Planta 142, 239–251 (1978)

Planta

Regulators of Cell Division in Plant Tissues

XXV. Metabolism of Zeatin by Lupin Seedlings*

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Abstract. [^{3}H]zeatin was supplied through the transpiration stream to de-rooted lupin (*Lupinus angustifolius* L.) seedlings. The following previously known metabolites were identified chromatographically: 5′-phosphates of zeatin riboside and dihydrozeatin riboside, adenosine-5′-phosphate, zeatin riboside, zeatin-7-glucopyranoside, zeatin-9-glucopyranoside, adenine, adenosine and dihydrozeatin. Five new metabolites were purified; four of these contain an intact zeatin moiety. Two were identified unequivocally, one as L-β-[6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-purin-9-yl]alanine, a metabolite now termed lupinic acid, and the second as O-β-D-glucopyranosylzeatin. These two compounds were the major metabolites formed when zeatin solution (100 μM) was supplied to the de-rooted seedlings. The radioactivity in the xylem sap of intact seedlings, supplied with [^{3}H]zeatin via the roots, was largely due to zeatin, dihydrozeatin and zeatin riboside. When [^{3}H]zeatin (5 μM) was supplied via the transpiration stream to de-rooted *Lupinus luteus* L. seedlings, the principal metabolite in the lamina was adenosine, while in the stem nucleotides of zeatin and adenine were the dominant metabolites. O-Glucosylzeatin and lupinic acid were also detected as metabolites. The level of the latter varied greatly in the tissues of the shoot, and was greatest in the lower region of the stem and in the expanding lamina. Minor metabolites also detected chromatographically were: (a) dihydrolupinic acid, (b) a partially characterized metabolite which appears to be a 9-substituted adenine (also formed in *L. angustifolius*), (c) glucosides of zeatin riboside and/or dihydrozeatin riboside, and (d) O-glucosyldihydrozeatin. While lupinic acid supplied exogenously to *L. luteus* leaves underwent little metabolism, chromatographic studies indicated that O-glucosylzeatin was converted to its riboside, the principal metabolite formed, and also to adenosine, zeatin and dihydrozeatin. A thin-layer chromatography procedure for separating zeatin, dihydrozeatin, zeatin riboside and dihydrozeatin riboside is described.

Key words: Cytokinin metabolism — O-glucosylzeatin — Lupinic acid — *Lupinus*.

* XXIV = Gordon et al., 1975

Abbreviations: Me_3Si = trimethylsilyl, TLC = thin-layer chromatography, UV = ultraviolet

Introduction

Very little information is available concerning the distribution in the shoot of cytokinins supplied exogenously through the intact root or via the stem base of de-rooted seedlings (for review see Letham, 1978). In plants exhibiting sequential leaf senescence and abscission (as e.g. in lupin), cytokinin distribution studies would be of particular interest because differential cytokinin supply to the leaves may be responsible for, or involved in, these phenomena. However translocation studies using radioactive cytokinins are of limited value if the identity of the translocated radioactivity is not known. The present investigations of cytokinin metabolites in lupin were initiated to provide a chemical basis for physiological studies of cytokinin translocation and metabolism in relation to sequential leaf senescence and abscission and other phenomena in lupin seedlings.

In a preliminary publication (Parker et al., 1975), we reported the purification of two metabolites of zeatin from *Lupinus angustifolius*. One was identified as O-β-D-glucopyranosylzeatin, a metabolite not previously reported, while the other appeared to be a purine-amino acid conjugate, although the exact structure was not established. In the present paper and in a related chemical paper (Duke et al., 1978),

0032-0935/78/0142/0239/$02.60

the purification, properties, structure and chemical synthesis of zeatin metabolites formed in lupin seedlings are discussed.

Materials and Methods

Chemicals

[G-^{3}H]Zeatin (160 mCi/mmol) was prepared as described by Letham and Young (1971) and for some studies was diluted with unlabelled compound to give a specific activity of 16 mCi/mmol. The [^{3}H]lupinic acid and O-glucosyl-[^{3}H]zeatin supplied to lupin leaves were the ^{3}H-labelled metabolites purified as described below and then diluted with synthetic compound prepared according to Duke et al. (1978).

Dihydrolupinic acid was synthesized by the following method. A solution (20 ml) of DL-lupinic acid (20 mg; for synthesis see Duke et al., 1978) in ethanol-acetic acid (1:1, v/v) was stirred with 10% palladium on carbon (12 mg) for 12 h under an atmosphere of hydrogen. The two major products were separated by preparative TLC on silica gel (solvent A) to yield a low-R_f compound (6 mg) and a high-R_f compound (7 mg). These were further purified by cation exchange chromatography and crystallization from ethanol-water. Dihydrolupinic acid (low R_f compound) was obtained as colourless crystals, m.p. 208° C; λ_{max} at pH 3, 6 and 11:266.5, 270.5 and 270.5 nm respectively. The molecular ion was not evident in the mass spectrum, the two ions of highest mass being at m/e 290 ($M^+ - H_2O$) and 264 ($M^+ - CO_2$; measured mass 264.1695, calculated for $C_{12}H_{20}N_6O$=264.1698). The tris-trimethylsilyl derivative of the product showed a molecular ion at m/e 524 (measured mass 524.2777, calculated for $C_{22}H_{44}N_6O_3Si_3$=524.2782) and prominent ions at m/e 509 ($M^+ - CH_3$), 407 ($M^+ - COOMe_3Si$) and 307 ($M^+ - CH(NHMe_3Si)$-$COOMe_3Si$ with H transfer). The high-R_f product of the hydrogenation reaction formed a bis-Me_3Si derivative which showed a molecular ion at m/e 436 and prominent ions at m/e 421 ($M^+ - CH_3$), 393 ($M^+ - C_3H_7$), 379 ($M^+ - C_4H_9$), 319 ($M^+ - COOMe_3Si$), 219 ($M^+ - CH(NHMe_3Si)COOMe_3Si$ with H transfer), 176 (219 − C_3H_7) and 162 (219 − C_4H_9). This spectrum was consistent with the product being β-[6-(3-methylbutylamino)purin-9-yl]alanine, formed by hydrogenolysis of the side-chain hydroxyl group.

Uptake of Labelled Compounds, Tissue Extraction and Preparation of Xylem Sap

Nine-d-old lupin seedlings (*Lupinus angustifolius* L., cv. New Zealand Blue) were grown from seed (source: Wright Stephenson & Co., Auburn, N.S.W., Australia) in a greenhouse with natural light at 15–25° C. The roots were excised from these seedlings and the stem bases were placed in an aqueous solution (100 μM) of [^{3}H]zeatin (16 mCi/mmol) in continuous light from fluorescent lamps and a gentle air current. [G-^{3}H]zeatin solution (160 mCi/mmol, 5 μM) was also supplied to 14-d-old de-rooted *Lupinus luteus* L. seedlings (growth conditions as above; source of seed: Westralian Farmers Co-op., Perth, W.A., Australia) by the same method except that the seedlings were not exposed to an air current. With the exception of leaves supplied with O-glucosyl-[^{3}H]zeatin which were extracted with 80% methanol at 2° C, all extractions were performed as outlined below. The tissue was dropped into 80% methanol (12 ml/g tissue) at 60–65° C, held at this temperature for 5 min, cooled rapidly to 20° C, and then homogenized. The clarified extracts were evaporated (<40° C) and the residue was dissolved in 50% ethanol (1.7 ml/g tissue) for chromatography.

For collection of xylem sap, lupin seedlings grown for 17 d in sand were deprived of water for 48 h. The sand was then moistened with 2 mM phosphate buffer (pH 6.0) which contained [G-^{3}H]zeatin at 12 μM. The seedlings were decapitated mid-way along the hypocotyl 9 h later. The exudate which appeared soon after decapitation was collected on filter paper and discarded. The droplets of sap which subsequently formed were collected over 4 d on small filter-paper wedges; these were placed in 50% methanol after each collection. The combined eluates were evaporated and dissolved in 50% methanol for chromatography.

