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CYTOKININ METABOLISM AND THE MODULATION OF CYTOKININ ACTIVITY IN RADISH

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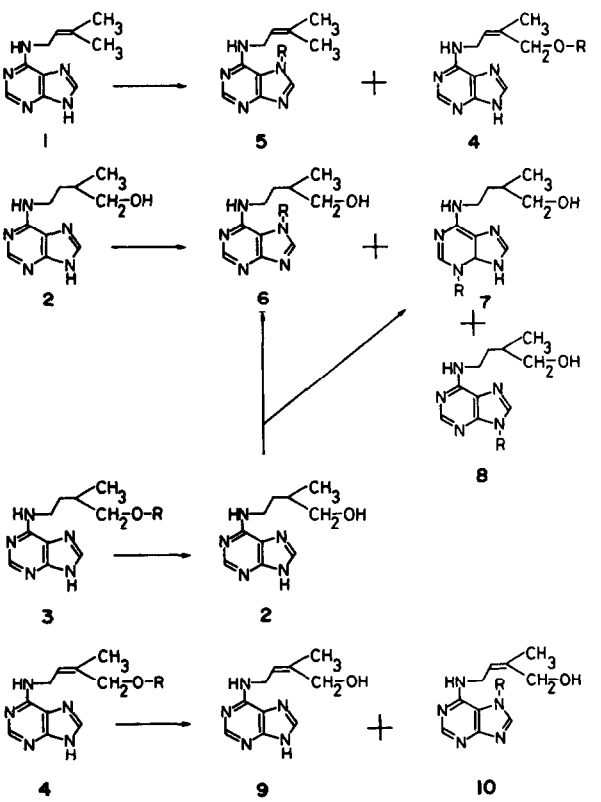
Key Word Index—*Raphanus sativus*; Cruciferae; radish; cytokinin metabolism; cytokinin-*O*-glucosides; storage forms; mass spectrometry; high performance liquid chromatography.

Abstract—The metabolism of zeatin, N^6-(Δ^2-isopentenyl) adenine, dihydrozeatin, zeatin-*O*-glucoside and dihydrozeatin-*O*-glucoside has been studied using derooted radish seedlings. The metabolites were identified by UV and GC/MS. The patterns of metabolism are compared and provide evidence that the *O*-glucosyl conjugates may be storage forms of the cytokinins.

INTRODUCTION

Externally applied zeatin (Z) (**9**), dihydrozeatin (DHZ) (**2**) and N^6-(Δ^2-isopentenyl) adenine (2iP) (**1**) are rapidly metabolized by all plant tissues [1–6]. In most cases there is a balance between conjugative metabolism and the products of oxidative N^6-side-chain cleavage. Though limited amounts of the biologically active 9-ribosides are formed by some tissues, the most abundant *N*-conjugation products (namely 7-β-D- and 9-β-D-glucopyranosides and 9-alanyl derivatives) exhibit little activity in bioassay [7]. These compounds are also extremely stable in the tissues where they have been studied (both as metabolites and when applied externally) and are regarded as detoxification or inactivation products [8–10]. *O*-Glucoside metabolites are also of widespread occurrence [1, 4, 6]. These compounds are highly active in bioassay [7], but it is possible that this activity is derived from hydrolysis to their parent bases by β-glucosidase enzymes. The *O*-glucosides are not substrates for cytokinin oxidase [11] and are quite stable in some tissues (e.g. *Lupinus* [10] and *Phaseolus* [2] leaves). However, unlike the *N*-glucosyl conjugates, they are readily metabolized in other systems (e.g. *Vinca rosea* crown gall [12]). The *O*-glucosides are abundant endogenous cytokinins [4, 6] and have been shown to accumulate and be rapidly metabolized at different stages of plant development [13]. For these reasons, the *O*-glucosides are strong candidates for the role of cytokinin storage forms.

