culture medium. Crown-gall tumour tissues seem to produce unusually high levels of a diversity of endogenous cytokinins (16 cytokinins were identified

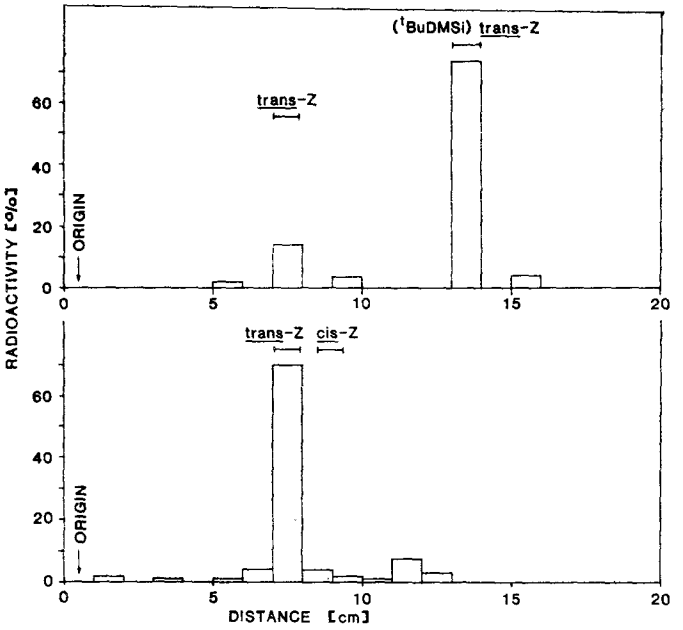

Fig. 2B. TLC analysis of [^{3}H] zeatin (resulting from chemical degradation of [^{3}H]ZR purified by HPLC as shown in Fig. 2A) before (lower Fig.) and after formation of ^{t}BuDMSi derivative (upper Fig.).

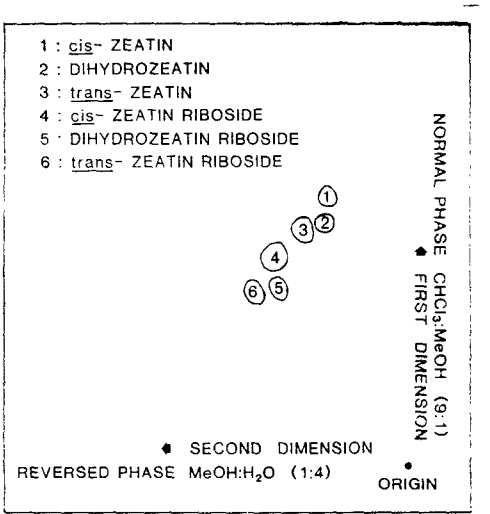

Fig. 3. Resolution of a mixture of closely related cytokinins by combination of normal and reversed-phase TLC. The plate (Kiesel gel 60 GF_{254}, 15 µM) was developed in the first dimension and then impregnated with a 6 % solution (v/v) of dimethylpolysiloxane (DMP-5X, Sigma) in petroleum ether (BP 100 to 120 °C) before development in the second dimension.