Chromatographic and Electrophoretic Methods

The following materials were used for TLC: Camag D-5 Silica Gel (Camag A.G., Muttenz, Switzerland) which was used only with solvent H for separation of zeatin and zeatin riboside from the corresponding dihydro compounds; Merck PF_{254} Silica Gel 60 (E. Merck, Darmstadt, Germany) for all other silica-gel TLC; and Serva cellulose (Serva Feinbiochemica, Heidelberg, Germany). Woelm green fluorescent indicator (M. Woelm, Eschwege, Germany) was incorporated into the Camag Silica Gel and Serva cellulose (0.4 and 0.8%, respectively) prior to spreading the layers. Camag Silica-gel plates were washed prior to use by allowing methanol to flow to the top of the layer. Borate-impregnated silica-gel layers were prepared by slurrying Merck Silica Gel with 0.05 M sodium tetraborate. For preparative TLC, layers were 1 mm thick; the thickness of all other layers was 0.25 mm. For final purification of metabolites before mass spectrometry, chromatography was performed on Schleicher & Schüll 2040b washed paper (Schleicher & Schüll, Dassel, Germany); the paper was rewashed exhaustively with 20% ethanol prior to use. In all other paper chromatography, Schleicher & Schüll 598L paper was used. All electrophoresis was carried out on Whatman No. 3mm paper (W. and R. Balston, Maidstone, U.K.) at 40 V/cm.

The following solvent systems were used for chromatography (proportions are by volume):

A, butan-1-ol-14 N ammonia-water (6:1:2, upper phase)
B, butan-1-ol-acetic acid-water (12:3:5)
C, butan-1-ol saturated with water, used in an atmosphere containing ammonia
D, ethyl methyl ketone-acetic acid-water (16:1:4)
E, ethyl methyl ketone-acetic acid-water (4:1:2)
F, water-saturated butan-1-ol saturated with $Na_2B_4O_7 \cdot 10H_2O$
G, chloroform-methanol (9:1)
H, methyl acetate-ethanol-2,2-dimethoxypropane (90:10:1) with 5 drops of formic acid per 100 ml. The formic acid and dimethoxypropane were added to the tank 5–10 min before use and TLC was performed at 3° C. At 20° C the separation was still obtained, but the spots were more diffuse.

When used with Camag Silica Gel, solvent H gave a satisfactory separation of zeatin, dihydrozeatin, zeatin riboside and dihydrozeatin riboside, the layers being developed twice using freshly prepared solvent on both occasions. The separation on Merck PF_{254} Silica Gel was less satisfactory, and no separation at all was obtained on pre-coated Merck HPTLC Silica-gel 60 F_{254} plates.

Columns for cation exchange chromatography were packed with Whatman P1 (floc) cellulose phosphate (W & R. Balston, Maidstone, U.K.) and were used in the NH_4^+ form equilibrated to pH 3.0.

Radioactivity in chromatogram zones was determined by elution and liquid scintillation counting as described previously (Gordon et al., 1974).

Characterization of Chromatographic Fractions and Metabolites

Enzymic Degradations. Incubation with *Escherichia coli* alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo., USA, type III; 0.3 mg/ml, pH 9–10) for 3 h at 35° C was used to convert nucleotides to nucleosides. Lupinic acid (3 µg) was incubated with L-amino acid oxidase (2 µg; Boehringer, Mannheim) in Tris-HCl buffer (30 µl, 0.05 M, pH 7.3) for 6 h at 30° C. The incubation mixture was examined directly by TLC. The enzyme preparation used did not degrade D-[^{14}C]alanine. Hydrolysis of glucoside metabolites with β-glucosidase was performed as described previously (Letham et al., 1975).

Degradation of Nucleoside 5'-Phosphates to Bases. An aqueous solution of the nucleotide fraction (0.5 ml) containing added sodium periodate (5 mg) was kept at 30° C for 10 h when cyclohexylamine (100 µl) was added. After being held at 35° C for 3 h, the solution was evaporated in vacuo at 40° C; the residue was dissolved in water (1 ml) which was then extracted with butan-1-ol (1.5 ml). Labelled purine bases in the extract were identified by TLC.

Oxidation of Fractions Containing Zeatin. $KMnO_4$ solution (0.5%) was added in 10-µl volumes to an aqueous solution of the fraction until a definite pink colour persisted. After 5 min, allyl alcohol was added to destroy the excess permanganate. The solution was then diluted with 2 volumes of ethanol, acidified with acetic acid, and left at 2° C for 18 h. MnO_2 was removed by centrifuging and the supernatant subjected to TLC.

Cytokinin Bioassay. The radish cotyledon bioassay (Letham, 1971) was used to assess the cytokinin activity in zones of paper chromatograms of extract of seedlings supplied with zeatin. Identically prepared extract of seedlings supplied with water instead of zeatin solution was used to give a control chromatogram for bioassay. This served as a basis for calculating increments in cotyledon weight induced by metabolite-containing zones.

Mass Spectra. Metabolite spectra were obtained with an AEI MS-902 instrument operated at an ionization voltage of 70 eV, solutions of the metabolites being evaporated onto the tip of the probe.

Purification of the Major Metabolite in Paper-chromatogram Zone 2

Lupinus angustifolius seedlings were supplied with [^{3}H]zeatin solution (100 µM) as described above. Extract equivalent to 17 g of upper shoot (see Results) was subjected to preparative paper chromatography (15 sheets of 46 cm width; solvent A). The zone containing the metabolite (R_f 0.18–0.23; cf. Figure 2A) was excised from each chromatogram and eluted exhaustively by allowing 0.15 N acetic acid to flow down it. The combined eluates were evaporated in vacuo (<40° C) and subjected to preparative TLC on silica gel (solvent A). The UV-absorbing zone at R_f 0.12–0.16 contained 78% of the radioactivity and was eluted with 80% methanol for rechromatography on silica gel (solvent B). This yielded a single UV-absorbing zone (R_f 0.20) with more than 80% of the rechromatographed radioactivity. The evaporated eluate of this zone was dissolved in water and the solution (1.0 ml, pH 3) was percolated through a column of cellulose phosphate (4.0 ml) which was then washed with 0.05 N acetic acid (24 ml) and finally eluted with 0.3 N NH_4OH (36 ml). More than 75% of the radioactivity applied to the column was in the eluate which was evaporated. The final purification was by chromatography on washed paper (solvent C), the chromatogram being developed twice. The resulting major UV-absorbing zone was eluted with 60% redistilled ethanol to yield purified metabolite L2 (45 µg) for chemical characterization.