In radish tissue there is little oxidative side-chain cleavage of externally applied cytokinins [3, 8, 9, 14], thus conjugation provides the only mechanism by which the expression of their biological activity can be controlled. Accordingly, when Z (**9**) was fed to derooted radish seedlings [8] and radish roots [14] zeatin-7-β-D-glucopyranoside (Z7G) (**10**) was the major metabolite. When supplied in massive doses (*ca* 1 mg per seedling), equal quantities of zeatin-*O*-β-D-glucopyranoside (ZOG) (**4**) and Z7G (**10**) were found (Scott, I. M., personal communication). On the other hand, benzyladenine (BA) [9, 15] and DHZ (**2**) [3] gave their corresponding 3-, 7- and 9-β-D-glucopyranosides (**6–8**) when fed to derooted radish seedlings. These 3- (**7**) and 7-glucosides (**6**) are unique in nature, the only known compounds of this type being ribofuranosides, namely 3-ribosyl uric acid from bovine blood [16] and compounds related to vitamin B_{12} [17].



Scheme 1. Metabolic products of 2iP (**1**), DHZ (**2**), DHZOG (**3**) and ZOG (**4**) in derooted radish seedlings. R = β-D-glucosyl moiety.

Detailed studies on the structural requirements necessary for 7-glucosylation to occur have been conducted by Letham *et al.* [18]. On feeding a variety of different purine compounds to derooted radish seedlings, it was found that acceptance of a 7-glucosyl residue was dependent upon the presence of an N^6-substituent of 5–7 carbon atoms, but was not restricted to compounds with high cytokinin activity. Since most naturally occurring cytokinins have a 5-carbon N^6-substituent, 7-glycosylation is probably confined to biologically active structures in nature. Indeed, for those cytokinins which exhibit little activity in bioassay [e.g. zeatin-9-glucoside (Z9G), 9-alanylzeatin (9-AlZ) and, of course, Z7G (**10**)] 7-glucosylation is not possible without prior hydrolysis.

The enzymology of *N*-glucosylation has been studied in detail using a partially purified glucosyltransferase preparation from radish cotyledons [19, 20]. Whilst only UDPG and TDPG could serve as glucose donors, a wide variety of naturally occurring and synthetic cytokinins were able to serve as acceptor substrates [20]. The ratios of 7-/9-glucoside products were determined [e.g. *trans*-Z (**9**) = 10.5, BA = 1.65, DHZ (**2**) and ZOG (**4**) = only a trace of 9-glucoside], but these figures bear little relation to those observed when these compounds were externally applied [e.g. externally applied Z (**9**) does not give its 9-glucoside [8], yet DHZ (**2**) does [3]]. No 3-glucosides were formed by any of the substrates indicating that a separate enzyme may be involved here.

In this paper we report on the comparative metabolism of five naturally occurring cytokinins in derooted radish seedlings in order to understand further the structural requirements necessary for *N*-glucosylation *in vivo*.

RESULTS AND DISCUSSION

Derooted radish seedlings were supplied with [8-^{14}C]-Z (**9**), DHZ (**2**), ZOG (**4**), dihydrozeatin-*O*-glucoside (DHZOG) (**3**) and 2iP (**1**). After 3 days, the seedlings were harvested and the metabolites purified by HPLC and identified by UV and GC/MS. These data are presented in Table 1. In all the experiments variable quantities of polar metabolites (possibly nucleotides) were detected, but were not identified.

Zeatin metabolism

At 'low' levels of application, as with previous studies [8, 14], the principle metabolite and sole *N*-glucosylation product was Z7G (**10**). However, when applied at very high levels (over 500 nmol/seedling), both zeatin-9-riboside (ZR) and ZOG (**4**) became increasingly important metabolites.

Dihydrozeatin metabolism

The metabolism of DHZ (**2**) by derooted radish seedlings has been discussed in detail elsewhere [3], but the data presented in this paper have been extended to include a feed with a 'low' DHZ concentration (80 nmol/seedling) and is included for the sake of comparison.

DHZ (**2**) was metabolized in the same manner as BA [9, 15], giving the corresponding 3-, 7- and 9-β-D-glucosides as the major metabolites. This result was unexpected both because of the close structural similarity between Z (**9**) and DHZ (**2**) and because work with the previously discussed cell-free system [20] would suggest that DHZ (**2**) was even less likely than Z (**9**) to form its corresponding 9-glucoside. There was little difference between the distribution of metabolites at the two levels of application.

