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The Biosynthesis of Cytokinins in Crown-Gall Tissue of *Vinca rosea*

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Abstract. The crown-gall tissue of *Vinca rosea* converts labelled adenine into cytokinins. The principal initial products appear to be ribosylzeatin phosphates; zeatin and ribosylzeatin are also produced in appreciable quantities. The efficiency of conversion of adenine into cytokinins suggests a pathway of synthesis independent of turnover of tRNA. Isopentenyl adenine or its derivatives do not appear to be intermediates in the conversion of adenine to zeatin compounds. Cytokinins in *V. rosea* turnover rapidly and further metabolism of zeatin derivatives seems to result in their conversion into glucosides which are the main cytokinin active compounds in the tissue.

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

Introduction

Many plant tissues metabolise exogenously supplied cytokinins to a variety of products, some of which are inactive as cytokinins (Hall, 1973; Whitty and Hall, 1974). Since it is likely that endogenous cytokinins are degraded by the same enzyme systems a mechanism for their constant renewal must exist. Although the instantaneous levels of cytokinins are presumably determined by the relative rates of biosynthesis and degradation, virtually nothing is known about the rate or mechanism of biosynthesis of free cytokinins.

Cytokinin-active bases occur in the tRNA of many species (Hall, 1973; Burrows, 1975) and are formed by attachment of an isopentenyl side-chain to the N^6-nitrogen atom of a previously unmodified adenine in the RNA (Chen and Hall, 1969; Murai et al., 1975). Free cytokinins might be formed therefore by the breakdown of cytokinin-containing RNA.

Whilst free cytokinins might arise from those present in tRNA there is no direct evidence that they do so. The metabolism by tobacco of an adenosine derivative modified in the ribose ring to a cytokinin analogue even though it is not incorporated into tRNA (Chen et al., 1976) and the conversion of AMP into isopentenyl adenine by extracts of *Dictyostelium discoideum* (Taya et al., 1978) suggests the existence of pathways for the production of free cytokinins independent of tRNA turnover. Further, though more circumstantial, evidence for such pathways is provided by the small quantities of cytokinins present in tRNA (Short and Torrey, 1972), the known low rate of turnover of tRNA in many tissues (Trewavas, 1970; Hall, 1973; Klemen and Klämbt, 1974) and differences in the chemical nature of the free and tRNA-bound cytokinins (Hall, 1973; Burrows, 1978).

Vinca rosea crown-gall tissue readily converts adenine into zeatin and ribosylzeatin (Peterson and Miller, 1976) and the rate of this process is apparently modified by changing the nitrogen balance of the nutrient supply. In order to investigate further the mechanism and control of cytokinin biosynthesis a more detailed qualitative and quantitative investigation of cytokinin production in this tissue has been undertaken.

Materials and Methods

Plant Materials

Vinca rosea crown-gall callus, kindly supplied by Dr. C.O. Miller, was grown on modified White's medium containing 0.75% agar (Miller, 1974).

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Abbreviations: HPLC = high performance liquid chromatography; AMP = adenosine monophosphate; TLC = thin-layer chromatography; GLC = gas-liquid chromatography