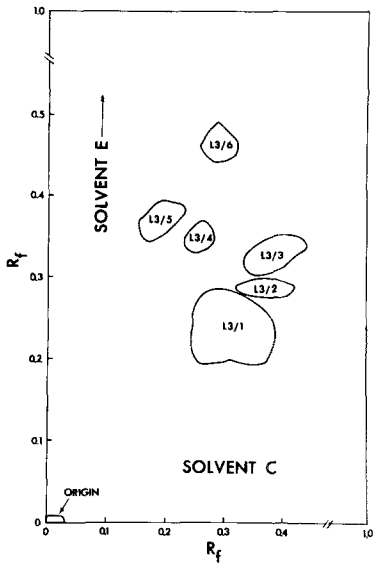


Fig. 1. The location of the UV-absorbing components on a two-dimensional paper chromatogram of a fraction derived from the eluate of zone 3 (see Fig. 2A). Solvent C: butan-1-ol saturated with water; solvent E: ethyl methyl ketone-acetic acid-water (4:1:2)

Purification of Principal Metabolites in Paper-chromatogram Zone 3

Strips containing the radioactivity termed zone 3 (Figure 2A) were cut from the paper chromatograms on which the preparative separation of zone 2 had also been achieved. The combined eluates (0.15 N acetic acid) were evaporated in vacuo and the residue was subjected to preparative TLC on silica gel (solvent A). About 76% of the radioactivity was in a UV-absorbing zone at R_f 0.19 which was eluted with 80% methanol. The evaporated eluate was dissolved in water (4.0 ml), adjusted to pH 3.0, and chromatographed on a column of cellulose phosphate (6 ml) which was washed with 0.05 N acetic acid (36 ml) and eluted with 0.3 N NH_4OH (54 ml). Further purification of the eluate was achieved using a two-dimensional paper chromatography system (solvent E followed by solvent C) which resolved the mixture into six UV absorbing spots (Fig. 1). These were designated L3/1–L3/6 and their relative radioactivities in order were 26.40, 7.50, 2.30, 0.13, 0.03 and 1.00. Spot L3/1 appeared to be a mixture of incompletely separated metabolites which differed greatly in their intensity. Spots L3/2–6 were of very weak intensity and were just detectable under UV light. Spot L3/2 was eluted with ethanol and rechromatographed on washed paper yielding purified metabolite L3/A (15 µg). Spots L3/3–6 were not examined further because of the very limited amounts present.

The separation of the components in spot L3/1 proved very difficult but was finally achieved by TLC on borate-impregnated silica gel (solvent F). This yielded two UV-absorbing zones of R_f 0.01 (zone A) and R_f 0.13 (zone B), the latter accounting for 80% of the radioactivity. The zones were eluted with 0.15 N acetic acid and borate was removed from the eluates by passage through small columns of cellulose phosphate; these columns were then

washed with 0.05 N acetic acid and eluted with 0.3 N NH_4OH. The evaporated eluates were dissolved in 50% ethanol for final purification by paper chromatography (solvent C).

By the above procedure, the zone of R_f 0.01 yielded a single UV-absorbing spot which was eluted with 50% ethanol to give purified metabolite L3/B (80 µg). However, the zone of R_f 0.13 yielded two UV-absorbing spots, R_f 0.28 and 0.34. The former, more intense one was eluted with ethanol to yield purified metabolite L3/C (40 µg) while the latter gave purified metabolite L3/D (15µg).

Results

Chromatographic Characterization of Zeatin Metabolites formed by Shoots of Lupinus angustifolius

Nine-d-old, de-rooted seedlings of *L. angustifolius* were supplied with ^3H-labelled zeatin (100 µM) through the transpiration stream for 21 h. Lupin seedlings of this age have a pair of large fleshy cotyledons, two fully developed foliage leaves and a cluster of young emerging leaves at the shoot tip. Before extraction, the shoots were divided into three sections: (a) the shoot above the cotyledons, (b) the axis below the cotyledons, and (c) the cotyledons themselves. Segment (a) consisted of both leaf tissue (ca. 80% of fresh weight) and stem (ca. 20% of fresh weight). Henceforth, extracts of sections (a), (b) and (c) will be referred to as upper shoot, stem and cotyledon extracts, respectively.

The distribution of radioactivity over paper chromatograms (solvent A) of upper-shoot and stem extract is presented in Figure 2. The chromatograms of these extracts showed four peaks of radioactivity at the R_f values of 0.06, 0.20, 0.30 and 0.77, as well as a pronounced shoulder of radioactivity associated with the R_f 0.30 peak. The major difference between the two distributions is that the peak at R_f 0.20 is relatively more intense in upper-shoot extract than in stem extract. Extracts were also prepared from the upper shoots of de-rooted seedlings deprived of water after zeatin uptake until flaccid. These extracts and also those prepared from the cotyledons of unwilted seedlings yielded chromatograms with a radioactivity distribution similar to that of Figure 2A. However, in the case of cotyledons, the total radioactivity extracted per unit tissue weight was ca. $^1/_{10}$ of that extracted from the other two sections of the shoot.

Zones from a preparative chromatogram of the upper-shoot extract were eluted for investigation of the identity of the metabolites present. These zones are indicated in Figure 2A and are termed zones 1, 2, 3, 4 and 5.

Radish cotyledon cytokinin bioassays of paper

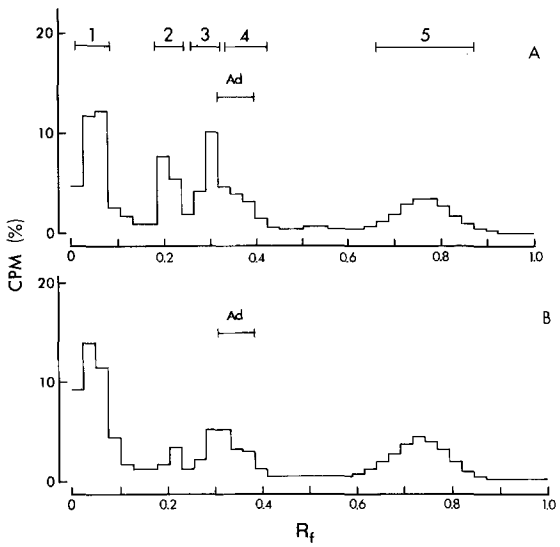


Fig. 2A and B. The distribution of radioactivity over paper chromatograms of extracts of upper shoot **A** and stem **B** of lupin seedlings which had been supplied with [^3H]zeatin. The barred line designated Ad denotes the position of cochromatographed adenosine. The barred lines 1–5 in **A** denote zones eluted for further investigation and purification. The chromatograms were developed with solvent A, butan-1-ol-14 N NH_4OH-water (6:1:2)

chromatograms of upper-shoot extract and of "control" chromatograms (see Methods) indicated that zones 1–5 accounted for about 87% of the cytokinin activity due to the extracted zeatin metabolites and, if present, unmetabolized zeatin. The activity of the zones increased in the following sequence: 4, 2, 1, 3 and 5. Since zone 5 was the location of cochromatographed zeatin and zeatin riboside, the activity of this zone may be due at least partly to these compounds. However the activity in the other zones indicated that a diversity of metabolites with cytokinin activity were formed by the seedlings.

Zone-1 Metabolites. When the alkaline phosphatase hydrolyzate of zone 1 was chromatographed on paper (solvent A), 80% of the radioactivity occurred at R_f 0.70 and was coincident with cochromatographed zeatin riboside. Two small peaks of radioactivity were also present; one cochromatographed with adenosine while the other was at the origin. In a two-dimensional system involving paper chromatography (solvent A) followed by high-voltage electrophoresis (0.025 M borate, pH 9.2), peaks of radioactivity were again coincident with marker zeatin riboside and adenosine.

The procedures used above do not distinguish between zeatin riboside and dihydrozeatin riboside. To determine whether 5′-phosphates of these ribosides were present in zone-1 eluate, the eluate was degraded with periodate using a procedure which specifically

cleaves nucleoside 5′-phosphates to the corresponding free bases. The reaction product was chromatographed on silica gel using solvent H (the TLC system developed to separate zeatin from dihydrozeatin). The relative amounts of radioactivity which cochromatographed with adenine, zeatin and dihydrozeatin were ca. 1.0, 6.0 and 3.4, respectively. Thus, from chromatographic evidence, the radioactivity in the zone-1 eluate was largely zeatin riboside-5′-phosphate together with lesser amounts of dihydrozeatin riboside-5′-phosphate and adenosine-5′-phosphate, and metabolites unchanged in R_f by phosphatase treatment, which are presumably non-nucleotides.

