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Incorporation of ^{3}H-adenine into free cytokinins by cytokinin-autonomous tobacco callus tissue

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SUMMARY

Cytokinin-autonomous tobacco callus was incubated in defined mineral medium containing ^{3}H-adenine for 60 minutes. Radioactivity was incorporated into the four predominant free cytokinins, ribosyl-trans-zeatin, trans-zeatin, N^{6}-(Δ^{2}-isopentenyl) adenosine and N^{6}-(Δ^{2}-isopentenyl) adenine. The bases were more abundant than their respective ribosides, N^{6}-(Δ^{2}-isopentenyl) adenine being the most abundant cytokinin. No discrete peaks of radioactivity could be detected on the HPLC column eluate corresponding to the elution volumes of cis-zeatin and ribosyl-cis-zeatin.

INTRODUCTION

Cytokinins occur naturally both as bases adjacent to the first letter of the anticodon triplet in specific tRNA species and as free bases, ribonucleosides and ribonucleotides (see reviews[1,2,3]).

Opinion is not unified as to the biosynthetic routes involved in generating these free cytokinins. The obvious alternative mechanisms are that the free cytokinins either (1) arise by biosynthesis de novo or (2) are by-products released intact during turnover of the cytokinin-containing tRNA species. It is also possible that both pathways are employed during plant growth and development. Data in support of the two opposite hypotheses have been published. Leineweber and Klämbt[4] and Klemen and Klämbt[5] support the hypothesis that tRNA acts as the source of free cytokinins and

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Chen et al[6] and Burrows[7] claim that free cytokinins can arise by biosynthesis de novo. Direct evidence in support of this latter hypothesis is reported in this paper and involves the study of the incorporation of ^{3}H-adenine into free cytokinins by cytokinin-autonomous tobacco callus.

METHODS AND MATERIALS

Cytokinin-autonomous tobacco callus, ca 150 g, grown in mineral medium[8] minus kinetin was harvested after ca 20 days growth and incubated in 100 ml similar medium, omitting the agar and with a reduced sucrose concentration (0.1%), containing 10 μCi ^{3}H-adenine (specific activity 25 Ci/mmol). After 60 min the tissue was harvested and homogenised in an Ultra-Turrax homogeniser (full speed, 2 min) in 1.5 volumes of cold N perchloric acid and left for 1 h at 2°C to inactivate completely any phosphatase present. The resultant slurry was centrifuged at 10,000 g for 15 min and the supernatant was decanted. The pellet was re-extracted a further twice with equal volumes of cold N perchlorate and the combined supernatants were spiked with 100 g each of io^{6} ado,* io^{6} ade, i^{6} ado and i^{6}ade neutralised to pH 5.0 with KOH. The mixture was centrifuged at 10,000 g for 20 min and the supernatant was lyophilised, dissolved in 10 ml distilled water, adjusted to pH 9.0 and extracted three times with 0.5 volumes of water-saturated 1-butanol. The butanol extracts were combined and lyophilised, and the solid material was extracted twice with 2.0 ml dry methanol. The methanol extracts were combined and lyophilised, and the solid material was dissolved in 2.0 ml of 35 per cent aqueous ethanol and chromatographed on a Sephadex LH-20 column (44 x 2.0 cm) equilibrated and eluted with the same solvent. The regions of the column eluates known to contain the four cytokinins were combined separately and lyophilised, and each was further analysed by reverse phase HPLC[11] on Lichrosorb RP8 semi-preparative columns (20 cm x 10 mm id) equilibrated and eluted with H_2O/MeOH (70:30 for the io^{6} ado and io^{6} ade fractions and 40:60 for the i^{6} ado and i^{6} ade fractions). The column eluates were monitored at 260 nm. The fractions were collected directly into plastic scintillation vials as indicated in the figure. The vials were placed in an oven overnight at 35° to remove most of the eluant. The scintillation fluor (Packard M197) was then added and the samples were counted in an Intertechnique SL30 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Discrete peaks of radioactivity co-eluted with both t-io^{6}ado (Figure 1a) and t-io^{6}ade (Figure 1b). No discrete peak of radio-

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Abbreviations used:

Ribosyl-trans-zeatin; t-io^{6}ado; ribosyl-cis-zeatin; c-io^{6}ado; trans-zeatin; t-io^{6}ade; cis-zeatin; c-io^{6}ade; N^{6}-(Δ^{2}-isopentenyl) adenosine; i^{6}ado; N^{6}-(Δ^{2}-isopentenyl) adenine; i^{6}ade.

