Metabolism and compartmentation of dihydrozeatin exogenously supplied to photoautotrophic suspension cultures of *Chenopodium rubrum*

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**Abstract.** [3H]Dihydrozeatin supplied to photoautotrophically growing cell suspension cultures of *Chenopodium rubrum* was rapidly taken up and metabolized by the cells. The predominant metabolites in extracts of the cells were [3H]dihydrozeatin-O-glucoside and [3H]dihydrozeatin riboside-O-glucoside. Both these compounds could be shown to be compartmented within the vacuole, whereas [3H]dihydrozeatin and [3H]dihydrozeatin riboside, which were both present to a minor extent in cell extracts, were localized predominantly outside the vacuole. Analysis of the culture medium at the end of the 36-h incubation period showed that there had been an efflux of [3H]dihydrozeatin metabolites out of the cells. Whereas [3H]dihydrozeatin riboside was found to be the major extracellular [3H]dihydrozeatin metabolite, the O-glucosides of neither this compound nor [3H]dihydrozeatin could be detected in the medium. The differential compartmentation of [3H]dihydrozeatin metabolites found with the *C. rubrum* suspension-culture system is discussed with respect to possible mechanisms governing the metabolism of cytokinins in plants cells.

**Key words:** Cell cytokinin culture – Compartmentation (hormones) – Cytokinin (metabolism) – Dihydrozeatin – Vacuole (hormone storage).

**Introduction**

Cytokinin applied to plant tissue is known to be rapidly metabolized, whereby either the N6-side chain of the compound is cleaved by cytokinin oxidase or various conjugates are formed (McGaw and Horgan 1985). These conjugates include products of both ring and isoprenoid-side-chain substitution, in which glucose is the most frequently observed substituent (Letham and Palni 1983; McGaw et al. 1984). This metabolic activity has been interpreted to represent a mechanism to control the cellular level of “active” cytokinins by generating products exhibiting little biological activity (Palni et al. 1984).

Whereas both side-chain cleavage and ring substitution appear to result in the irreversible destruction of cytokinin activity, O-glucosylation results in an only temporary biological inactivation of cytokinins (van Staden and Davey 1979; McGaw et al. 1984).

Although some evidence for the role of O-glucosides as storage forms of cytokinins has been presented (Palmer et al. 1981 a, b), no information as to either the intracellular compartmentation or the enzymes controlling this storage pool is available. On the other hand, glucoside conjugates of abscisic acid (ABA; Bray and Zeevaart 1985; Lehmann and Glund 1986); of gibberellin A1 (GA1; Garcia-Martinez et al. 1981) and of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D; Schmitt and Sandermann 1982) have been shown to be specifically compartmented within the vacuole.

In the present paper the metabolism of [3H]dihydrozeatin ([dH]Z), which is known to be converted preferentially to O- and N-glucosides in plant tissue (Wang et al. 1977; Palmer et al. 1981 a, b, c; McGaw et al. 1984; Forsyth and van Staden 1986), has been investigated with photoautotrophically growing cell suspension cultures of *Chenopodium rubrum*. This plant material facilitates a simple application of cytokinins and is well suited to
the study of the intracellular compartmentation, in particular with respect to the vacuole (Ziegler and Beck 1986).

**Material and methods**

**Cell suspension culture; application of cytokinin.** Cell suspension cultures of *Chenopodium rubrum* (Hüsemann and Barz 1977) were grown photoautotrophically as described by Campbell et al. (1984) for 39 d. At this time 1.79 gg \[^3H\](dill)Z dissolved in methanol were obtained using a DU-50 spectrophotometer (Beckman, München, FRG).

**Extraction of the cultured cells.** Cells were harvested as described by Campbell et al. (1984) and thoroughly washed with water on a sintered-glass filter. The cells were extracted with ethanol (4 ml of ethanol were added to each gram of harvested cells) overnight at 4°C without stirring. The extract was filtered, and the ethanol was evaporated under reduced pressure at 40°C. The aqueous remaining solution was adjusted to a pH of 3.5 and frozen. Upon thawing the resulting suspension was centrifuged at 12000 g for 1 h. The supernatant was adjusted to a pH of 3.1 and separated into anionic-plus-neutral and cationic fractions by cation-exchange on cellulose phosphate as described by Palmer et al. (1981 a). Both the cationic fraction and the anionic-plus-neutral fraction after treatment with alkaline phosphatase were then further purified by affinity chromatography on disposable octadodecyl silica (ODS) columns (bed volume 3 ml: Baker, Gross-Gerau, FRG) using ethanol as the eluent.