Zone-2 Metabolites. This peak of radioactivity, which appeared to be associated with only weak cytokinin activity, occurred at an R_f different from that of any identified metabolite of zeatin. TLC studies indicated that the radioactivity of zone 2 was largely accounted for by one new compound. The retention of the metabolite on ion-exchange columns of cellulose phosphate (NH_4^+ form) at pH 3 and DEAE-cellulose (HCO_3^- form) at pH 9 indicated that the metabolite was amphoteric. The isolation of the metabolite, termed L2, is outlined in Methods.

Zone-3 Metabolites. Because of the considerable cytokinin activity associated with this zone, it was of particular interest. The metabolites, which possessed an R_f value slightly less than that of adenosine on paper in solvent A (Figure 2A), differed chromatographically from any previously identified zeatin metabolites. When zone 3 eluate was chromatographed on silica gel using solvent A, the radioactivity was resolved into three distinct peaks at R_f 0.22, 0.40 and 0.59. These peaks accounted for 59, 16 and 14%, respectively, of the eluted radioactivity. Only the metabolites in the major peak of radioactivity (R_f 0.22) were present in sufficient amount to permit chemical characterization. Their purification was undertaken by the procedures detailed in Methods, and yielded four metabolites, L3/A, L3/B, L3/C and L3/D.

Zone-4 Metabolites. This is the region to which the 7- and 9-glucosides of zeatin are known to move during paper chromatography in solvent A. TLC of the eluate on silica gel (solvent A) indicated that 30% of the radioactivity cochromatographed with adenine (R_f 0.47), 27% with adenosine (R_f 0.27) and 26% with zeatin-7- and -9-glucopyranosides (not resolved; R_f values = 0.17). TLC on silica gel (solvent B) indicated that the 7-glucoside (R_f 0.40) and the 9-glucoside (R_f 0.47) were present in approximately equal amounts as metabolites. Hence chromatographic evidence indicated that the major metabolites in the zone-4 eluate were adenine, adenosine, and zeatin-7- and -9-glucopyranosides.

Table 1. TLC characteristics of zeatin and related compounds on Camag Silica Gel. The values listed are the distances travelled after double development at 3° C with solvent H, i.e. methyl acetate-ethanol-2,2-dimethoxypropane (90:10:1) with 5 drops of formic acid per 100 ml. In each development, the solvent travelled 16 cm from the origin

Compound	Travel distance (cm)
Zeatin	3.6
cis-Zeatin	4.1
Dihydrozeatin	2.9
Zeatin riboside	5.4
cis-Zeatin riboside	5.75
Dihydrozeatin riboside	4.6
Adenine	2.6

Zone-5 Metabolites. During TLC of the zone-5 eluate on silica gel (solvent A), 65% of the radioactivity cochromatographed with zeatin and 14% with zeatin riboside. However, this system does not distinguish between zeatin, *cis*-zeatin or dihydrozeatin, nor does it separate the corresponding ribosides. When zone-5 eluate was chromatographed on silica gel (solvent G), the majority of the radioactivity cochromatographed with zeatin and dihydrozeatin; negligible radioactivity moved with *cis*-zeatin. Permanganate oxidation of the zone-5 eluate followed by TLC (solvent A) resulted in a peak of radioactivity which cochromatographed with dihydrozeatin indicating the presence of this metabolite which, unlike zeatin, is not appreciably oxidized. Hence the eluate was examined by the TLC system (silica gel, solvent H) which separates zeatin, dihydrozeatin, zeatin riboside and dihydrozeatin riboside (see Table 1). The percentage of zone-5-eluate radioactivity which cochromatographed with dihydrozeatin and zeatin was found to be 22.0 and 33.5%, respectively. Satisfactory separation of zeatin riboside and dihydrozeatin riboside was not obtained on these particular chromatograms because of the presence of interfering material, but 11% of the radioactivity cochromatographed with zeatin riboside and/or dihydrozeatin riboside. These conclusions were substantiated by two-dimensional TLC on silica gel (solvent H followed by solvent A) and by permanganate oxidation. After permanganate oxidation, the radioactivity attributed to zeatin exhibited the chromatographic behaviour of adenine; however the radioactivity which cochromatographed with dihydrozeatin was unaffected by this treatment. Thus from chromatographic evidence the major labelled components of

Table 2. Radioactivity attributable to zeatin metabolites formed by *L. angustifolius*. The extract of upper shoots was chromatographed on paper using solvent A (butan-1-ol-14 N NH_4OH-H_2O, 6:1:2) and the 5 major zones of radioactivity were eluted. The eluates were examined by TLC procedures to detect the metabolites listed

Metabolite	Paper-chromatogram zone from which metabolite was derived	% of radioactivity
Zeatin riboside-5′-phosphate	1 (R_f 0.06)	7.0
Dihydrozeatin riboside-5′-phosphate	1 (R_f 0.06)	2.0
Adenosine-5′-phosphate	1 (R_f 0.06)	2.0
L2	2 (R_f 0.20)	10.0
L3/A	3 (R_f 0.30)	3.0
L3/B	3 (R_f 0.30)	0.6
L3/C	3 (R_f 0.30)	9.7
L3/D	3 (R_f 0.30)	2.1
Zeatin-7-glucopyranoside	4 (R_f 0.38)	1.0
Zeatin-9-glucopyranoside	4 (R_f 0.38)	0.9
Adenine	4 (R_f 0.38)	2.4
Adenosine	4 (R_f 0.38)	2.2
Dihydrozeatin	5 (R_f 0.77)	4.0
Zeatin riboside and/or dihydrozeatin riboside	5 (R_f 0.77)	2.1
Unmetabolized zeatin	5 (R_f 0.77)	6.1

zone 5 were zeatin, dihydrozeatin, zeatin riboside and/or dihydrozeatin riboside.

A summary of the data obtained from studies of the metabolites present in the five paper-chromatogram zones is presented in Table 2.

Table 3. UV spectral characteristics of the purified metabolites of zeatin in *Lupinus angustifolius*

Metabolite	Aqueous ethanol[a]		0.2 N ethanolic NH_4OH[a]		0.1 N acetic acid	
	λmax	λmin	λmax	λmin	λmax	λmin
L2	270.5	230.5	270.5	233.0	266.5	231.5
L3/A	268.0	230.5				
L3/B	259.5	227.5	259.5	228.5	257.5	227.0
L3/C	269.0	230.0	275.5 285.5[b]	241.0	274.0	233.5
L3/D	267.5	230.5				

[a] 75% ethanol for all spectra except those of L2 which were taken in 25% ethanol

[b] Shoulder

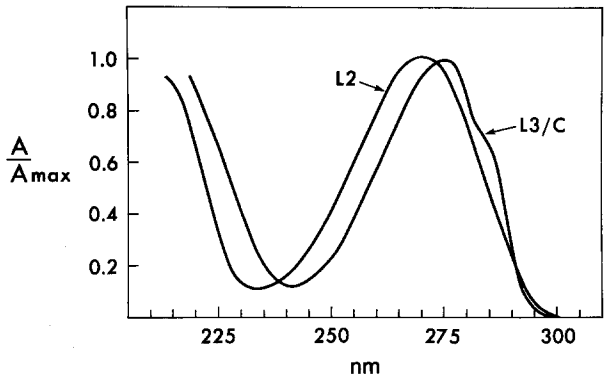


Fig. 3. The UV spectra of metabolites L2 and L3/C in 0.2 N ethanolic NH_4OH (L2 in 25% ethanol, L3/C in 75% ethanol).

