EVIDENCE FOR THE BIOSYNTHESIS OF TRANSFER RNA-FREE CYTOKININ

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Received 15 March 1976

1. Introduction

i^6Ade, io^6Ade and their ribosides occur naturally either free or as constituents of tRNA [1–7]. These compounds and closely related derivatives constitute a group of plant hormones, the cytokinins, which promote plant cell division and differentiation. The precursor of the isopentenyl side chain of i^6Ado in tRNA has been shown to be mevalonate or Δ^2-isopentenylpyrophosphate [8–13]. Although free cytokinins have been detected in various organisms, the question of whether the free cytokinins are produced by the turnover of cytokinin-containing tRNA and/or by an alternative biosynthetic pathway is yet to be resolved.

This paper reports the biosynthesis of i^6Ade and its derivatives by supplying [8-^{14}C] Ade and a ^{14}C, ^{3}H-labeled adenosine analog, 2-*O*-[1-(R)-(9-adenyl)-2-(hydroxy) ethyl] glycerol, to cytokinin-autotrophic tobacco tissue cultures. The tissue is capable of synthesizing i^6Ade and its derivatives from adenine and from the adenosine analog. Cleavage of the glycosidic bond of this analog was not detected. The analog was neither a substrate for adenosine deaminase (EC 3.5.4.4) nor was it incorporated into tRNA. These results suggest that an alternative 'tRNA-free' cytokinin biosynthetic pathway may be functional in the plant tissue.

Abbreviations: i^6Ade, N^6-(Δ^2-isopentenyl)adenine; Ade, adenine; Ado, adenosine; io^6Ade, 6-(4-hydroxy-3-methyl-2-butenylamino)purine; $Ado^{ox\text{-}red}$ which has been first oxidized with periodate and then reduced with $NaBH_4$; $i^6Ado^{ox\text{-}red}$ or $io^6Adeo^{ox\text{-}red}$ which has been similarly treated as for adenosine above. HPLC, high pressure liquid chromatography.

2. Materials and methods

[8-^{14}C]Ado (4.7 mCi/mmol), [8-^{14}C]Ade (5.0 mCi/mmol) and [^{3}H]$NaBH_4$ (300 μCi/μmol) were obtained from New England Nuclear Corporation. Ado, i^6Ado and calf-intestinal adenosine deaminase (type I) from Sigma Chemical Company, and io^6Ade and io^6Ado from Calbiochem. [^{14}C, ^{3}H]$Ado^{ox\text{-}red}$ (^{14}C, 4.7 mCi/mmol; ^{3}H, 32.5 mCi/mmol) unlabeled $io^6Ado^{ox\text{-}red}$ and $i^6Ado^{ox\text{-}red}$ were prepared as previously described [14]. Autonomous tobacco pith tissue cultures originally supplied to us by Dr J. E. Fox of Kansas University was grown on a medium without cytokinin and auxin [15].

Paper chromatography was carried out on Whatman No.1 or No.3 MM paper in a descending fashion. Solvents used were (v/v): (A) 2-propanol–water–concentrated NH_4OH (7:2:1); (B) ethylacetate–1-propanol–water (4:1:2); (C) 95% ethanol–0.1 M $(NH_4)_3BO_3$, pH 9.0 (1:9); (D) 1-butanol–water–concentrated NH_4OH (86:14:5). High pressure liquid chromatography was performed in a Waters Associates Model ALC 202/R401 liquid chromatograph equipped with an M-6000 pump. The column was a stainless steel tube (60 × 0.2 cm) packed with

Corasil II. (Waters Associates, Milford, Massachusetts), and was eluted with degassed solvent (20% ethanol–80% acetonitrile) at room temperature (22–24°C), 500 psi. The effluent was monitored at 254 nm and the flow-rate at 0.5 ml/min.

Radioactivity was measured as previously described [16]. A Cary model 14 spectrophotometer was used to measure absorbance of the adenine derivatives and the nucleosides.