Zeatin-O-glucoside and dihydrozeatin-O-glucoside metabolism

Cell-free studies [20] have indicated that radish glucosyltransferase preparations are capable of glucosylating ZOG (**4**), presumably giving zeatin-*O*-glucoside-7-glucoside (though this product was not identified by mass spectrometry). It was unexpected, therefore, to find that both ZOG (**4**) and DHZOG (**3**) were hydrolysed to their aglycones prior to *N*-conjugation. Thus ZOG (**4**) was metabolized in the same manner as Z (**9**) giving Z7G (**10**). Similarly, DHZOG (**3**) gave DHZ7G (**6**) and smaller quantities of DHZ3G (**7**). No DHZ9G (**8**) was detected in this experiment.

Unlike the DHZ (**2**) and Z (**9**) feeds, a considerable amount of *O*-glucoside remained unmetabolized after 3 days and, on increasing the level of applied ZOG (**4**) from

Table 1. The products and their proportions of various cytokinin feeds to derooted radish seedlings. Unidentified products are not listed

Cytokinin fed	nmol/ seedling	Unmetabolized material	Products (% of total in parentheses) Metabolites
Z	100	n.d.	Z7G (88)
	500	Z (12)	Z7G (46), ZOG (11), ZR (8)
	2000	Z (27)	Z7G (32), ZOG (13), ZR (12)
DHZ	80	DHZ (14)	DHZ-glucosides (55)
	120	DHZ (6)	DHZ7G (57), DHZ9G (5), DHZ3G (8)
ZOG	80	ZOG (20)	Z7G (36), Z (7)
	160	ZOG (59)	Z7G (28)
DHZOG	120	DHZOG (35.3)	(+)-DHZ7G (22), (−)-DHZ7G (31.6), DHZ3G (5.4)
2iP	80	n.d.	2iP7G (75), ZOG? (8)
	160	n.d.	2iP7G (67), ZOG? (3)

n.d. = not detected.

80 to 160 nmol/seedling, the proportion of unmetabolized material rose dramatically (Table 1).

N^6-(Δ^2-*Isopentenyl)adenine metabolism*

2iP (**1**) was metabolized in the same manner as Z (**9**), giving N^6-(Δ^2-isopentenyl) adenine-7-glucoside (2iP7G) (**5**) as the major metabolite and sole *N*-glucosylation product. This compound has also been demonstrated as a metabolite of 2iP (**1**) in cytokinin-dependent tobacco cell suspensions [5, 21].

Whilst the Δ^2-double bond is obviously important in determining the synthesis of other *N*-glucosylation products (viz. DHZ), the side-chain alcohol group appears to have no effect. No trace of unmetabolized 2iP (**1**) was detected, but ZOG was tentatively identified as a minor metabolite.

At the two levels of application (80 and 160 nmol/seedling), there was little difference in metabolic pattern.

The 7- and 9-glucosides are not generally active in bioassay [7], thus the expression of biological activity of applied cytokinins will be modulated via the production of these metabolites. In contrast, the *O*- and 3-glucosides are biologically active [7, 15], but this is probably due to hydrolysis back to their respective aglycones by β-glucosidase enzymes (to which 7- and 9-glucosides are resistant).

The results provide further evidence for the view [4, 5, 7, 10, 11] that the *O*-glucosides may be storage forms of the cytokinins. Firstly, unlike Z (**9**), DHZ (**2**) and 2iP (**1**), the *O*-glucosides were not 7-glucosylated. Secondly, substantial quantities remained unmetabolized when applied at 'low' concentrations. The limited metabolism that does occur is presumably initiated by β-glucosidases (i.e. hydrolysis of the *O*-glucosyl moiety) generating the free bases which are then inactivated by *N*-glucosylation.

Apart from shedding some light on the roles of the various conjugated cytokinins, the results are useful in that they may reveal the presence or absence of certain enzyme systems and their substrate specificity. However, the quite different metabolic patterns observed for the two levels of ZOG (**4**) and at 'high' levels of Z (**9**) application emphasize the difficulty in extrapolating data gained from external application of cytokinins to the *in vivo* situation.

EXPERIMENTAL

Plant tissue. Raphanus sativus L. cv Yates Long Scarlet, Market strain, was obtained from Arthur Yates Co. Ltd., Auckland, New Zealand. Ten days after sowing, the seedlings were uprooted and the roots surgically removed.