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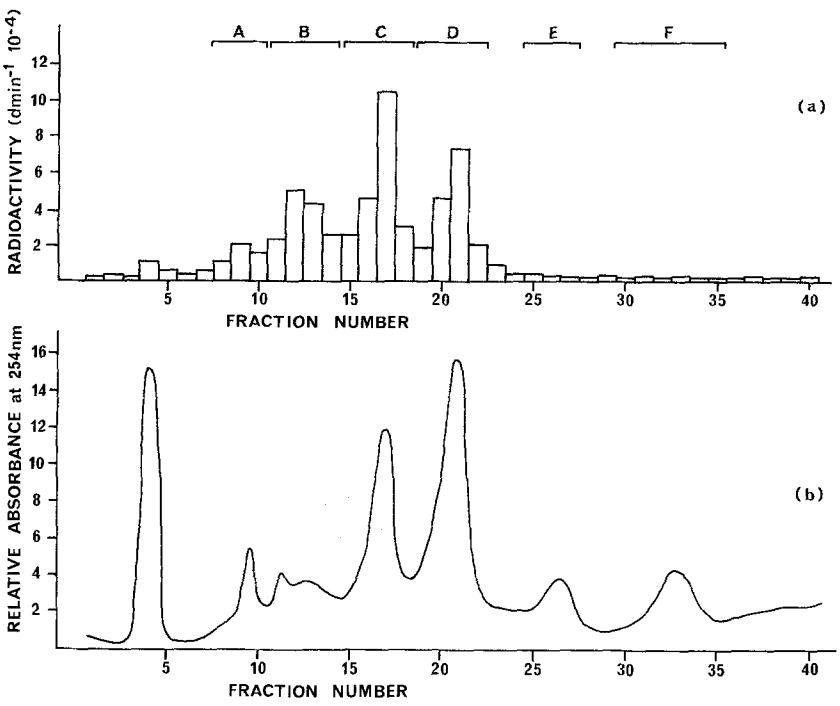

Fig. 1 a and b. Elution of n-butanol soluble cytokinins from column of Sephadex LH-20 (2.5 × 85 cm) by 35% ethanol. 10 g *V. rosea* crown gall were supplied with $1.9 \cdot 10^5 ds^{-1}$[U-^{14}C] adenine and the cytokinins and other purines were extracted after 8 h as described in Materials and Methods.
a Shows radioactivity.
b u.v. absorbance ($\lambda = 254$ nm). Known cytokinins eluted in the following regions. *A*, no known cytokinins *B*, glucosylzeatin, glucosylribosylzeatin; *C*, ribosylzeatin; *D*, zeatin; *E*, isopentenyladenosine; *F*, isopentenyladenine

Metabolic Studies

These were performed using tissue grown for 8 weeks after transplanting. 10 g crown-gall tissue was transferred aseptically to a 100 ml conical flask containing 10 ml normal nutrient medium (without agar) and $1.9 \cdot 10^5$ ds^{-1}[U-^{14}C] adenine ($1 \cdot 10^{10}$ ds^{-1} $mmol^{-1}$, Radiochemical Centre, Amersham, UK). Several such flasks were set up on a reciprocating shaker (approx. 60 rev min^{-1}) at 25° C. After the appropriate time the tissue was removed by filtration on a Buchner funnel and the metabolites present in the tissue were examined.

The numerical results described are derived from one large experiment but the system is both qualitatively and quantitatively very reproducible.

Analysis of Metabolites

Exhaustive identification of all the metabolites of adenine was not feasible. The identity of zeatin and ribosylzeatin resulting from metabolism of radioactive adenine by *V. rosea* crown-gall has been established beyond doubt (Peterson and Miller, 1976). Ribosylzeatin, glucosylzeatin and glucosylribosylzeatin have also been identified as endogenous cytokinins in this tissue using a variety of chemical and physical techniques (Miller, 1974; Peterson and Miller, 1977; Morris, 1977).

All of the major metabolites of adenine i.e., adenosine, adenine nucleotides etc. are more polar than adenine itself and in the chromatographic system used migrate behind adenine. In consequence relatively simple chromatographic procedures are very effective for the purification and identification of compounds more mobile than adenine such as the cytokinin bases and ribosides. Accordingly analysis of metabolites was conducted as follows.