Mass spectra were measured with a CH-5 single focusing mass spectrometer. The sample was admitted to the source via a direct insertion probe. The probe temperature could be precisely regulated between 10°C and 500°C. The electron impact source was adjusted to an electron beam energy of 70 eV and an accelerating voltage of 3000 V. Line spectra were drawn from the mass spectrum; the most abundant ion was set to 100%.

3. Results and discussion

The structure of $i^6Ado^{ox\text{-}red}$, an oxidized-reduced product of i^6Ado, was confirmed by mass spectral analysis to be 2-*O*[1(R)-(9-N^6-(Δ^2-isopentenyl)adenyl-2-hydroxyethyl] glycerol (fig.1). This compound had a molecular ion m/e 337 [i^6Ado(335) + 2]. Peak at m/e 322 (M-15) is due to loss of CH_3; 294(M-43), loss of $C(CH_3)_2$ and H; 306 (M-31) and 246 (M-91), loss of CH_2OH and $C_3H_7O_3$ from 2-*O*-β-hydroxyethylglycerol moiety; 203, free N^6-(Δ^2-isopentenyl)adenine; 188, free N^6-(Δ^2-isopentenyl)adenine less CH_3; 160 (M-177), loss of 2-*O*-β-hydroxyethylglycerol and $C(CH_3)_2$. Further fragmentation of the N^6-(Δ^2-isopentenyl)adenine side chain yield ions at m/e 148, 135 and 119. The breakdown of the 2-*O*-β-hydroxyethylglycerol yields ion at m/e 103, 60 and 45.

The uptake of [^{14}C, ^{3}H] $Ado^{ox\text{-}red}$, [8-^{14}C] Ado, and [8-^{14}C] Ade by autonomous tobacco tissue was compared to see if the absorption rate of these compounds is related to their metabolism. Twenty-eight day old autonomous tobacco pith tissues (three pieces per flask, about 3.7 to 4.6 g; five flasks for each treatment) were placed onto semi-solid basal media containing 7.5×10^{-6} M [^{14}C, ^{3}H] $Ado^{ox\text{-}red}$, [8-^{14}C] Ado or [8-^{14}C] Ade. The uptake of these radioactive materials was measured by determining