Chemicals. 8-^{14}C-Labelled Z, 2iP, DHZ, ZOG and DHZOG (11.7 mCi/mmol) were synthesized by condensing [8-^{14}C]-6-chloropurine (Amersham) with the relevant side-chain amine [22]. The labelled cytokinins were purified by HPLC [23] prior to use.

Derivatization of the cytokinin metabolites. (a) *Per-(trimethylsilyl) derivatives.* TMSi derivatives were prepared by heating the dried (P_2O_5) sample in a sealed capillary tube with *N,O*-bis-(trimethyl)-acetamide (5 μl) and dry pyridine (5 μl) at 70° for 1 hr. (b) *Permethyl derivatives.* The samples were placed in Reactivials and dried (P_2O_5) overnight before mixing 50 μl 0.08 M dimethylsulphinyl carbanion in DMSO (prepared by dissolving 200 mg potassium *t*-butoxide in 10 ml DMSO). After 10 min, MeI (10 μl) was added. After 30 min at room temp., H_2O (50 μl) was added and the derivatized cytokinins partitioned into $CHCl_3$ (100 μl). The $CHCl_3$ soln was evapd and redissolved in EtOAc prior to GC/MS.

Gas chromatography/mass spectrometry. The derivatized cytokinins were injected onto a 10 m × 0.3 mm i.d. BP1 capillary GC column (film thickness 0.5 μm) and run with He at 55 kPa. The ionizing *V* was 70 eV and the temps. of the source, separator and inlet were, respectively, 190°, 250° and 240°. The scan speed was 1 sec/decade and the temp. of the GC column was taken from 35° to 280° (ballistic) and then to 300° at 8°/min for the TMSi glucosides, from 35° to 240° (ballistic) and then to 300° at 8°/min for the methylated glucosides and from 35° to 165° (ballistic) and then to 240° at 8°/min for the derivatized bases.

Identification of glucosides. The presence of a glucose residue in the 2iP7G and the DHZ glucosides was confirmed by hydrolysis of these compounds using a cation exchange resin. The hydrolysate was subjected to TLC, the resulting glucose being detected with glucose oxidase [15].

Feeding experiments. The labelled cytokinins were supplied in the following quantities: Z (100, 500 and 2000 nmol/seedling), 2iP and ZOG (80 and 160 nmol/seedling), DHZ (80 and 120 nmol/seedling) and DHZOG (120 nmol/seedling). In all cases, these were fed to two derooted seedlings that had been placed in glass vials containing H_2O (1 ml). When the soln had been taken up (2–3 days), the seedlings were harvested and extracted immediately.

Extraction and purification of the cytokinin metabolites. The seedlings were macerated in MeOH (100 ml) and the resulting liquor was filtered and evapd at red. pres. before being redissolved in H_2O (10 ml) and applied to a SEP-PAK C_{18} Cartridge (Waters Associates), which was washed with H_2O (20 ml) then MeOH (20 ml). The combined washings were evapd to dryness and subjected to HPLC using an analytical column (150 mm × 4.5 mm i.d.) of Apex ODS reversed-phase material. The column was eluted at 2 ml/min with various linear gradients of MeCN or MeOH with H_2O at pH 7 (triethylammonium bicarbonate) [23]. Fractions were collected and portions of these were counted in a liquid scintillation spectrometer. In all cases between 80 and 90% of the applied radioactivity was recovered at this stage.

Normal-phase HPLC was carried out with a Partisil PAC column (250 mm × 4.5 mm i.d.) using 90% MeCN–10% H_2O (pH 7.0) at 2 ml/min.