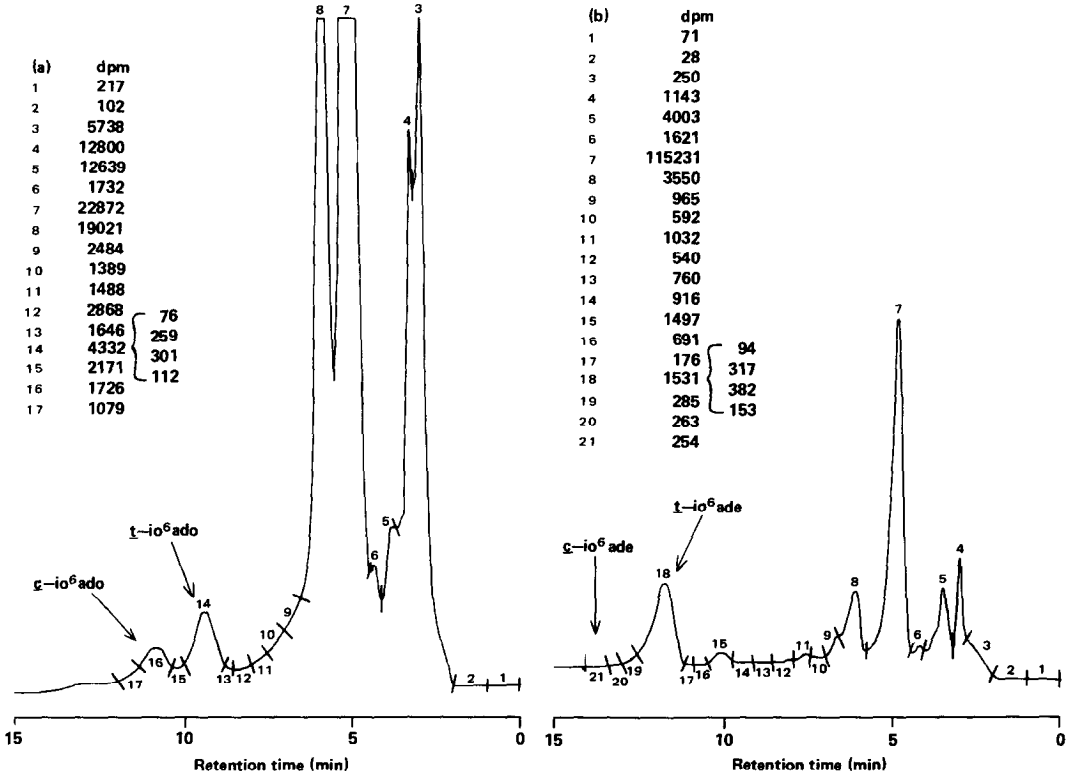

Figure 1: The distribution of the radioactivity in the UV-absorbing material eluting from the HPLC of the io^{6}ado and io^{6}ade fractions obtained following Sephadex LH-20 chromatography of an extract of cytokinin autonomous tobacco callus incubated for 60 min in the presence of ^{3}H-adenine.

(a) io^{6}ado fraction, 30 µl injection out of a total of 120 µl; rechromatography, repeat injection.

(b) io^{6}ade fraction, 10 µl injection out of a total of 150 µl; rechromatography, 20 µl out of a total of 100 µl.

activity was detected in the elution volumes corresponding to the cis-isomers. The eluate in fractions 14 and 18 (Figure 1a and b respectively) was collected and rechromatographed under identical conditions. The radioactivity in each half of the cytokinin peak and in an equal volume of eluate immediately adjacent to the peak was determined. The reduced radioactivity (Figure 1a and b) illustrates that some of the radioactivity in fractions 14 and 18 was