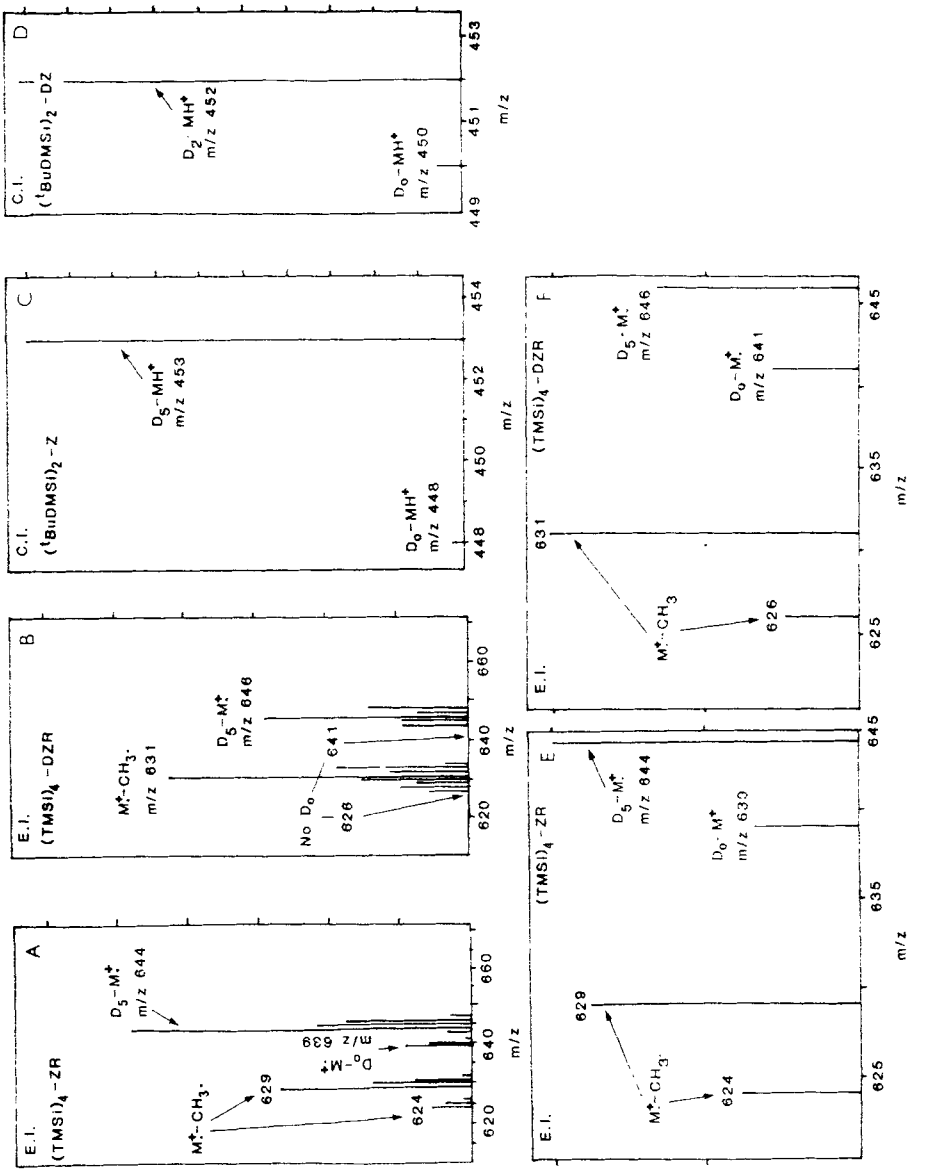

Fig. 4. Selected peak clusters in the mass spectra of cytokinins isolated from tobacco genetic tumour tissue taken during combined GC-MS. Molecular ion regions in the E. I. mass spectra of TMSi derivatives of cytokinin ribosides, (A) ZR and (B) DZR (note the absence of ions from the endogenous compound); and S.I.M. profiles of cytokinin nucleotides following enzymic hydrolysis, (E) ZR and (F) DZR. C.I. — S.I.M. profiles of ^{t}BuDMSi derivatives of cytokinin bases (C) Z and (D) DZ. GC-MS conditions: E.I. and C.I. (NH_3 at 1 torr) spectra recorded at 70 eV and ion source temp. of 150 °C, and at 140 eV and 120 °C, respectively. GC was run at 200 °C with 20 °C min^{-1} increase for cytokinin ribosides (A, B, E and F), and at 250 °C isothermal for cytokinin bases (C and D). SGE capillary column (50 meter length, 0.33 mm I. D.; bonded phase, vitreous silica BP1) was used with - SGE on-column injector.

unequivocally in *Datura* crown-gall), however, the first analysis of endogenous cytokinins in a genetic tumour line reported here would suggest that only a few cytokinins are produced in such tissues, and the levels produced