The tissue in the funnel was washed with 20 ml water and suspended in 100 ml Bieleski No. 1 solvent overnight at −20° C to inhibit phosphatases (Bieleski, 1964). The tissue was removed by filtration and treated in a Virtis blender (half speed, 2 × 30 s) with 100 ml Bieleski No. 2 solvent at −20° C. After standing overnight at −20° C the residue was filtered off, washed with a little solvent and the filtrate combined with the first Bieleski extract. 100 µg each of the unlabelled standards adenine, adenosine, zeatin, ribosylzeatin, isopentenyladenine and isopentenyladenosine was added. The combined Bieleski extracts were evaporated to dryness at 30° C in vacuo. The residue was suspended in 20 ml water, adjusted to pH 8, filtered and the filtrate extracted with water saturated n-butanol (6 × 20 ml). Materials extracting into n-butanol (including purine bases, ribosides and glucosides) were further analysed as in a) below whilst the nucleotides remaining in the aqueous layer were examined as described in b).

a) Purine Bases, Ribosides, Glucosides

The butanol layers were combined and evaporated in vacuo at 30° C. The residue was dissolved in 1.5 ml 35% ethanol, quantitatively applied to a column of Sephadex LH-20 (2.5 × 85 cm) and eluted with the same solvent. 30 ml fractions were collected and an aliquot counted in a Beckman LS-200 Scintillation Counter using a PPO, toluene, triton-X100 scintillator. A u.v. trace ($\lambda =$ 254 nm) of the eluate allowed detection of the added standards. A typical elution profile is shown in Fig. 1. Fractions indicated were combined and evaporated at 30° C in vacuo. Previous experience suggested that the fractions would contain the standards described in the legend to Fig. 1.

Fractions A–D were examined by TLC. An aliquot of each was applied, together with each of the standards which it lacked, to the corner of a 2D-TLC plate and developed in Solvents A and B. Radioactive areas were located using a radiochromatogram spark chamber (Birchover Instruments, Letchworth, UK). Areas of silica gel corresponding to the appropriate standards were removed from the plate, and eluted with aqueous ethanol (50% or 80% depending on sample). Radioactivity and u.v. spectra of the eluate were determined. From the u.v. spectrum recovery of the standards was calculated and used to correct the radioactivity for losses occurring during purification.

Radioactivity running with adenine, adenosine, zeatin or ribosylzeatin on the 2D-plates also cochromatographed as a single spot with the appropriate standard on TLC on silica gel developed

in Solvents C or D. These solvents resolve the cis- and trans-isomers of zeatin and ribosylzeatin; radioactivity was present only in trans-zeatin and in trans-ribosylzeatin. GLC of the trimethylsilyl derivative of the ribosylzeatin confirmed its identity as trans-ribosyl zeatin rather than the cis-isomer or dihydroribosyl zeatin and suggested that this compound was radiochemically pure. Preliminary analysis of the zeatin fraction by HPLC in several solvent systems (details to be published) suggested that it did contain impurities and that the values quoted for radioactivity present in zeatin are considerable overestimates.

Fractions E and F were treated as A–D but because of their low radioactivity were subjected to TLC only in Solvent A.

In addition half of fraction B was treated with β-glucosidase (Boehringer – from sweet almond; suspension in 3.2 M ammonium sulphate – 10 µl in 0.25 ml 0.1 M sodium acetate pH 5.4) for 24 h at 37° C. 50 µg of each of the usual six standards was added and the samples examined by 2D TLC as above. Before glucosidase treatment samples from tissue incubated with adenine for 24 h or 72 h showed a large complex radioactive spot with low Rf in solvent B. This was thought to contain inosine, zeatin- and ribosylzeatin glucosides together with other unidentified compounds. After treatment with β-glucosidase radioactivity in this region was much reduced and more localised and new radioactive spots co-chromatographing with zeatin and ribosyl zeatin were found. These were eluted and radioactivity and u.v. absorbance determined as above. This procedure determines recovery of zeatin and ribosyl zeatin from the TLC plate but does not allow for efficiency of butanol extraction or β-glucosidase treatment so that quoted values for the radioactivity of the glucosides are certainly underestimates. Such a procedure was necessary because authentic samples of the glucosides were not available.

