# RAPHANATIN, AN UNUSUAL PURINE DERIVATIVE AND A METABOLITE OF ZEATIN

C.W. Parker and D.S. Letham

Research School of Biological Sciences,
Australian National University,

and

D.E. Cowley and J.K. MacLeod

Research School of Chemistry,
Australian National University,
Canberra, Australia

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SUMMARY: When zeatin was supplied to radish seedlings with roots excised, a number of metabolites were formed. These included adenine, adenosine, adenosine 5'-phosphate, zeatin riboside and zeatin riboside 5'-phosphate. However the major metabolite was a glucoside of zeatin which differed from 9-β-D-glucopyranosylzeatin. This metabolite termed raphanatin, which is active as a cytokinin, occurred mainly in the cotyledons of the seedlings.

The phytohormone zeatin, 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine, was originally isolated from sweet corn (1,2), but it and related compounds are now known to occur in numerous plant species (3). Zeatin, the *cis* isomer of zeatin and related compounds [e.g. 6-(3-methylbut-2-enylamino)purine] also occur as bases in some species of tRNA (4, 5). Little is known of the metabolites formed from zeatin when it is supplied exogenously to plant tissues. The only information available is that reported by Sondheimer and Tzou (6) who presented evidence that zeatin is converted by bean axes to 6-(4-hydroxy-3-methylbutylamino)purine, a compound previously isolated from lupin seeds (7). Preliminary results of an investigation of metabolites

formed from zeatin by radish seedlings are reported herein.

## MATERIALS AND METHODS

[G-$^3$H]zeatin (231 mC/mM) was prepared according to Letham and Young (8). The roots were excised from 9-day-old radish (Raphanus sativus cv. Long Scarlet) seedlings* and the cut ends of the hypocotyls were placed in [$^3$H]zeatin solution (7μM or 56μM, see Results). The de-rooted seedlings (henceforth referred to as "seedlings") were then left at 24° for varying times.

Inactivation of phosphatase and extraction were performed by a modification of the method of Bieleski (9). Briefly the seedlings were rinsed with distilled water, frozen in liquid nitrogen and dropped into methanol-chloroform-formic acid - water (12:5:1:2 by vol.) at -20°. After 18 hours at this temperature, the tissue and solvent were homogenized and then centrifuged. The pellet was re-extracted with methanol-water-formic acid (60:40:1 by vol.). The combined extracts were evaporated in vacuo and the residue dissolved in 50% ethanol for chromatography.

Chromatographic solvents used were: butan-1-ol—conc. $NH_4OH$ (d = 0.88)—water, 6:1:2 by vol., upper phase (solvent A); butan-1-ol—acetic acid—water, 12:3:5 by vol. (solvent B); butan-1-ol—conc. $NH_4OH$—water—ethanol, 6:1:2:1 by vol. (solvent C); ethanol—conc. $NH_4OH$—water, 18:1:2 by vol. (solvent D).
Radioactivity on chromatograms was determined by liquid scintillation counting of eluates. In all thin-layer

* At this stage, the seedlings consisted of roots, hypocotyl and cotyledons only.

chromatography (t.l.c.), Merck $PF_{254}$ silica gel was used.

The new compound 9-β-D-glucopyranosylzeatin (m.p. 234-235°) was prepared by condensing 6-chloro-9-β-D-glucopyranosylpurine (10) with 4-amino-2-methylbut-trans-2-en-1-ol (11) in refluxing butan-1-ol (c.f. ref. 12).

## RESULTS AND DISCUSSION

Radish seedlings were supplied with [$^3$H]zeatin solution (7 μM) for 3 and 8 hours; a further group of seedlings was transferred to water for 15 hours after taking up zeatin for 8 hours. Since conversion of zeatin to nucleotides was likely, the seedlings were extracted after inactivation of phosphatase (see Methods). Extract from each group of seedlings was chromatographed on paper using solvent A. On all chromatograms, radioactivity was distributed over almost the entire length. However on chromatograms for seedlings supplied with zeatin for 3 hours, two pronounced peaks of radioactivity were centred at $R_f$ 0.40 and 0.74, while two further peaks occurred very near the origin (see Fig. 1A). The peak at $R_f$ 0.74 was due to zeatin, while zeatin riboside 5'-phosphate was the major labelled compound at and near the origin. When seedlings were supplied with zeatin for 8 hours, the radioactivity associated with the peak at $R_f$ 0.40 greatly exceeded that due to zeatin and approximately equalled that occurring near the origin. Seedlings transferred to water for 15 hours after an 8-hour labelling yielded an extract in which none of the radio-activity could be attributed to zeatin (Fig. 1B). The radioactivity peak near the origin was now due principally to adenosine 5'-phosphate and unidentified compounds, while