Characterization of New Zeatin Metabolites Formed in Shoots of Lupin Seedlings

The UV spectral characteristics of metabolites L2, L3/A, L3/B, L3/C and L3/D are presented in Table 3, while complete UV spectra of L2 and L3/C in 0.2 N ethanolic NH_4OH are shown in Figure 3. Mass spectra of L2 and L3/C are recorded in Figures 4 and 5, respectively. The principal mass spectral characteristics for L3/A are (m/e values with relative intensities in parentheses): 250 (15), 220 (21), 204 (19), 203 (14), 202 (62), 201 (17), 188 (14), 186 (14), 185 (8), 162 (33), 160 (25), 149 (23), 148 (100), 136 (35), 135 (78), 120 (15), 119 (29). The principal m/e values for L3/D were: 203 (10), 202 (58), 201 (12), 188 (10), 186 (10), 185 (8), 160 (18), 148 (16), 136 (24), 135 (100), 120 (9), 119 (18), 108 (34); the two ions of highest m/e were 220 (3) and 219 (2). L3/B yielded an extremely weak spectrum, the only prominent ion above m/e 120 being 135 (74% of base peak at m/e 94). The structures of the above metabolites are discussed briefly below.

Metabolite L2. This gave a purple spot on thin-layer chromatograms when sprayed with ninhydrin and heated at 100° C, indicating the presence of an amino group in the molecule. During paper electrophoresis at pH 10.2, L2 behaved as an anion (mobility relative to AMP = 0.45), but at pH 6 and 7 it did not exhibit electrophoretic mobility. Below m/e 220, the mass spectrum closely resembled that of zeatin (Shannon and Letham, 1966). L2 was completely converted by L-amino acid oxidase to a product which did not react with ninhydrin (R_f of product on silica gel using solvent B, 0.35; cf. undegraded L2, R_f 0.25). These observations established that L2 was a zeatin-amino acid conjugate. The UV spectra for L2 (Table 3, Fig. 3) are characteristic of an N^6,9-disubstituted adenine. The mass spectrum of underivatized L2 (Fig. 4) did not exhibit a molecular ion, the ion of highest m/e (288) being derived from the M^+ of L2 by elimination of H_2O. However, the mass spectrum of the bis-Me_3Si derivative of L2 did show a definite molecular ion (m/e 450). A detailed discussion of this spectrum and other chemical structural evidence, in-

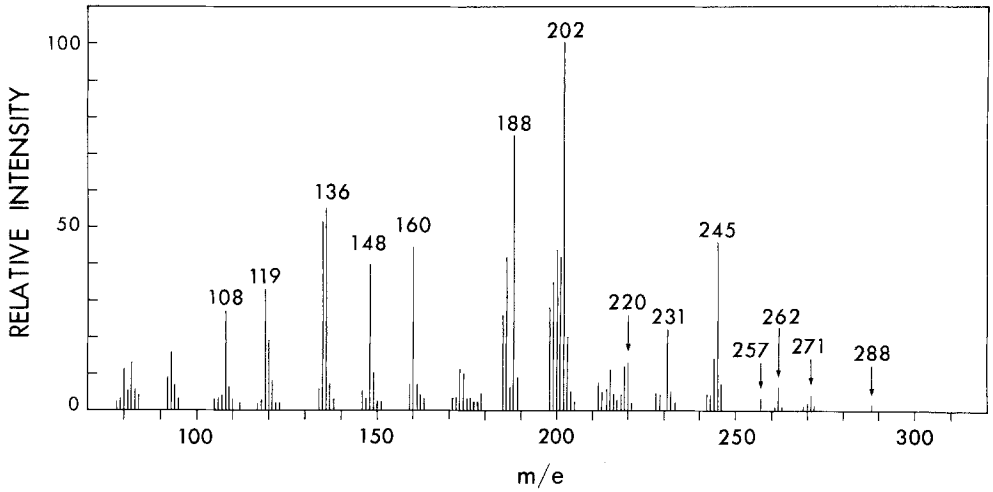


Fig. 4. The mass spectrum of metabolite L2

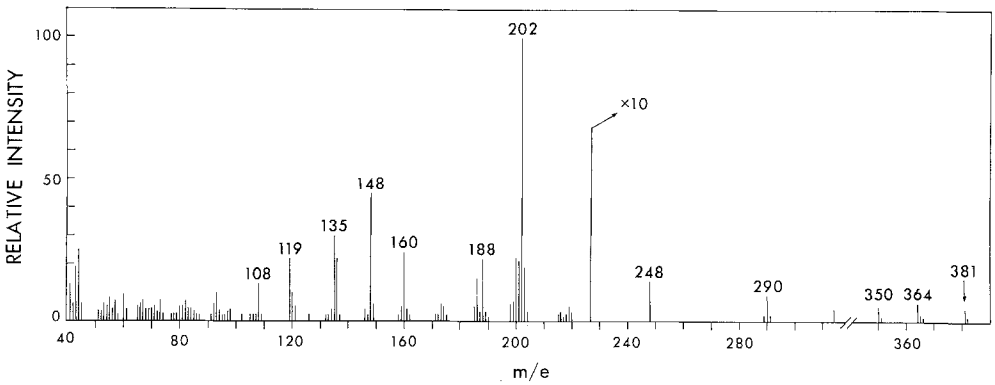
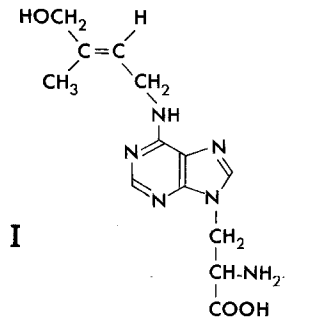


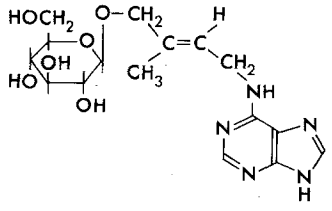
Fig. 5. The mass spectrum of metabolite L3/C

cluding an unambiguous synthesis of I, will be detailed elsewhere (Duke et al., 1978; for a preliminary report see MacLeod et al., 1975). The above studies established that L2 possessed structure I. L2, L-β-[6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-purin-9-yl]alanine, has been termed lupinic acid since it was first purified from *Lupinus angustifolius* seedlings supplied with zeatin.

Metabolite L3/C. L3/C exhibited UV spectra (Table 3) characteristic of an N^6-monosubstituted adenine. The mass spectrum (Fig. 5) shows a very weak molecular ion peak at m/e 381 and below m/e 220 the spectrum is similar to that of zeatin, indicating the presence of an intact zeatin moiety. However, a noticeable difference between the two spectra is the intensity of the peak at m/e 188; this is very pronounced in the spectra of zeatin, zeatin riboside and 7- and 9-β-D-glucopyranosylzeatin, but is relatively weak in



I



II

L3/C. Metabolite L3/C was rapidly hydrolyzed by β-glucosidase, but not by α-glucosidase, to yield a compound which was chromatographically indistinguishable from zeatin; hydrolysis with a polystyrene sulphonic-acid resin (H^+ form) yielded glucose which was identified by reaction with the specific enzyme glucose oxidase. These degradations and other chemical evidence, including an unambiguous synthesis of II, will be detailed elsewhere (Duke et al., 1978; for a preliminary report see Parker et al., 1975). The above studies established unequivocally that L3/C was O-β-D-glucopyranosylzeatin (II).

L2 and L3/C were the only two new zeatin metabolites from lupin extracts which were identified unambiguously. However the limited structural information available regarding L3/A, L3/B and L3/D is of interest and is outlined below.

Metabolite L3/A. Prominent peaks at m/e 162, 204, 160 and 202 in the mass spectrum of L3/A, together with the general pattern of peaks, indicated the presence of both zeatin and dihydrozeatin moieties. Hence, L3/A was probably a mixture of two compounds, one containing an intact zeatin moiety, the other an intact dihydrozeatin moiety. However, the prominent peak at m/e 250 indicated that the metabolite with the dihydrozeatin moiety was a glycoside. This peak is prominent in the spectra of synthetic O-β-D-glucopyranosyldihydrozeatin, 9-β-D-ribofuranosyldihydrozeatin and 9-β-D-glucopyranosyldihydrozeatin. It is representative of an ion which is evident in the mass spectra of all purine glycosides and is caused by the base moiety with an attached CHOH fragment of the sugar. A mass spectrum of the Me_3Si derivative of L3/A did not yield additional structural information.