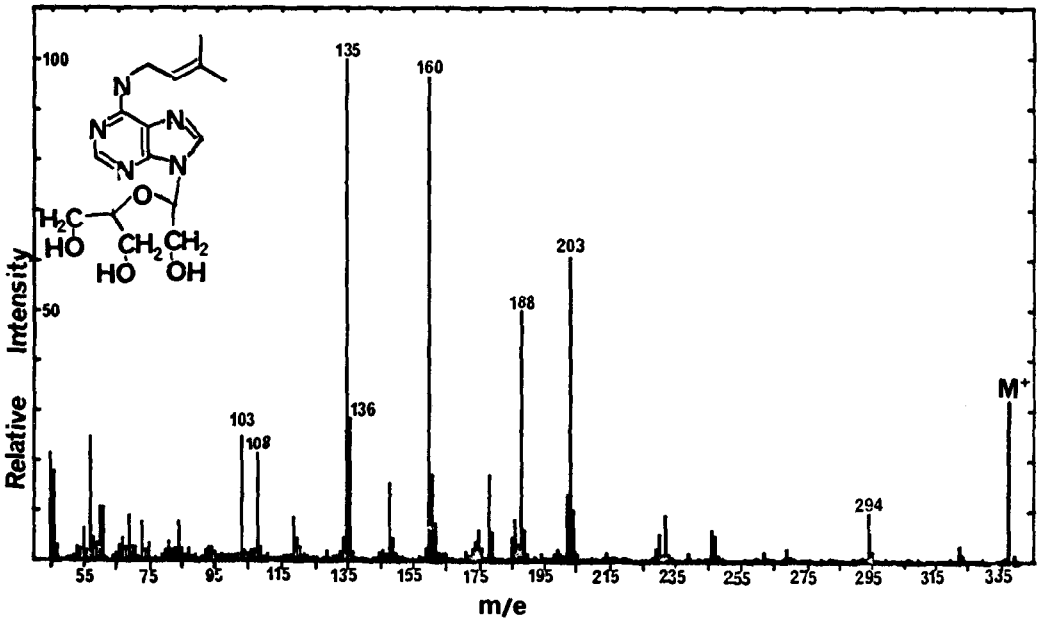

Fig.1. Mass spectrum of $i^6Ado^{ox\text{-}red}$. Major peak assignments m/e: 337, molecular ion; 322 (−15), loss of CH_3; (−43), loss of $C(CH_3)_2$ and H; 246 (−91), loss of $C_3H_7O_3$ from 2-*O*-β-hydroxyethylglycerol moiety; 203, free base; 188, free base less CH_3; 160 (−177), loss of 2-*O*-β-hydroxyethylglycerol and $C(CH_3)_2$.

the radioactivity present in the tissues after various periods of incubation. Any residual medium attached to the tissue was wiped off with Kimwipe paper. The average yield of fresh tissue for all time periods, from 2 to 28 days in the dark at room temperature (22–24°C), was from 3.9 to 6.8 g per flask. Radioactive materials in the tissues were extracted with 50 and 95% ethanol (20 ml ethanol/g of fresh tissue) and with ethylacetate–water (5:1, v/v; 15 ml/g of tissue). Insoluble material was removed by centrifugation. The ethanol and ethylacetate soluble fractions were combined, reduced to less than 4 ml in a flash evaporator (38°C) and the final volume was adjusted to 4 ml with water. Aliquots of the extracts were removed, and radioactivity was determined using Bray's solution [17]. Fig.2 shows a comparison of the results of the uptake of [^{14}C, ^{3}H]Ado$^{ox\text{-}red}$, [8-^{14}C]Ado and [8-^{14}C]Ade by the tissue cultures. The rate of [^{14}C, ^{3}H]Ado$^{ox\text{-}red}$ uptake by the tobacco tissues was about 30 and 43% that of [8-^{14}C]Ade and [8-^{14}C]Ado respectively on a fresh-weight basis over an experimental period of 12 days. Although reasons for the difference in absorption rate between Ado and Ado$^{ox\text{-}red}$ are not yet known, it is possible that the differences in metabolism and membrane permeability of these compounds may be related to the rate of uptake.

In order to identify the metabolic products, the radioactive materials extracted were chromatographed on Sephadex LH-20 columns and eluted with 35% aqueous ethanol [16,18]. After a 12-day incubation, three major doubled-labeled fractions (fig.3, Fl-3) were obtained from [^{14}C, ^{3}H]Ado$^{ox\text{-}red}$ metabolites. These amounted to 19.4, 58.8 and 21.8% of the relative ^{14}C c.p.m. The three radioactive fractions from the column were further purified by paper chromatography in various solvent systems. In solvent system A, the F-3 fraction was again separated into three bands (fig.4, Bl-3). Comparison of the paper chromatographic mobilities in solvents A-D and the ultraviolet absorption spectra of the purified Band 1-3 samples with control samples led to the partial