b) Nucleotides

Material not extracting into n-butanol from aqueous solution pH 8 includes the nucleotides. The aqueous sample was adjusted to pH 3 with acetic acid and passed through a small column of zerolit-225 (NH_4^+ form) at the same pH to remove traces of purine bases and ribosides. After evaporation to dryness the sample was treated with alkaline phosphatase (Sigma; type VII; from calf intestine; 25 µl) in 0.03 M ethanolamine hydrochloride pH 9.5 (7.0 ml) containing 10 mM $MgCl_2$. Adenine and adenosine (500 µg each) and zeatin, ribosylzeatin, isopentenyladenine, isopentenyladenosine (200 µg each) were added and the solutions evaporated to dryness. The residue was suspended in 20 ml water and adjusted to pH 8. Materials extracting into n-butanol (4 × 20 ml) were examined by 2D TLC in the usual way. Phosphatase treatment resulted in the production of radioactive adenosine and ribosylzeatin which were eluted from the silica gel. Recovery of these compounds was determined from the u.v. absorbance and used to correct radioactivity for losses during purification. Radioactivity running with ribosylzeatin on the 2D TLC plates cochromatographed with the trans-isomer in solvents C or D. Treatment of the residue from the phosphatase incubation with further enzyme did not release more than 15% of the radioactivity liberated by the first treatment and this was all adenosine.

Thin-layer Chromatography (TLC)

This was performed on 20 cm square glass plates coated with 0.5 mm thick layer of Merck GF_{254} silica gel and dried at room temperature.

Chromatography solvents were as follows:
Solvent A – n-butanol: acetic acid: water (12:3:5).
Solvent B – n-butanol: 14 M ammonia: water (6:1:2) – upper phase.
Solvent C – chloroform: methanol (9:1).
Solvent D – chloroform: acetic acid (4:1).

Table 1. The uptake and metabolism of $1.9 \cdot 10^5 ds^{-1}$[U-^{14}C]adenine by 10 g *Vinca rosea* crown gall tissue. Incubation and analysis of metabolites was performed as described in Materials and Methods

Time	Counts present in medium	Counts present in adenine in tissue	Counts present in adenosine in tissue	Counts present in adenine nucleotides in tissue
	total $dmin^{-1}$	total $dmin^{-1}$	total $dmin^{-1}$	total $dmin^{-1}$
0	$110 \cdot 10^5$	–	–	–
20 min	$68 \cdot 10^5$	$1.05 \cdot 10^5$	$0.12 \cdot 10^5$	$7.60 \cdot 10^5$
1 h	$25.50 \cdot 10^5$	$0.96 \cdot 10^5$	$0.33 \cdot 10^5$	$31.10 \cdot 10^5$
3 h	$4.40 \cdot 10^5$	$0.43 \cdot 10^5$	$0.68 \cdot 10^5$	$40.40 \cdot 10^5$
8 h	$4.18 \cdot 10^5$	$0.47 \cdot 10^5$	$0.53 \cdot 10^5$	$21.30 \cdot 10^5$
24 h	$4.69 \cdot 10^5$	$2.49 \cdot 10^5$	$1.85 \cdot 10^5$	$9.05 \cdot 10^5$
72 h	$5.70 \cdot 10^5$	$1.80 \cdot 10^5$	$0.93 \cdot 10^5$	$2.28 \cdot 10^5$

Results

A suspension of *Vinca rosea* rapidly takes up adenine from the surrounding medium. Table 1 shows the result of suspending 10 g *V. rosea* crown-gall in 10 ml medium containing $1.9 \cdot 10^5$ ds^{-1}[U-^{14}C]adenine. More than 90% of the supplied adenine is taken up within the first 3 h of the experiment and most of this is converted into adenine nucleotides. This occurs presumably by the action of adenine phosphoribosyl transferase as this enzyme catalyses the first step in the utilisation of free purines by most tissues (see for example Murray, 1971).