The various metabolites were purified and identified as follows:

(a) *Z feeds.* The MeOH extract from the 100 nmol/seedling feed was chromatographed on the reversed-phase HPLC system (5–9% of MeCN, 20 min) yielding the following radioactive fractions (% of total extractable radioactivity in parentheses): 1–3 min (12%) and 9–10 min (88%). The former was not identified, but the latter co-chromatographed with authentic Z7G (on reversed and normal-phase HPLC) and gave a UV λ_{max}^{MeOH} of 275.0 nm (which shifted to 278.1 nm on addition of a drop of HOAc. The compound was converted to its TMSi derivative for GC/MS, *m/z* (rel. int.): 741 $[M]^+$ (18.3), 726 (5.1), 652 (16.3), 638 (13.5), 61.0 (6.0), 450 (4.3), 406 (1.6), 361 (7.4), 331 (2.5), 320 (3.4), 218 (16.1), 217 (47.4), 204 (18.4), 203 (8.6), 202 (16.5), 201 (13.8), 188 (12.5), 156 (13.1), 147 (60.5), 143 (10.2), 133 (16.8), 129 (25.4), 117 (13.3), 103 (28.7), 101 (11.8), 75 (80.4), 73 (100). The compound was assigned zeatin-7-β-D-glucopyranoside [24].

The MeOH extract from the 500 nmol/seedling feed was subjected to reversed-phase HPLC (4–10% MeCN, 20 nm) giving four zones of radioactivity (% of total extractable radioactivity in parentheses): 1–3 min (23%), 9 min (46%), 13 min (11%), 14 min (12%) and 19–20 min (8%). Fraction 1–3 min was unidentified and fraction 9 min was identical (UV, GC/MS) to the

Z7G above. Fractions 13 and 14 min were purified further on reversed-phase HPLC and were identified as ZOG and Z, respectively [giving identical UV and GC/MS (as permethyl derivatives) to authentic samples]. Fraction 19–20 min was similarly identified as ZR giving an identical MS to that of authentic TMS-ZR.

The 2000 nmol/seedling feed was processed as above giving the following metabolites (% of total extractable radioactivity in parentheses): unidentified polar metabolite (16%), Z7G (32%), ZOG (13%), Z (27%) and ZR (12%).

(b) *DHZ feeds.* The purification of the metabolites from the 120 nmol/seedling feed has been reported elsewhere [3]. The following products were conclusively identified by chemical degradation, UV [25] and GC/MS of their TMSi derivatives [26] (% of total extractable radioactivity in parentheses): DH7G (57%), DHZ9G (5%), DHZ3G (8%) and unmetabolized DHZ (6%). Unidentified polar and possibly nucleotide material accounted for the balance (24%). When DHZ was applied at the 80 nmol/seedling level, the extract was chromatographed as before [3] on reversed and normal-phase HPLC, giving DHZ glucosides (55%) and DHZ (14%) with the unidentified polar metabolites accounting for 31% of the total extractable radioactivity.

(c) *ZOG feeds.* The MeOH extract from the 80 nmol/seedling feed was subjected to reversed-phase HPLC (4–6% MeCN, 20 min then to 15%). The following fractions were radioactive (% of total extractable radioactivity in parentheses): fractions 1–3 min (37%), 9–10 min (36%), and 16–19 min (27%). Fraction 1–3 min, as for the other feeds, remained unidentified. Fraction 9–10 min was bulked and re-chromatographed using the same HPLC system as above and gave a single peak with UV λ_{max}^{MeOH} of 274.4 nm which shifted to 277.7 nm on addition of a drop of HOAc. The compound was converted to its TMSi derivative for GC/MS. The spectrum was identical to that of TMSi zeatin-7-β-D-glucopyranoside [24]. Fraction 16–19 min was bulked and re-chromatographed on reversed-phase HPLC (7–11% MeCN, 20 min), giving two peaks of radioactivity: fractions 5–6 min and 7 min, which both gave UV λ_{max}^{MeOH} values of 268.4 nm. The former (20% of total extractable radioactivity) co-chromatographed with ZOG and was converted to its permethyl derivative for GC/MS. The spectrum was identical to that reported for the permethyl derivative of ZOG [26]. The second peak of radioactivity (fraction 7 min) (7% of the extractable radioactivity) co-chromatographed with reference Z on HPLC and was converted to its TMSi derivative for GC/MS. The spectrum was identical to that of authentic TMSi-Z.

When fed at 160 nmol/seedling level, the following metabolites were identified by UV spectroscopy, GC/MS and co-chromatography on HPLC (% of total extractable radioactivity in parentheses): fractions 1–3 min (13%), 9–10 min = Z7G (28%) and 16–19 min = ZOG (59%). No Z was detected in this experiment.