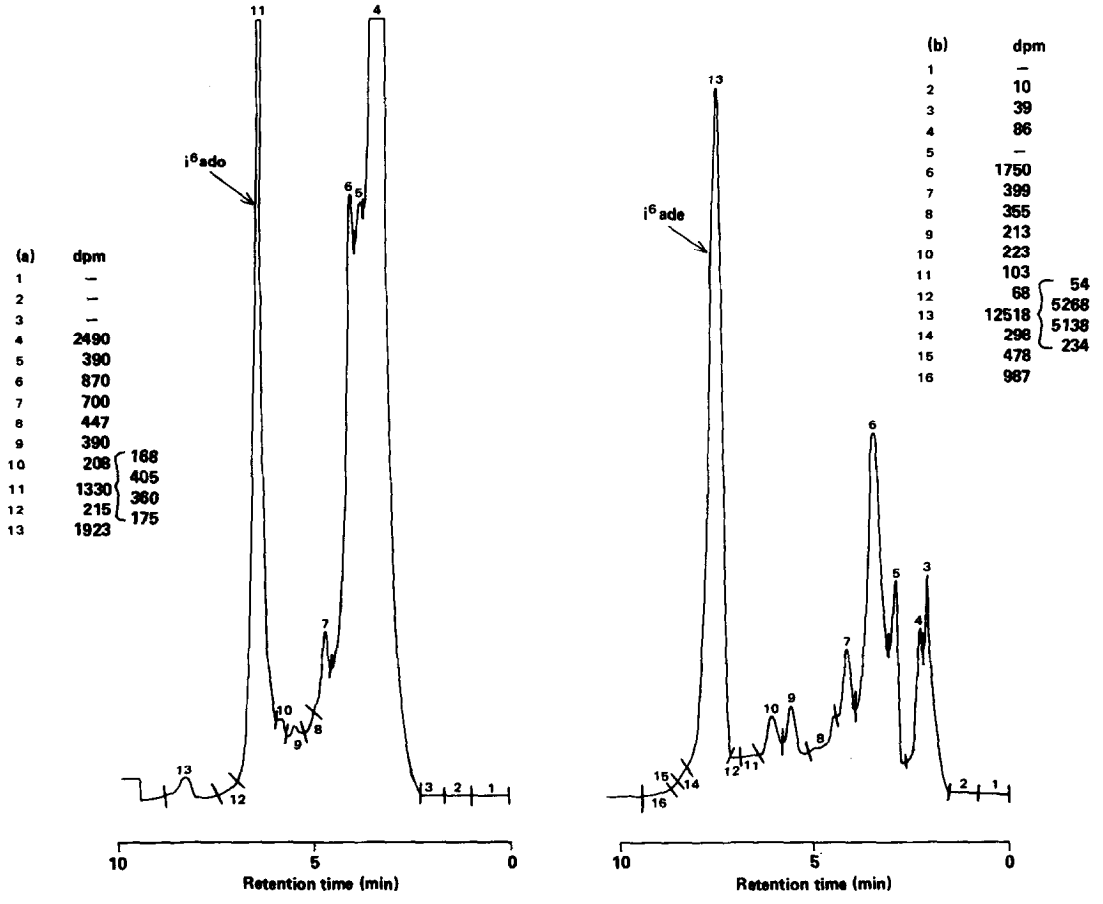

Figure 2: As for Figure 1 with the exception that this figure represents the HPLC of the i^{6}ado and i^{6}ade fractions from the Sephadex LH-20 columns.

(c) i^{6}ado fraction, 20 µl injection out of a total of 150 µl; rechromatography, repeat injection.

(d) i^{6}ade fraction, 20 µl injection out of a total of 150 µl; rechromatography, repeat injection.

due to compounds other than t-io^{6}ado and t-io^{6}ade respectively. The distribution of radioactivity within the peaks of t-io^{6}ado and t-io^{6}ade is coincident with the uv profile. The major peaks of radioactivity in fractions 5 and 6 (Figure 1a) and fraction 7 (Figure 1b) co-elute with adenosine and adenine respectively. Peaks of radioactivity also co-elute with i^{6}ado and i^{6}ade (Figure 2a and b respectively). Rechromatography of the eluate in fractions 11 and

13 (Figure 2a and b) under identical conditions confirms that the distribution of radioactivity within these peaks is also coincident with the uv-profile. The most abundant cytokinin synthesised was i^6ade (78,045 dpm), the other cytokinins were less abundant, i^6ado (5,692 dpm), t-io^6ade (3,495 dpm) and t-io^6ado (2,240 dpm). The chromatographic data strongly support the claim that the observed radioactivity is associated with the respective cytokinins and is not due to any other contaminating material. The identity of the other radioactive compounds in the column eluates is unknown.

It is unlikely that these cytokinins are the by-products of tRNA turnover for the following reasons: (1) The *cis*-isomer of zeatin predominates in the tRNA and in this study the incorporation of ^{3}H-adenine is specifically into the *trans*-isomers, the predominant 'free' isomer. (2) in tRNA the predominant cytokinin is the 'oxidised form' t-io^6ado, in this study it is the 'reduced form' i^6ade. Therefore if one attributes all the free cytokinins to tRNA turnover one must support the statement by claiming that tRNA species containing i^6ado must turnover at a rate up to *ca* one hundred times that for tRNA species containing i^6ado. No data is available pertaining to the relative stabilities of these tRNA species.

Very recent published evidence[13] suggested that 5'-AMP was the substrate for the alkylating enzyme in the biosynthesis *de novo* of i^6ade by the slime mould *Dictyostelium*. The *in vivo* incorporation of ^{3}H-adenine into i^6ade reported above for tobacco callus cannot differentiate between 5'-AMP and adenine as the substrate for the alkylating enzyme. It is conceivable that 5'-AMP is the substrate for the alkylating enzyme. The resulting cytokinin nucleotide is then rapidly degraded to i^6ade during the incubation period as was shown to be the case in *Dictyostelium*[13]. Any breakdown of a cytokinin nucleotide during the extraction procedure was minimised

by the inclusion of the perchloric acid step[9]. No adenosine or adenine was detected in extracts of tobacco callus spiked with 5'-AMP immediately prior to homogenising. Adenine was the major metabolite of 5-AMP when the perchloric acid step was omitted.

Experiments are in progress to determine both the substrate for the alkylating enzyme and the possible contribution to the pool of free cytokinin by tRNA turnover.

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