Metabolite L3/B. The mass spectrum of L3/B exhibited a prominent peak at m/e 135 suggesting the presence of an adenine moiety in the molecule. The UV spectra (Table 3) are characteristic of 9-substituted adenines and are almost identical to those of adenosine. However, TLC studies clearly established that L3/B was neither adenosine nor deoxyadenosine, the R_f values for L3/B, adenosine and deoxyadenosine on silica gel in solvent B being 0.22, 0.41 and 0.40, respectively. Hence L3/B appeared to be considerably more polar than these two common adenine nucleosides. L3/B was hydrolyzed with a sulphonic-acid resin under conditions previously used to hydrolyze cytokinin glucosides to glucose (Letham et al., 1975). Elution of the resin (conc. NH_4OH) yielded a UV-absorbing product which was identified as adenine by UV and mass spectra and by TLC.

The above evidence establishes that L3/B is a 9-substituted adenine. However, attempts to elucidate the structure of the substituent were unsuccessful. Hydrolysis with acid resin yielded a product which after TLC reacted with the anisaldehyde-sulphuric acid spray for sugars to give a grey-brown colour. This colour reaction did not correspond with that of any common sugar. TLC and use of a glucose oxidase spray reagent (Letham et al., 1975) confirmed that glucose was not a hydrolysis product. Mass spectrometry of the Me_3Si derivative of L3/B failed to yield significant structural information. L3/B migrated towards the anode during paper electrophoresis in borate buffer (pH 9.2); however, it did not migrate in phosphate buffer at this pH. Hence the substituent group in L3/B probably contains a *vic*-glycol grouping.

Metabolite L3/D. In the mass spectrum of L3/D, the molecular ion peak was not evident, but the spectrum indicated the presence of an intact zeatin moiety in the compound. As in the mass spectrum of L3/C, the peak at m/e 188 was relatively weak and hence the oxygen in the zeatin side chain may carry a substituent. The small amount of sample available was sufficient to obtain a UV spectrum only in neutral ethanol; this did not provide sufficient data to draw conclusions regarding the location of the substituent on the zeatin moiety. A mass spectrum of the Me_3Si derivative of L3/D failed to provide further structural information.

The above structural evidence establishes that *L. angustifolius* seedlings form several minor zeatin metabolites in which the zeatin moiety is conserved, in addition to the major metabolites L2 and L3/C.

Zeatin Metabolites in Leaves, Stems and Apices of Lupinus luteus

In the experiments reported above, zeatin was supplied at 100 μM, which is probably an unphysiological concentration. Fourteen-d-old de-rooted seedlings of *Lupinus luteus* (*L. angustifolius* seed was not available) were therefore supplied with [^{3}H]zeatin at 5 μM via the transpiration stream for 30 h. Such seedlings have two pairs of leaves; one pair is mature while the other pair is still expanding, having reached about one-third of the final weight. The extractable radioactivity in the various portions of the seedlings is presented in Table 4. The lower portion of the stem (below the cotyledon) which directly contacted the zeatin solution contained the highest level of extractable radioactivity followed, in order of decreasing radioactivity, by the upper stem (stem between cotyledons and base of petioles of mature leaves),

Table 4. The content of extractable radioactivity and [^{3}H]lupinic acid in different parts of de-rooted *Lupinus luteus* seedlings supplied with [^{3}H]zeatin through the transpiration stream. The content of lupinic acid was determined by two-dimensional TLC on cellulose

Part of seedling	Mean weight (mg)	Total radioactivity extracted (cpm mg^{-1} tissue)	Lupinic acid (cpm mg^{-1} tissue[a])
Mature lamina	93	2,705	101
Expanding lamina	28	2,445	174
Mature petiole	18	2,150	47
Petiole of expanding leaf	5	1,945	43
Upper stem	25	2,811	52
Lower stem	53	3,420	183
Apical region	10	1,920	24

[a] 240 cpm is equivalent to 1 ng of lupinic acid

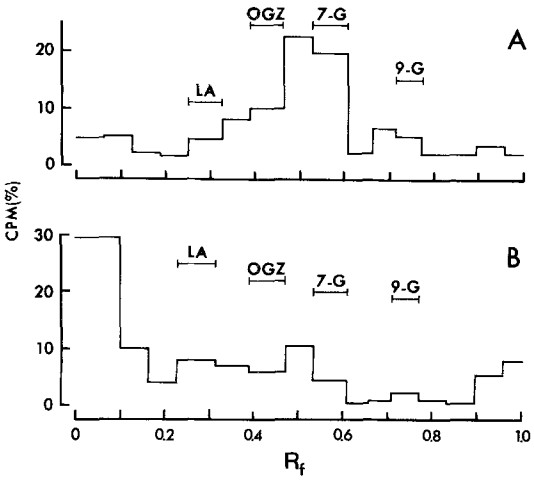


Fig. 6A and B. The distributions of radioactivity over cellulose thin-layer chromatograms of extracts of mature lamina **A** and upper stem **B**. These tissues were derived from *L. luteus* seedlings supplied with [^{3}H]zeatin (5 μM). The chromatograms were developed in one dimension with solvent D followed by A. Barred lines denote the location of cochromatographed lupinic acid (LA), O-glucosylzeatin (OGZ), zeatin-7-glucopyranoside (7-G) and zeatin-9-glucopyranoside (9-G)

mature laminae, expanding laminae, petioles of both leaves, and the apical region of the shoot (stem above base of petioles of mature leaves including apical bud).

Lupinic Acid and O-Glucosylzeatin as Metabolites. The content of ^{3}H-labelled lupinic acid in extracts of the above tissues was readily determinable. Each extract was mixed with non-radioactive synthetic DL-lupinic acid (to serve as a chromatographic marker) and subjected to two-dimensional TLC on cellulose (solvent A followed by D). The lupinic acid spots were then eluted with water for liquid scintillation counting. When such eluates were subjected to further TLC on silica gel, all the ^{3}H cochromatographed with marker lupinic acid. However, the ^{3}H in eluates of O-glucosylzeatin spots on the same cellulose chromatograms was not entirely accounted for by this compound as evidenced by subsequent TLC on silica gel. Although such studies provided strong evidence that both O-glucosylzeatin and lupinic acid were present in the lupin extracts, quantitative determination of O-glucosylzeatin was not possible by the simple procedure used for lupinic acid. The level of lupinic acid per unit fresh weight of tissue was greatest in the lower stem and in the expanding lamina, and least in the apical region (Table 4). The ^{3}H present as lupinic acid, expressed as a percentage of the total extractable ^{3}H, ranged from 1.3 (apical region) to 7.1 (expanding lamina). The various tissues of the shoot thus vary considerably in their ability to accumulate lupinic acid; in some tissues, lupinic acid is a minor metabolite. Two-dimensional TLC on cellulose followed by TLC on silica gel established that in extracts of mature laminae, 4.1% of the radioactivity was attributable to O-glucosylzeatin, a contribution only slightly greater than that made by lupinic acid (cf. Table 4).