(d) *DHZOG feed.* The MeOH extract from this feed (120 nmol/seedling) was subjected to reversed-phase HPLC (4–6% MeCN, 20 min). Three zones of radioactivity were distinguished (% of total extractable radioactivity in parentheses): fractions 1–3 min (5.7%), 11–12 min (59.0%) and 19–22 min (35.3%). Fraction 1–3 min was not identified. Fraction 11–12 min was re-chromatographed on the normal-phase system (90% MeCN) giving two peaks of radioactivity (% of total extractable radioactivity in parentheses): fractions 8–9 min (31.6%) and 11–12 min (5.4%). The former gave a UV λ_{max}^{MeOH} of 275.0 nm and was converted to its TMSi derivative for GC/MS. This compound, by analogy with previous HPLC retention times [3], was identified as the *S*-(−)-DHZ-7-β-D-glucopyranoside antipode. The second fraction of radioactivity in the normal-phase HPLC run (fraction 11–12 min) gave a typical 3-substituted purine UV spectrum [25]; λ_{max}^{MeOH} = 292.5 nm and was converted to its TMSi derivative for GC/MS. This compound was assigned DHZ-3-β-D-glucopyranoside [3].

Fraction 12 min from the original reversed-phase HPLC run (22% of total extractable radioactivity) gave a single peak on the normal-phase system (8–9 min) with the same UV and GC/MS as the above (−)-DHZ7G. This compound was assigned *R*-(+)-DHZ-7-β-D-glucopyranoside. No DHZ9G was detected in this experiment.

Fraction 19–22 min (35.3% of total extractable radioactivity) from the reversed-phase HPLC run was re-chromatographed on the same system (5% MeCN) and gave a single peak of radioactivity which co-chromatographed with authentic DHZOG (eluted in 13–14 min). This compound was converted to its permethyl derivative for GC/MS. The spectrum was identical to that of authentic methylated DHZOG.

(e) *2iP feeds.* The MeOH extract from the 80 nmol/seedling feed was chromatographed on the reversed-phase system (5–9% MeCN, 20 min then to 15%). The following fractions were shown to contain radioactivity (% of total extractable radioactivity in parentheses): fractions 1–3 min (14%), 7 min (3%), 16–17 min (75%) and 22 min (8%). Fractions 1–3 min and 7 min were not identified. The latter co-chromatographed with Z7G, but it was not possible to obtain a mass spectrum of this compound. Fraction 16–17 min was bulked and re-chromatographed on the reversed-phase column (5–9% MeCN, 20 min). All the radioactivity eluted in fractions 16–17 min and the compound gave a UV λ_{max}^{MeOH} of 274.5 nm which shifted to 278.8 nm on addition of a drop of HOAc; typical of 7-substituted purines [25]. The compound was shown to be a glucoside and gave a mass spectrum (TMSi derivative) identical to that of N^6-(Δ^2-isopentenyl) adenine-7-β-D-glucopyranoside [21], *m/z* (rel. int.): 653 $[M]^+$ (25.5), 638 (233), 610 (6.2), 581 (1.2), 570 (2.2), 450 (6.9), 361 (4.5), 346 (2.9), 332 (2.8), 318 (4.9), 305 (3.3), 271 (5.2), 257 (4.0), 232 (13.2), 217 (63.3), 204 (25.0), 203 (24.2), 191 (10.6), 160 (18.7), 147 (48.7), 133 (12.0), 129 (15.2), 103 (12.2), 75 (26.0), 73 (100). Fraction 22 min co-chromatographed with reference ZOG eluting in fraction 16–17 min on another reversed-phase system (5–15% MeCN, 20 min). This fraction was permethylated and subjected to GC/MS. Contamination and the weak $[M]^+$ ion inherent in *O*-glucoside spectra [26] prevented a conclusive identification, but the base peak at *m/z* 230 and retention times on HPLC and GC suggest that this compound was probably ZOG.

The 160 nmol/seedling feed was extracted and chromatographed on the reversed-phase HPLC system in the same manner as above. The distribution of metabolites was as follows: (% of total extractable radioactivity in parentheses): fractions 1–3 min (26%), 7 min (4%), 16–18 min (67%) and 22 min (3%).

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