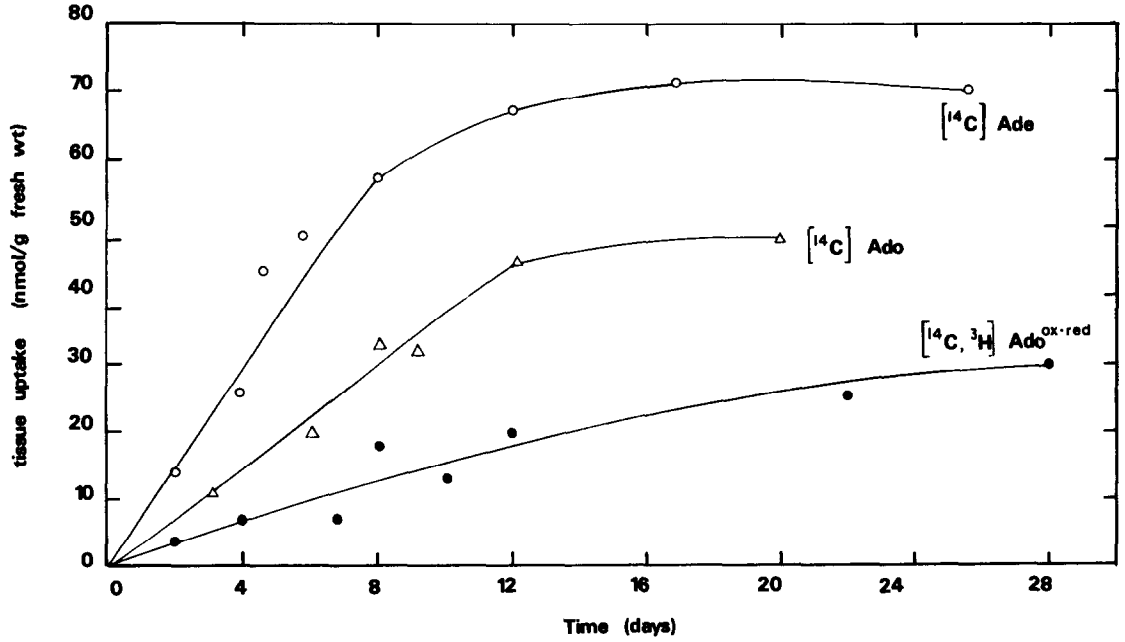

Fig.2. Comparison of the uptake of [^{14}C, ^{3}H]Ado$^{ox\text{-}red}$, [8-^{14}C]Ade and [8-^{14}C]Ado by autonomous tobacco pith tissue cultures. Twenty-eight day old autonomous tobacco tissues (three pieces per flask) were placed onto semi-solid basic media containing radioactive Ade or its derivatives (7.5×10^{-6} M). At the indicated times radioactive materials in the tissue (five flasks) were extracted with 50 and 95% ethanol (20 ml/g wet tissue) and with ethylacetate–water (5:1, v/v; 15 ml/g wet tissue). Ethanol and ethylacetate was evaporated from the combined extracts. Aliquots of the extracts were removed and radioactivity was determined in Bray's solution [17].

