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Regulators of Cell Division in Plant Tissues. XXX. Cytokinin Metabolism in Relation to Radish Cotyledon Expansion and Senescence

D. S. Letham and B. I. Gollnow

Department of Developmental Biology, Research School of Biological Sciences, The Australian National University, PO Box 475, Canberra City, ACT, 2601, Australia

Received December 4, 1984; accepted May 2, 1985

Abstract. Kinetic studies of formation of glucosides of 6-benzylaminopurine (BAP) in excised radish cotyledons indicated that the 3-, 7-, and 9-glucosides (N-glucosides) were each formed directly from BAP. The 7- and 9-glucosides of BAP and the 7-glucoside of zeatin exhibited great stability in the cotyledons, but the 3-glucoside was converted to free BAP and to the 7- and 9-glucosides of BAP. When ^{3}H-labeled zeatin was supplied to developed cotyledons, at high concentrations (100 μM), 7-glucosylzeatin was the principal metabolite, but an appreciable proportion of the extracted ^{3}H was due to O-glucosylzeatin. In immature cotyledons, as used in the radish cotyledon cytokinin bioassay, this O-glucoside was shown to be converted into zeatin 7-glucoside probably via free zeatin.

Metabolism of BAP and zeatin in radish cotyledons was studied in relation to cytokinin-induced cotyledon expansion. Cytokinin N-glucosides were not metabolites responsible for the observed cytokinin-induced expansion, and were not detoxification products, or deactivation products formation of which was coupled with cytokinin action. However, the free base, its riboside, and nucleotide were possible active forms of BAP associated with cotyledon expansion. The possible significance of cytokinin N-glucosides is discussed.

Senescent and nonsenescent cotyledons differed in their metabolism of BAP, zeatin, and zeatin riboside. Senescence was associated principally with a reduction in ability to form 7-glucosylzeatin, enhanced metabolism to adenine derivatives, and an inability to form appreciable amounts of 3-glucosyl-BAP.

A two-dimensional thin layer chromatography (TLC) system, based on adjoining layers of cellulose and silica gel, for separating zeatin metabolites

is described. This does not completely separate zeatin and zeatin riboside from the corresponding dihydro-compounds. A reversed phase TLC method for achieving these separations is also reported.

The 7-glucoside of zeatin was first detected in derooted radish seedlings to which zeatin had been supplied through the transpiration stream. The glucoside metabolite was concentrated in the cotyledon laminae in which it was the major metabolite (Parker and Letham 1973) and its identity as 7-β-D-glucopyranosyl-zeatin (7GZ) was confirmed by unambiguous chemical synthesis (Cowley et al. 1978). While 7GZ was the only N-glucoside metabolite of zeatin identified in radish seedling extracts, 6-benzylaminopurine (BAP) was found to be converted into 7- and 9-β-D-glucopyranosyl-BAP (7G-BAP and 9G-BAP), the major metabolites, and also into the 3-glucopyranoside (3G-BAP) (Letham et al. 1975). 7G-BAP and 9G-BAP were also detected as metabolites of BAP in immature cotyledons excised soon after seed germination (Wilson et al. 1974) Although 3G-BAP was a minor metabolite, it exhibited cytokinin activity which greatly exceeded that of 7G-BAP and 9G-BAP (Letham et al. 1975, 1983). 7- and 9-Glucosides have now been detected as metabolites of exogenous cytokinins in several tissues and 7GZ, 9GZ, and dihydro-9GZ are also known to occur normally in plants (Letham and Palni 1983). In particular, 7GZ has been detected and quantified by mass spectrometry in dry radish seed (Summons et al. 1977). Recently, 3-, 7-, and 9-glucosides have been identified as metabolites of dihydrozeatin in radish cotyledons (McGaw et al. 1984). The physiological significance of these N-glucosides of cytokinins is unknown.

Exogenous cytokinins are known to promote markedly the expansion of cotyledons of diverse species including radish and cytokinin-induced radish cotyledon expansion involves promotion of both cell division and cell enlargement (Goodwin 1978; Gordon and Letham 1975; Letham et al. 1983). Cytokinins also retard the senescence of discs excised from radish cotyledons (Letham et al. 1983). In the present contribution, the formation of cytokinin 3-, 7-, and 9-glucosides and other metabolites has been studied in radish cotyledons in relation to cotyledon expansion and senescence. The stability and metabolism of N-glucosides of BAP and zeatin and of O-β-D-glucopyranosyl-zeatin (OGZ) in radish cotyledons have been related to the ability of these compounds to promote expansion of the cotyledons.

Materials and Methods

Chemicals

The following cytokinins were synthesized by procedures detailed in the cited references: [2,8-^{3}H]zeatin, 270 mCi/mmol (Letham and Young 1971); [8-^{3}H]zeatin riboside [8-^{3}H]9RZ, 330 mCi/mmol (Summons et al. 1980); [G-^{3}H]BAP, 25 and 600 mCi/mmol (Wilson et al. 1974); 3G-BAP, 7G-BAP, and 9G-BAP (Letham et al. 1975, Cowley et al. 1978); glucosides of zeatin and 9RZ (Cowley et al. 1978; Duke et al. 1978, 1979); ^{2}H-labeled OGZ (Summons et al. 1979).