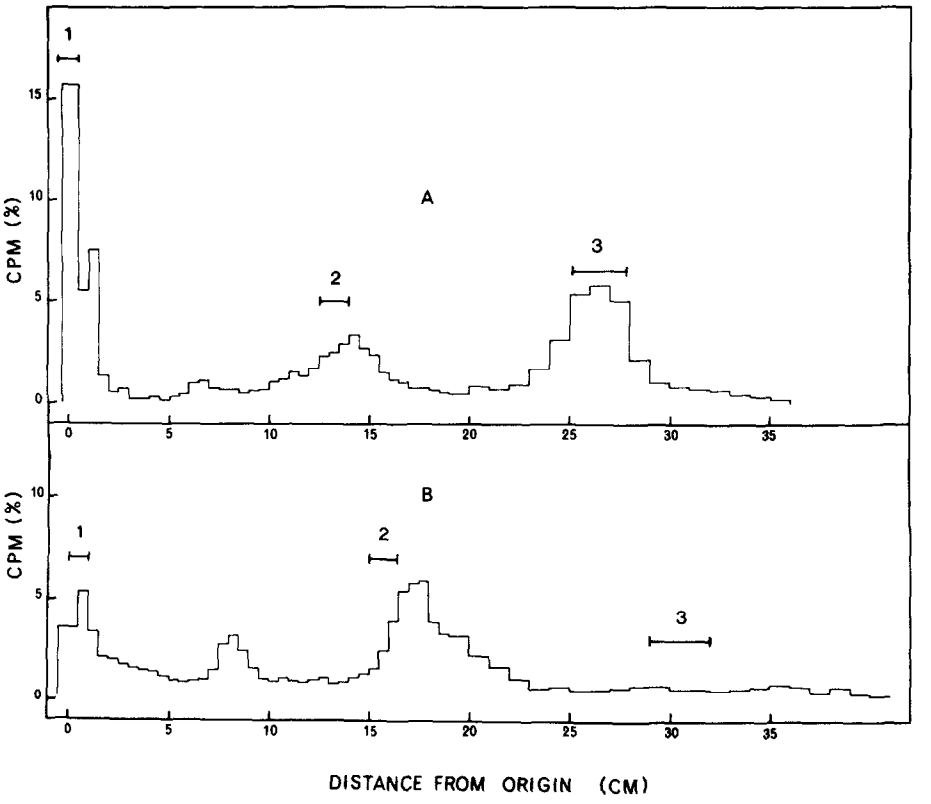


Figure 1. Histograms showing the distribution of radioactivity on paper chromatograms (solvent A) of extracts of radish seedlings supplied with [$^3$H]zeatin.
A: seedlings supplied with zeatin for 3 hours.
B: seedlings supplied with zeatin for 8 hours and then left in water for 15 hours. The positions to which co-chromatographed markers moved are indicated by barred lines; 1 AMP, 2 adenosine, 3 zeatin. The radioactivity eluted from each zone is expressed as a percentage of the total radioactivity.

the main peak occurred at $R_f$ 0.43, just ahead of co-chromatographed adenosine. Chromatographic studies indicated the major labelled compound in this peak was not a known purine or purine riboside. It is referred to henceforth as X.

Compound X occurred mainly in the cotyledons where it was the principal metabolite and was readily detectable 30 minutes after the hypocotyl ends contacted the zeatin solution. In

contrast, zeatin nucleotide was the major metabolite in the hypocotyls of the seedlings. X exhibited activity in the radish-cotyledon cytokinin bioassay (13) and was isolated by the following method. Radish seedlings were supplied with labelled zeatin (56 μM) for 8 hours, then transferred to water for 15 hours and finally extracted with 80% methanol. Evaporation of the extracts yielded a residue which was suspended in water and centrifuged. The resulting aqueous solution was adjusted to pH 6.2 and passed through a column of DEAE-cellulose ($HCO_3^-$ form) which was washed with water. The combined effluents were adjusted to pH 3.3 and percolated through a column of cellulose phosphate ($NH_4^+$ form; equilibrated to pH 3.3) which was washed with water and then eluted with 0.3N $NH_4OH$. Evaporation of the eluate yielded a residue which was extracted with ethanol to give a soluble fraction containing X. Two dimensional t.l.c. on silica gel and autoradiography detected at least 11 other labelled compounds in this fraction; three of these were zeatin riboside, adenine and adenosine. The radioactivity associated with X greatly exceeded that of any other labelled compound. X was purified from the ethanol-soluble fraction by three preparative t.l.c. separations using solvents B, C and D in which X exhibited $R_f$ values of 0.40, 0.37 and 0.37 respectively. Finally chromatography on exhaustively washed paper using solvent A yielded chromatographically homogeneous X. To isolate unlabelled X for mass spectrometry, the above procedure was repeated using seedlings supplied with unlabelled zeatin; the yield was approximately 70 μg from 1,600 seedlings (210 g). X exhibited the following u.v. spectral characteristics: $\lambda_{max}$(EtOH) 277.5 nm; $\lambda_{max}$(0.1N HAc) 282 nm; $\lambda_{max}$(0.2N ethanolic $NH_4OH$) 277.5 nm. The principal

peaks in the mass spectrum occurred at m/e 381, 364, 219, 203, 202 (base peak), 201, 200, 199, 198, 188, 186, 185, 174, 173, 160, 148, 136, 135, 120, 119 and 108; $M^+$ 381.164 (calc. for $C_{16}H_{23}N_5O_6$, 381.165). Below m/e 219, the spectrum was almost identical to that of zeatin (14). The mass spectrum indicated that X consisted of an intact zeatin moiety joined to a $C_6H_{11}O_5$ residue. This suggested that X was a zeatin-hexose sugar conjugate. Acid hydrolysis of X yielded glucose which was identified by use of the specific enzyme, glucose oxidase. Hence X is a zeatin glucoside. 9-β-D-Glucopyranosylzeatin was synthesized as a model compound and was found by t.l.c. and u.v. spectroscopy to differ from X. Although qualitatively very similar, the mass spectra for the two compounds showed some significant peak intensity differences. The location of the glucosidic linkage in X is not definitely known at present, but the u.v. spectra suggest it is at position 7 of the purine ring. The synthesis of 7-glucosylzeatin is in progress.

Naturally occurring compounds, with a sugar residue attached to a purine ring at positions other than 9, are rare. Although at least one naturally occurring pyrimidine glucoside is known (namely vicine, in which the glycosidic linkage is at position 5 and not 1 as in normal pyrimidine ribosides), the occurrence in Nature of a purine glucoside has not previously been established. Because of the unusual structure of X and since it is probably an important metabolite of zeatin, reference to it by a trivial name would be convenient. Since it was first detected in Raphanus sativus, the name raphanatin is now assigned to this compound.

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