Adenosine, Dihydrolupinic Acid, L3/B and Nucleotides as Metabolites. To provide information regarding the identity of metabolites other than lupinic acid and O-glucosylzeatin, extracts of mature laminae, expanding laminae, the apical region and the upper stem were chromatographed on cellulose in one dimension using two solvents, D followed by A (this system gives an excellent separation of lupinic acid, O-glucosylzeatin, zeatin-7-glucopyranoside and zeatin-9-glucopyranoside; see Fig. 6). The two first-mentioned extracts gave chromatograms with similar distributions of ^{3}H, as did the two last-mentioned. On all four chromatograms, a pronounced peak of radioactivity (R_f 0.50) was located immediately in front of cochromatographed O-glucosylzeatin and, in the case of mature lamina extracts (Fig. 6A), this extended into the region of the zeatin-7-glucopyranoside marker. The labelled compounds in this peak derived from mature-lamina and upper-stem extracts were examined by TLC on silica gel. In both cases, about 90% of the radioactivity was accounted for by adenosine, which was therefore the major metabolite in mature laminae.

On the chromatograms of extracts of mature laminae (Fig. 6A), of expanding laminae and of the apical region, about 10% of the total radioactivity

was located in a zone (R_f 0.36) between cochromatographed lupinic acid and O-glucosylzeatin. TLC (silica gel and cellulose) and paper-electrophoresis studies (0.025 M phosphate, pH 10.0; 0.025 M borate, pH 9.2) of the R_f-0.36-zone eluates from laminae indicated that about 19 and 12% of the eluate ^3H was accounted for by the partially characterized metabolite L3/B and by dihydrolupinic acid, respectively. The best separation of these compounds was achieved on silica gel using solvent D (R_f values: L3/B, 0.20; dihydrolupinic acid, 0.10); with solvent A, the two compounds were not separable, both exhibiting an R_f of 0.18.

Chromatograms of extracts of the apical region and the upper stem (Fig. 6B) exhibited a prominent peak of radioactivity near the origin which accounted for about 30% of the total ^3H. In contrast, only minor peaks were present near the origin of chromatograms of extracts of mature (Fig. 6A) and expanding laminae. The eluates of the origin peaks from mature lamina and upper stem were hydrolyzed with alkaline phosphatase and then subjected to TLC. Of the total ^3H in the lamina hydrolyzates, 50 and 3% cochromatographed with adenosine and with zeatin riboside, respectively; the corresponding percentages for the stem hydrolyzates were 30 and 18. Hence in upper stems, nucleotides of adenine and zeatin are major metabolites. In mature laminae, nucleotides of adenine are the dominant nucleotide metabolites, but these make only a small contribution to the total ^3H extracted from the tissue.

New Glucoside Metabolites. When extracts of mature and expanding laminae, of the apical region, and of upper stems were subjected to TLC on silica gel (solvent A), about 9% of the radioactivity occurred between cochromatographed lupinic acid and the origin region (i.e. between R_f 0.03 and 0.15), while about 13% was located between R_f 0.20 and 0.27. The ^3H in these zones was not attributable to any of the metabolites so far mentioned (lupinic acid, O-glucosylzeatin, zeatin-7-glucopyranoside, zeatin-9-glucopyranoside, dihydrolupinic acid and L3/B all have almost identical R_f values in this system, ca. 0.18). However, when the eluates of the zones with R_f 0.08–0.15 from the four extracts were hydrolyzed with β-glucosidase and the butanol extracts of the hydrolyzates subjected to TLC on silica gel and cellulose (solvent A), in each case a peak of radioactivity cochromatographed with zeatin riboside and dihydrozeatin riboside (not separated in this system). Hence the lupin extracts appeared to contain glucosides of zeatin riboside and/or dihydrozeatin riboside as minor metabolites. Chromatographic studies with synthetic O-glucosyl derivatives of zeatin riboside and dihydrozeatin riboside (Letham et al., 1977) showed that these glucosides would move to the R_f 0.08–0.15 zone. The eluate of the zone of R_f 0.20–0.27 from extract of mature laminae was also examined for new metabolites. During TLC (cellulose, solvent A), 51% of the radioactivity of the eluate cochromatographed with synthetic O-glucosyldihydrozeatin. The presence of this metabolite was confirmed by hydrolysis with β-glucosidase; this yielded a labelled product which cochromatographed with dihydrozeatin in several systems, including solvent H on silica gel. Zeatin was not a hydrolysis product.

Metabolites of Zeatin in Xylem Sap and Roots of Lupin Seedlings

Xylem sap was collected over a period of 4 d from 17-d-old *Lupinus angustifolius* seedlings. The collection commenced about 11 h after the sand in which the seedlings were growing had been watered with a [^3H]zeatin solution (12 µM). TLC of the sap on silica gel using solvents A and H indicated that most of the radioactivity was attributable to zeatin, dihydrozeatin and zeatin riboside, in the proportions of 5:2:1. A small amount (about 8%) of the ^3H was probably accounted for by nucleotides.

The roots of the same seedlings were extracted after collection of sap. Paper chromatography (solvent A) resolved the radioactivity into three major zones with R_f values of 0.10, 0.17 and 0.39. Using the chromatographic and paper electrophoretic methods already described, the major metabolite in the peak at R_f 0.17 was identified as lupinic acid; the radioactivity peak at R_f 0.39 was largely attributable to zeatin-7- and -9-glucosides present in the ratio of 3:1. The metabolite(s) at R_f 0.10 were not identifiable because of the limited amount of extract available. O-Glucosylzeatin, if present at all, was only a minor metabolite.

The Metabolic Fate of Lupinic Acid and O-Glucosylzeatin Supplied to Lupin Leaves

The ends of the petioles of excised leaves of 18-d-old *L. luteus* seedlings were placed in a solution of [^3H]lupinic acid (50 µM) or of O-glucopyranosyl-[^3H]zeatin (27 µM) for 27 h and then all leaves were transferred to water. After 18 and 72 h, samples of the leaves were extracted and the extracts were subjected to one-dimensional TLC on cellulose (solvent D followed by A). These TLC studies indicated that exogenously supplied lupinic acid was not rapidly metabolized (in extracts prepared at both times, over 80% of the

^{3}H cochromatographed with authentic lupinic acid), a result confirmed by paper electrophoresis at pH 10.2. If adenosine, free zeatin and zeatin riboside were present in the extracts, their contribution to the total radioactivity was less than 0.2%. However a large proportion of the O-glucosylzeatin supplied to the lupin leaves was metabolized even after 18 h in water. The distributions of radioactivity over the cellulose chromatograms of extracts prepared at 18 and 72 h were similar and three zones of radioactivity were evident. A broad zone, termed zone A, contained 90% of the ^{3}H and its centre was coincident with cochromatographed O-glucosylzeatin; one minor zone cochromatographed with zeatin (zone B, 3% of ^{3}H) and another (4% of ^{3}H) was at the origin.

TLC of zone A eluate on silica gel (solvent A) resolved the ^{3}H into three very distinct zones which cochromatographed with O-glucosylzeatin riboside (R_f 0.13), O-glucosylzeatin (R_f 0.18) and adenosine (R_f 0.30). For the extract prepared at 72 h, the radioactivity in these peaks represented 52, 22 and 17% respectively of the total recovered from the silica gel chromatograms. The eluates of the zones with R_f 0.13 and 0.18 were hydrolyzed with β-glucosidase. TLC studies indicated that the products formed were zeatin riboside and zeatin respectively. The above observations provided strong evidence that the major metabolite of exogenous O-glucosylzeatin in lupin leaves was the corresponding riboside. Adenosine also appeared to be a metabolite and this was confirmed by paper electrophoresis (borate buffer, pH 9.2) and by hydrolysis to adenine (1 N HCl at 100° C). Chromatographic studies using synthetic O-glucosyldihydrozeatin and its riboside as markers established that if these compounds were formed at all as metabolites, their contribution to the extractable radioactivity was less than 9% of that of the corresponding unsaturated compounds. The O-glucosyldihydro compounds can be separated conveniently from the corresponding unsaturated compounds by TLC on cellulose using solvent A. (R_f values: O-glucosylzeatin and its riboside, 0.15; O-glucosyldihydrozeatin and its riboside, 0.21).