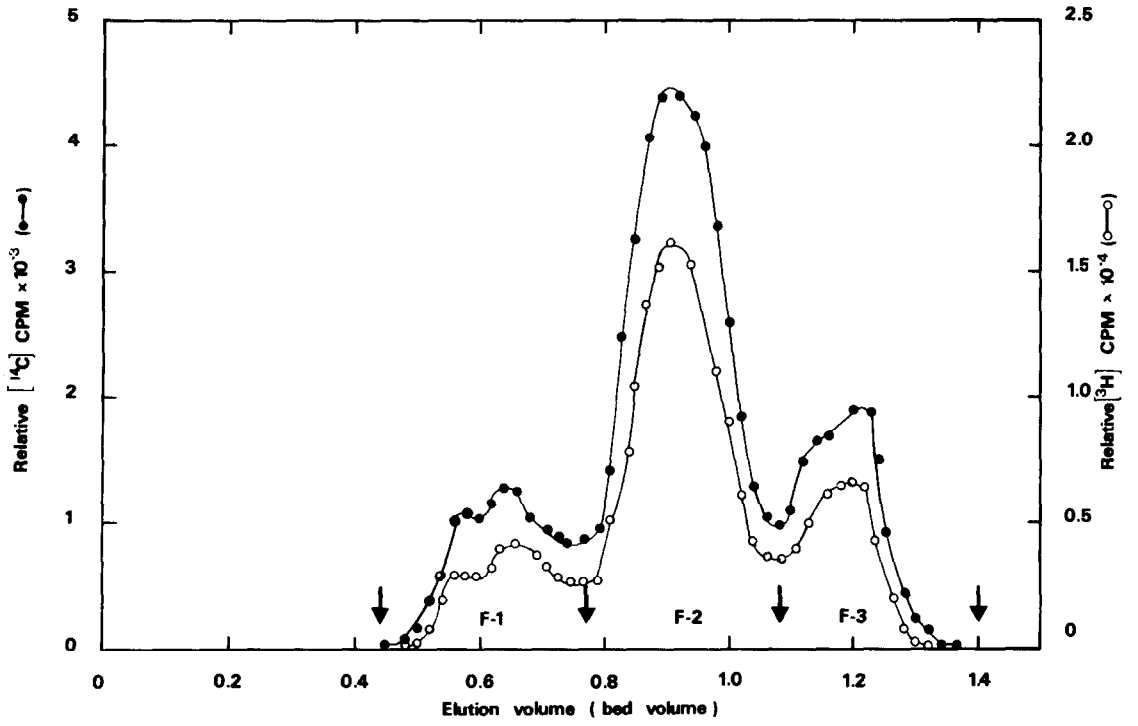

Fig.3. Elution profile of [^{14}C, ^{3}H]Ado$^{ox\text{-}red}$ metabolites on Sephadex LH-20 columns (1.5 X 23 cm) eluted with 35% ethanol. The elution volume 1.0 represents one column volume.

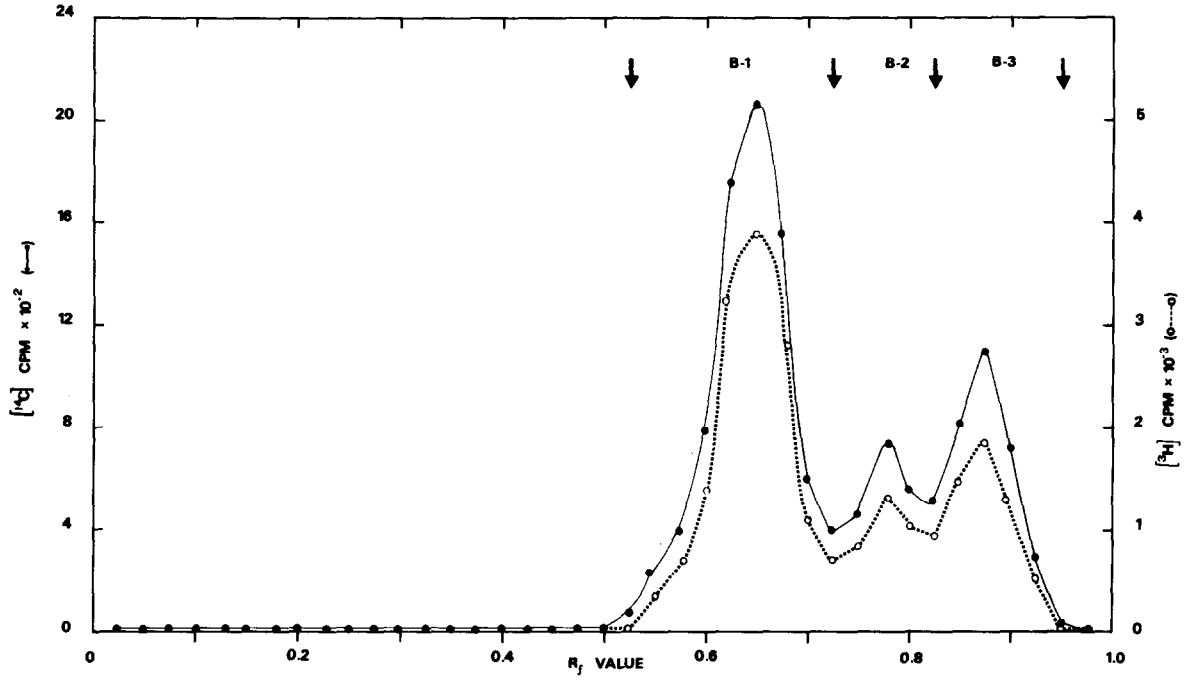

Fig.4. Further separation of radioactive F-3 (fig.3) on Whatman No.3 paper, solvent A.