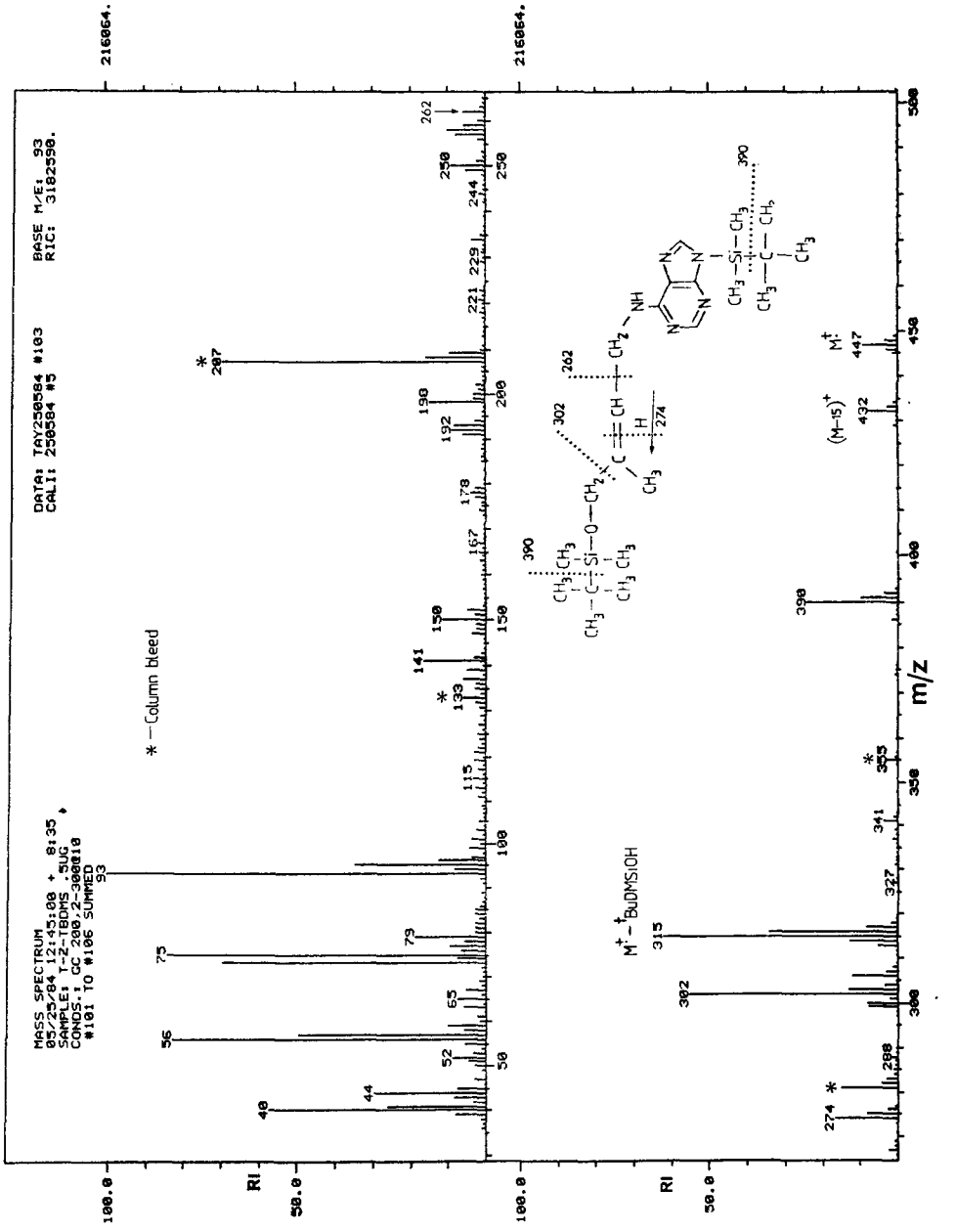

Fig. 5. E.I. mass spectrum of (^{t}BuDMSi)$_2$-zeatin taken during combined GC-MS. The spectrum was recorded on a VG Micromass 7070F instrument at 70eV with ion source temp. of 200 °C. GC run was from 200 °C (isothermal 2 min) to 300 °C at 10 °C min^{-1}. GC column (2 meter × 2 mm ID., 3 % OV−101 on Gas Chrom Q100−120). The spectrum is partially rationalized to define major fragment ions.

are considerably less than those reported for crown-gall tumours. It is possible that cytokinin production is under more rigorous (and "normal"?) control in genetic tumour tissues. The relative importance of tRNA turnover in free cytokinin production is still unclear (LETHAM and PALNI 1983). Although there is evidence to support the direct route, the possibility that free cytokinins are tRNA derived cannot be neglected (PALNI and HORGAN 1983). *cis*-ZR is the dominant cytokinin in plant tRNAs, while the considerably more active *trans*-ZR and its derivatives are major free cytokinins in higher plants. In this connection it is most desirable to know the stability of *cis*- and *trans*-ZR *in vivo*, and to examine if the two isomers are biologicaly interconvertable. The data from metabolisms tudies clearly establish that there is no conversion of *cis*-ZR to *trans*-ZR and *vice versa*. Similar observations were made with the two isomers of Z in *Amaranthus caudatus* cotyledonary explants (unpublished work). The results further suggest a direct biosynthetic route for cytokinin formation. The relative proportion of [^{3}H] adenine incorporation into Z, ZR and Z nucleotide(s) in *Datura* crown-gall would indicate that the pathway of free cytokinin production in this system is similar to that reported for crown-gall tissue of *Vinca rosea* (PALNI *et al.* 1983b). However, lack of sufficient [^{3}H] adenine incorporation into cytokinins in our preliminary work with tobacco genetic tumour tissues may be due to slower rate of cytokinin turnover. It is hoped that further work in this system would give us some understanding of cytokinin production and its control in "normal" higher plant systems.

Two techniques developed and used in this work are noteworthy. A mixture of the two isomers of Z (*cis*-, *trans*-), DZ, and the corresponding ribosides is difficult to resolve by chromatographic procedures commonly used. By combining normal-phase (first dimension) and reversed-phase (second dimension) TLC all of the six compounds can be easily separated from each other in a single step (Fig. 3). The use of TMSi and more recently permethyl derivatives is common for GC-MS analysis of cytokinins. However, formation of multiple derivatives, non-quantitative yields (particularly with bases) and the unstable nature of derivatives are problems commonly observed with TMSi derivatives. Although permethyl derivatives are stable, the manipulations involved cause problems at sub-microgram levels. We have found that ^{t}BuDMSi derivatives (HOCART *et al.* 1985) avoid these problems in the analysis of cytokinin bases. These can be made in quantitative yields as single derivatives and are stable through further TLC and HPLC analysis (HOCART *et al.* 1985). A representative mass spectrum of (^{t}BuDMSi)$_2$-zeatin has been partially rationalized as shown in Fig. 5. The present paper illustrates successful use of these new techniques in the analysis of biological samples.

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