The conversion of adenine into zeatin and ribosylzeatin occurs in *V. rosea* crown-gall (Peterson and Miller, 1976), a finding confirmed by the data shown in Table 2. The incorporation of ^{14}C into these compounds is however only about 15% of the total found in cytokinins. Most of the radioactivity is present in ribosylzeatinphosphates[1] which are the major initial products of cytokinin synthesis in *V. rosea*. Peterson and Miller did not investigate the nucleotides nor did they take precautions to prevent breakdown of phosphate esters so that some of their zeatin riboside might have arisen in this way.

The radioactivity present in zeatin, ribosylzeatin and ribosylzeatin phosphates rises simultaneously to reach a maximum between 8 h and 24 h after the

[1] These compounds yield ribosylzeatin on treatment with alkaline phosphatase and are probably 5′-nucleotides (although attachment of the phosphate to other positions cannot at present be excluded). This might include 5′-mono, di and triphosphates although preliminary evidence based on ion-exchange chromatography suggests that only the monophosphate is present after 8 h of incubation

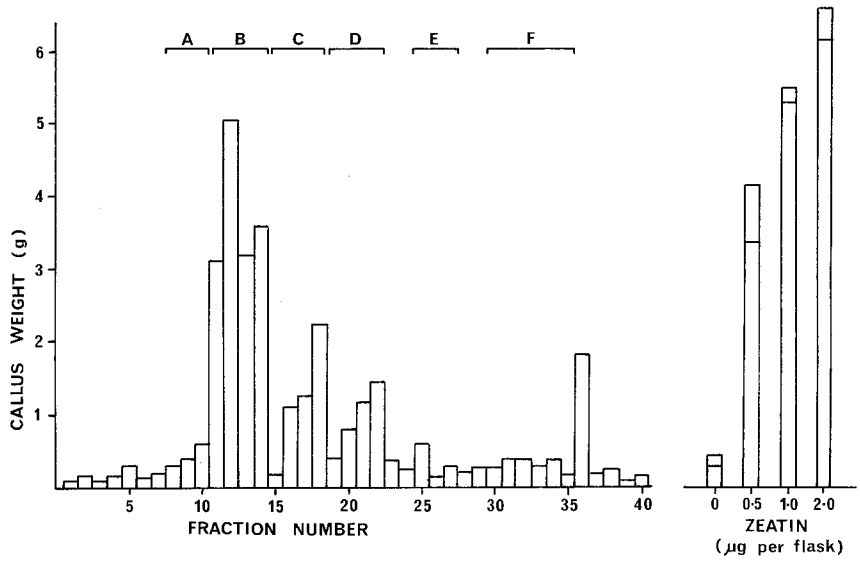

Fig. 2. Soybean callus bioassay of endogenous cytokinins in *Vina rosea*. 100 g of tissue were extracted in Bieleski's solvents and the material extracting into n-butanol from aqueous solution at pH8 was applied to a column of Sephadex LH-20 (2.5 × 85 cm) and eluted with 35% ethanol. One thirtieth of each fraction was used in the bioassay. Standards elute as described in the legend to Fig. 1

start of the incubation before declining rapidly. It seems that in *V. rosea* crown-gall cytokinins turnover rapidly and that the high endogenous levels of the hormones do not result from an absence of the enzymes which degrade them.

A large proportion of the cytokinin activity of *V. rosea* is present as glucosides. Figure 2 shows a typical bioassay of activity extracted from *V. rosea*. Peaks of activity elute coincidentally with zeatin and ribosylzeatin and the latter has been unequivocably identified from this tissue (Miller, 1974). Further biological activity elutes before ribosylzeatin. This fraction almost certainly contains N^6-(4-D-glucopyranosyl-3 methyl-2 transbutenyl) aminopurine and its riboside which have been identified in the tissue (Peterson and Miller, 1977; Morris, 1977).

As the radioactivity in zeatin, ribosylzeatin and ribosylzeatinphosphate declines it increases in cytokinin glucosides – Table 3. The latter compounds seem to be identical with the glucosides associated with high biological activity, for they elute together from Sephadex LH-20 in 35% ethanol, and on treatment with β-glucosidase yield compounds which cochromatograph with zeatin or with ribosylzeatin on silica gel plates in Solvents A and B.