**Extraction of the culture medium.** The filtered culture medium and the washing solutions were combined, filtered and passed through a 3 ml disposable ODS column (Baker), which was subsequently eluted with ethanol. The non-binding aqueous fraction was treated with alkaline phosphatase and re-chromatographed in the same manner.

**Preparation and fractionation of protoplasts.** Protoplast preparation and fractionation were carried out as described by Ziegler and Beck (1986) respective of *C. rubrum* culture cells. Extracts of protoplasts and vacuoles were passed through a disposable filter with a pore width of 0.45 μm (Acro LC 13; Gelman Sciences, Ann Arbor, Mich., USA) prior to further analysis.

**Purification and identification of \[^3H\](dill)Z-metabolite.** Aliquot portions of extract fractions were evaporated to dryness in a stream of air and the residues were dissolved in 1 ml of 10% acetonitrile. These extracts were co-chromatographed with unlabelled standards of (dill)Z and 9-β-D-ribofuranosyl dihydrozeatin ([dill]9RZ; Sigma, München, FRG) by high-performance liquid chromatography (HPLC). Samples were applied to analytical (125 mm long, 4.6 mm i.d.) or semi-preparative (250 mm long, 8 mm i.d.) columns of Hypersil ODS reversed-phase material (Shandon Southern, Runcorn, U.K.). The columns were eluted with a gradient of 10–40% acetonitrile in water at pH 7.0 (triethyl ammonium bicarbonate) for 40 min at flow rates of 1 ml·min⁻¹ (analytical HPLC) or 2 ml·min⁻¹ (semi-preparative HPLC). Fractions were collected every minute and absorbance of the standards at 265 nm was monitored with an Uvicon Spectrophotometer (720 LC; Kontron, Zürich, Switzerland). Radioactivity was determined with an HPLC radioactivity monitor (LB 505; Berthold, Wildbad, FRG) and by liquid scintillation counting using 0.2-ml aliquots of the fractions.

**Other analytical procedures:** Assays of α-mannosidase and phosphoenolpyruvate (PEP)-carboxylase were carried out as described by Ziegler and Beck (1986). β-Glucosidase treatment was carried out according to Wang et al. (1977) and alkaline-phosphatase treatment according to Palmer et al. (1981 a). Chlorophyll was extracted and determined as described by Ziegler and Beck (1986). Ultraviolet spectra of metabolites of \[^3H\](dill)Z were determined using a DU-50 spectrophotometer (Beckman, München, FRG).

**Results**

**Uptake of \[^3H\](dill)Z.** \[^3H\]Dihydrozeatin supplied to a photoautotrophically growing cell suspension culture of *C. rubrum* was rapidly taken up by the cells. Approximately 50% of the radioactivity was incorporated within the first 3 h, after which the rate of uptake decreased, resulting in a final proportion of up to 85% of the applied label being found in the cells after 36 h of incubation.

**Identification of derivatives of \[^3H\](dill)Z in the cell extract.** Approximately 65% of the radioactivity assimilated during the 36 h incubation period could be extracted from the cells. This label comprised 89.5% cationic material and 10.5% anionic-plus-neutral substances as revealed by ion-exchange chromatography on cellulose phosphate. One-fourth of the radioactivity of the anionic-plus-neutral fraction bound to ODS after treatment with alkaline phosphatase, and could thus be ascribed to \[^3H\](dill)Z metabolites with nucleotide character.

Separation of the cationic fraction of the cell extract by HPLC on ODS reversed-phase material yielded six radioactive peaks (Fig. 1). Only 3.4% of the extracted radioactivity proved to be still associated with (dill)Z (peak 4). 9-β-D-Ribofuranosyl dihydrozeatin could also be detected but accounted for only 1.5% of the recovered radioactivity (peak 6).

Two major peaks (3 and 5) exhibiting an absorbance maximum at 269 nm and representing 21.5% and 32.5%, respectively, of the radioactivity recovered from the cells, also eluted within the characteristic elution range of cytokinins of the (dill)-Z-type. The elution position of both these peaks shifted upon treatment with β-glucosidase. The smaller peak now exhibited the same retention time as (dill)Z and the larger one co-eluted with authentic (dill)9RZ (Fig. 2). Peak 3 could thus represent O-(dill)Z, whereas peak 5 could represent the O-glucoside of (dill)9RZ.

The ratio of the relative amounts of label in