TLC studies of the zone-B eluate on silica gel (solvents A and H) indicated the presence of both labelled zeatin and dihydrozeatin (ratio 5:3), but zeatin and dihydrozeatin ribosides were not detected. Hence cleavage of the glucoside linkage of O-glucosylzeatin to yield zeatin and dihydrozeatin is a minor metabolic reaction.

Discussion

The preliminary report (Parker et al., 1975) that O-β-D-glucopyranosylzeatin was a zeatin metabolite in lupin was the first evidence for the presence of this type of zeatin glucoside in plant tissues. The occurrence of O-glucosylzeatin as a metabolite has now been further substantiated in two species of *Lupinus*. The metabolite was identical in all respects to O-β-D-glucopyranosylzeatin synthesized by unambiguous methods (Duke et al., 1978). Subsequent to the initial work of Parker et al. (1975) this glucoside has been reported in diverse species. O-β-D-Glucopyranosylzeatin was tentatively identified as a zeatin metabolite in soybean tissue (Horgan, 1975), and has been identified unambiguously as a zeatin metabolite in *Populus alba* leaves which also convert zeatin to O-β-D-glucopyranosyldihydrozeatin and to the 9-riboside of this compound (Letham et al., 1977). Very recently O-glucosylzeatin and its 9-riboside have been reported to be endogenous cytokinins in crown-gall tumour tissue from *Vinca rosea* (Peterson and Miller, 1977; Morris, 1977). From the relative intensities of the m/e 204 and 217 ions in the mass spectrum of the Me_3Si derivative of O-glucosylzeatin, Morris (1977) assigned a correct pyranose ring structure to the glucose moiety of this compound. However, it is now known that these intensity data can in fact not be used to assign ring structure to such compounds (MacLeod et al., 1976). It should be noted that both Deleuze et al. (1972) and Laloue et al. (1975) assigned erroneous furanose ring structures to 7-glucoside metabolites of cytokinins by use of these intensity data (see Duke et al., 1975; MacLeod et al., 1976). In contrast, the relative intensities of ions at m/e 204 and 205 in the mass spectra of Me_3Si derivatives of purine glucosides are a reliable indication of sugar ring structure (MacLeod et al., 1976). Application of this criterion to the spectrum of Morris (1977) for O-glucosylzeatin indicates a glucopyranoside structure.

The purine-amino acid conjugate termed lupinic acid, which was identified as a metabolite of zeatin in lupin seedlings, is unusual structurally. It is the only plant product known in which an amino-acid moiety is conjugated to a nitrogen atom of the purine ring. The only other natural compound of this type is the spore germination inhibitor termed discadenine, which was isolated from the slime mould *Dictyostelium discoideum* and recently identified as 3-(3-amino-3-carboxypropyl)-6-(3-methyl-2-butenylamino)purine (Abe et al., 1976). This compound is thus a 3-substituted derivative of the well-known cytokinin, N^6-isopentenyladenine. Although endogenous compounds in which a purine-N carries an amino-acid moiety are unusual, pyrimidine-amino acid conjugates are known in plants, e.g. willardiine which has been isolated from *Acacia willardiana* and *Pisum sativum* (Lambein and Parijs, 1968). Furthermore, some plants

contain enzyme activity capable of catalyzing in vitro the addition of an alanine (2-amino-2-carboxyethyl) moiety to an N atom of certain ring systems other than purine (see e.g. Murakoshi et al., 1975). The alanine donor in these reactions is O-acetylserine which also appears to be the source of the alanine moiety in lupinic acid (Murakoshi et al., 1977). Serine at high, unphysiological concentrations promotes leaf senescence and suppresses the senescence-retarding activity of cytokinins (Shibaoka and Thimann, 1970). This could be a consequence of cytokinin conjugation to yield an alanine derivative with weak biological activity.

The physiological significance of the metabolites lupinic acid and O-glucosylzeatin is unknown. Both are active in the radish-cotyledon cytokinin bioassay; while lupinic acid exhibits only very weak activity, the activity of O-glucosylzeatin approaches that of zeatin. Comparative studies of the activity of these and related compounds will be reported elsewhere. While zeatin was rapidly metabolized in *Lupinus luteus* to adenosine as a major metabolite and to a diversity of other compounds, lupinic acid showed considerable metabolic stability. Even 72 h after the uptake of the ^{3}H-labelled acid had ceased, over 80% of the radioactivity was accounted for by undegraded lupinic acid; no labelled free zeatin or adenosine were detectable. O-Glucosyl-[^{3}H]zeatin was largely converted to the 9-riboside which with some undegraded O-glucosylzeatin accounted for about 75% of the extracted radioactivity 72 h after cessation of uptake. Hence lupinic acid and the O-glucosylzeatin moiety are structures with considerable metabolic stability. The O-glucosyl substituent must markedly inhibit cleavage, presumably by cytokinin oxidase (Whitty and Hall, 1974), of the isoprenoid side chain to yield adenine and adenosine. The adenosine detected as a minor metabolite of O-glucosylzeatin is possibly formed from zeatin (which is also a minor metabolite of O-glucosylzeatin), adenine being an intermediate. Cytokinin oxidase is known to degrade zeatin to adenine (Whitty and Hall, 1974). O-Glucosylzeatin, its riboside and lupinic acid may be storage forms or detoxification products of zeatin. 7-Glucopyranosylzeatin (raphanatin), now known to be an endogenous cytokinin (Summons et al., 1977), may also be a storage form of zeatin (Parker and Letham, 1973).

The various tissues of the lupin shoot differ markedly in their ability to accumulate particular metabolites of zeatin. Thus zeatin nucleotide was a major metabolite in the stem, but a very minor one in the leaf blades. This distribution of nucleotide metabolites resembles that in shoots of radish seedlings (Parker and Letham, 1973; Gordon et al., 1974). The levels of lupinic acid in the various shoot tissues also differ considerably (Table 4) and are not well correlated with the levels of total extractable ^{3}H. Thus the accumulation of lupinic acid does not appear to be closely related to the accumulation of other metabolites of zeatin.

Zeatin has previously been separated from dihydrozeatin by gas-liquid chromatography (Most et al., 1968) and by high-pressure liquid chromatography (Cole et al., 1974), but never by TLC. A TLC separation would be invaluable, especially for assessment of radioactivity in these compounds as was required in the present study of lupin metabolites. A method for separating zeatin, dihydrozeatin, zeatin riboside and dihydrozeatin riboside by silica-gel TLC was therefore devised. Of many solvent systems tried, the only one which effected a separation was methyl acetate-ethanol (9:1, v/v). Addition of traces of water to the solvents abolished the separation; however, separation could be enhanced by addition of formic acid and 2,2-dimethoxypropane, a water scavenger (see solvent H). Although 2,2-dimethoxypropane has been used previously in organic reactions, its use in chromatography has not been reported before.

Two techniques mentioned herein are of particular value for detection of [^{3}H]lupinic acid. They are (1) oxidation with L-amino-acid oxidase and subsequent TLC, and (2) electrophoresis at pH 10. These methods readily distinguish it from all other known cytokinin metabolites except dihydrolupinic acid.

Cytokinins have been implicated in the control of leaf abscission in lupin (Burrows and Carr, 1967), a plant which shows sequential leaf senescence and abscission. Cytokinin translocation and metabolism in relation to these phenomena in lupin merit study. It is unfortunate that cytokinin metabolism in lupin is so complex (16 metabolites have so far been at least partially characterized chemically) and that some minor metabolites remain unidentified. Nevertheless, the results of the present endeavour, and the chemical synthesis of the newly identified metabolites (Duke et al., 1978) to provide authentic chromatographic markers, should facilitate future physiological studies into the possible role of cytokinins in regulation of leaf senescence and abscission in lupin.

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Received 4 January; accepted 30 May 1978