The time over which the glucosides are produced suggests that they might be formed by direct glucosylation of zeatin and ribosylzeatin although their production from other unknown compounds cannot be ruled out. The ^{14}C appearing in the glucosides is considerably less than that found in zeatin or in ribosylzeatin but, as pointed out in the Materials and Methods, these values are probably underestimates because the lack of suitable standards prevented measurement of the recovery of the compounds. It is possible that a considerable proportion of the zeatin and ribosylzeatin becomes glucosylated.

Incorporation of radioactivity from adenine into isopentenyl adenine or isopentenyladenosine is very small; no more than 500 $dmin^{-1}$ were ever detected in

Table 2. Incorporation of [U-^{14}C]adenine into zeatin cytokinins in *Vinca rosea* crown gall tissue. Incubation and analysis of metabolites was performed as described in Materials and Methods

Time	Counts present in zeatin	Counts present in ribosyl zeatin	Counts present in ribosyl zeatin phosphates
	total $dmin^{-1}$	total $dmin^{-1}$	total $dmin^{-1}$
0	–	–	–
20 min	$0.30 \cdot 10^3$	$0.46 \cdot 10^3$	$10.70 \cdot 10^3$
1 h	$0.68 \cdot 10^3$	$2.36 \cdot 10^3$	$12.40 \cdot 10^3$
3 h	$1.90 \cdot 10^3$	$6.92 \cdot 10^3$	$62.90 \cdot 10^3$
8 h	$6.29 \cdot 10^3$	$17.70 \cdot 10^3$	$126.00 \cdot 10^3$
24 h	$5.68 \cdot 10^3$	$17.00 \cdot 10^3$	$112.00 \cdot 10^3$
72 h	$2.19 \cdot 10^3$	$3.98 \cdot 10^3$	$9.83 \cdot 10^3$

Table 3. Incorporation of [U-^{14}C] adenine into cytokinin glucosides in *Vinca rosea* crown gall tissue. Incubation and analysis of metabolites was performed as described in Materials and Methods

Time	Counts present in glucosyl zeatin	Counts present in glucosyl ribosyl zeatin
	total $dmin^{-1}$	total $dmin^{-1}$
0	–	–
20 min	–	–
1 h	–	–
3 h	–	–
8 h	–	$4.08 \cdot 10^3$
24 h	$3.09 \cdot 10^3$	$16.60 \cdot 10^3$
72 h	$4.40 \cdot 10^3$	$23.40 \cdot 10^3$

either compound. Neither could ^{14}C be detected in materials which yielded isopentenyladenosine on treatment with alkaline phosphatase i.e., in the nucleotide.

Discussion

A possible pathway for the synthesis of zeatin or ribosylzeatin is by attachment to the N^6 amino group of adenine or adenosine of an isopentenyl side chain and its subsequent hydroxylation. Conversion of isopentenyladenine (or its riboside) into zeatin (or ribosylzeatin) occurs in *Rhizopogon roseolus* (Miura and Miller, 1969) and in *Zea mays* (Miura and Hall, 1973).

The very small incorporation of radioactivity into isopentenyladenine, and its riboside and nucleotide suggests that if these compounds are obligatory intermediates in the production of zeatin derivatives then the enzymes catalysing their hydroxylation must be very active indeed. The alternative explanation – that isopentenyladenine is not involved in zeatin production is equally possible. Either explanation is consistent with the fact that despite isopentenyladenosine having been identified in *V. rosea* (Chen et al., 1976) very little biological activity behaves like isopentenyladenine or isopentenyladenosine. The latter compounds would elute from Sephadex LH-20 in fractions 25–27 and 30–35 respectively (Fig. 2). Work is at present being undertaken to establish whether *V. rosea* tissue is particularly active in converting exogenously supplied radioactive isopentenyladenine into zeatin.

Eight hours after incubation begins there are about $1.55 \cdot 10^5$ d min^{-1} in the cytokinins representing a conversion of about 1.4% of the supplied adenine into zeatin derivatives.[2] Cytokinins are found as a single base in only some of those tRNA species which recognise U as their initial codon letter. Presumably adenine supplied to the tissue will be incorporated into all nucleic acids which are undergoing appreciable turnover. The limited available data on the turnover of nucleic acids in higher plants suggest that tRNA turns over relatively slowly ($t^1/_2 = 3$–5 days; Klemen and Klämbt, 1974; Trewavas, 1970) at a rate comparable to that of rRNA which makes up the major proportion of RNA in the cell. It is not clear how the efficiency of conversion of adenine to zeatin compounds could be so great if the modification and turnover of tRNA were obligatory in the process. A pathway independent of tRNA turnover is indicated.

The simplest interpretation of the data is that a direct conversion of AMP into zeatin ribotide occurs, zeatin and ribosylzeatin being formed subsequently. This route would be analogous to that recently demonstrated for the conversion of AMP into isopentenyladenine by *Dictyostelium discoideum* (Taya et al., 1978) where the side chain is apparently attached only to the nucleotide. In *V. rosea* rapid interconversion of adenine and zeatin and their respective ribosides and ribotides takes place so that it is not possible to say that the only route transforming adenine into zeatin derivatives involves conversion of AMP into zeatin ribotide.

The possibility that the metabolism of tRNA in *V. rosea* crown-gall is abnormal cannot be ignored. Formation of appreciable quantities of cytokinin as a result of tRNA metabolism might occur if:

(i) the tissue contained abnormally large quantities of cytokinin-containing RNA species;

(ii) the cytokinin-containing tRNA in the tissue turned over abnormally rapidly;

(iii) there were a method of excising cytokinin bases from modified tRNA without breaking down the RNA completely.

Extraction, preliminary identification and quantification of the cytokinins present in the tRNA of *V. rosea* suggests that the amounts present are similar to those in other tissues (L.M. Palni and I. Sziraki – unpublished work).

The other two possibilities cannot be ruled out at present. Measurements of the turnover of total tRNA in the tissue may not help in view of the abnormally rapid turnover of a subpopulation of tRNAs in animal tumors (Borek et al., 1977). The only unambiguous answer will be obtained by direct measurement of the rate of turnover of cytokinin bases in individual species of tRNA under conditions such as those described here where the rates of production of free cytokinins can be measured. Such experiments are at present being undertaken.

The maximum synthesis of cytokinins as a result of supplying the adenine ($2.4 \cdot 10^{-6}$ g) is about $2.5 \cdot 10^{-8}$ g in 10 g of tissue i.e. $2.5 \cdot 10^{-9}$ g g^{-1} fresh wt. The endogenous level of ribosylzeatin alone in *V. rosea* crown-gall is estimated to be 3–$5 \cdot 10^{-7}$ g g^{-1} tissue (Fig. 2 and Miller, 1974) so that any observed metabolism of the cytokinins is unlikely to be the result of providing unphysiological concentrations of these compounds. Furthermore, since it is presumably the distribution of the enzymes needed for the biosynthesis of cytokinins which determines where they are formed, they should be produced only in their normal physiological location so that metabolism observed

[2] This is a minimum value. Preliminary studies suggest that some of the zeatin and ribosylzeatin formed is released into the medium during incubation and is therefore not included in the analysis

in this way ought to reflect the true capabilities of the tissue.

The incorporation of large amounts of ^{14}C from adenine into ribosylzeatin phosphates must prompt reconsideration of the effect of changing the nitrate/ammonia balance of the nutrient medium on cytokinin production in *V. rosea* (Peterson and Miller, 1976). The changes which they observed in fact occurred in only a small part of the total amount of cytokinins being produced and may be of less significance than previously thought. They might result, for example, from a small change in the activity of phosphatases in the tissue. On the other hand an investigation of the effect of such changes in nutrient supply on the total cytokinin production might be of value.

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