^{3}H-labeled 3-, 7-, and 9-glucosides of BAP (25 mCi/mmol) were prepared by

supplying [^{3}H]BAP to excised radish cotyledons via the transpiration stream. The glucosides were purified to radiochemical purity by TLC methods (Letham et al. 1975). [^{3}H]OGZ (132 mCi/mmol) was prepared by supplying zeatin to blue lupin seedlings and purifying the glucoside by published methods (Parker et al. 1978). [^{3}H]7GZ (110 mCi/mmol) was prepared enzymically from [^{3}H]zeatin using partially purified cytokinin 7-glucosyltransferase (Entsch et al. 1979).

Uptake of ^{3}H Cytokinins by Cotyledons and Extraction of Tissue

^{3}H-cytokinins were supplied to radish (*Raphanus sativus* L. cv. Long Scarlet) cotyledons at three stages of development: (a) about 12 h after bursting of the seed coat ("immature" cotyledons); (b) when they reached about 60% of their maximum size ("maturing" cotyledons); the leaf bud was just beginning to open on the seedlings used; and (c) when fully expanded and showing yellowing ("senescent" cotyledons). The immature cotyledons were excised aseptically from sterile seedlings which developed from surface sterilized seed after 48–58 h at 23°C on wetted filter paper in darkness. The cotyledons were then cultured under continuous weak light (700 lux from fluorescent tubes) in petri dishes (diameter 9.0 cm), each of which contained one circle of Whatman No. 1 filter paper and cytokinin solution (3 ml) prepared in 2 mM potassium phosphate buffer (pH 5.8). To provide maturing cotyledons, seedlings were grown for about 10 days in potting mixture in a green house under natural light. At this stage, the apical bud, from which the first leaves are derived, was just beginning to open. Senescent cotyledons were derived from seedlings grown for about 25 days under the above conditions. Maturing and senescent cotyledons were supplied with cytokinin through the transpiration stream in either of two ways: (1) the roots were excised from the seedlings and the cut ends of the hypocotyls were placed in an aqueous cytokinin solution after excision of the apical bud or leaves; (2) the petioles were excised at their bases which were then placed in cytokinin solution. The resulting derooted seedlings or excised cotyledons were left in light (as above) at 23°C.

Extracts of tissue were prepared as outlined below. After rinsing with water and weighing, the tissue was dropped into 80% methanol (20 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 to dryness under reduced pressure (bath temperature 35–40°C) and the residue was dissolved in 50% ethanol (1.0 ml/g tissue) for chromatography. When extracting cotyledons and seed to which [^{3}H]7GZ had been supplied, $CaCO_3$ (30 mg/g tissue) and zeatin and 9RZ (10 μg of each /g tissue) were added to the extracting solvent. After extraction with 80% methanol, some tissues were further extracted with NCS tissue solubilizer (Amersham/Searle; 10 volumes diluted with 1 volume of water) at 30°C for 4 days. Aliquots were neutralized for scintillation counting.

Materials for Chromatography

Normal phase TLC was performed on layers spread with either Merck silica gel 60 PF_{254} (E. Merck, Darmstadt, Germany) or Serva cellulose (Serva Fein-

biochemia, Heidelberg, Germany). For separation of cytokinin metabolites, Serva cellulose was found to be much superior to all other types of cellulose tested and yielded very compact spots. Woelm green fluorescent indicator (M. Woelm, Eschwege, Germany) was incorporated into the cellulose (0.8%) prior to spreading. Borate-impregnated silica-gel and cellulose layers were prepared by slurrying silica gel or cellulose with 0.05 M sodium tetraborate prior to spreading. For paper chromatography, Schleicher and Schull 598L paper was used. For reversed phase TLC, 0.3-mm layers of Merck silica gel 60 GF_{254} (15 μm particle size) were impregnated either with liquid paraffin (see Noodén and Letham 1984) or with the silicon oil dimethylpolysiloxane (DMPS-5X, Sigma Chemical Co.). To impregnate with the silicon oil, the dried layers were dipped in a 6% (w/v) solution of the oil in petroleum ether (b.p. 100–120°). The layers were then dried at room temperature.

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, ethyl methyl ketone/acetic acid/water (16:1:4); D, water-saturated butan-1-ol saturated with $Na_2B_4O_7 \cdot 10\ H_2O$; E, butan-1-ol/propan-1-ol/14 N ammonia/water (6:1:1:2); F, ethyl methyl ketone/propan-1-ol/14 N NH_4OH/water (10:1.1:1:1); G, methanol/water (1:4); H, methanol/water (3:7).

Determination of Radioactivity due to Metabolites

Radioactivity due to BAP metabolites other than BAP nucleotide was determined by two-dimensional (2D) TLC on silica gel (first dimension, solvent B; second dimension, solvent E run twice). Use of solvent E instead of solvent A, which was used previously for the second dimension (Letham et al. 1975), improved the separation of 3G-BAP from 7G-BAP. Otherwise the pattern of spots was very similar to that found previously (Letham et al. 1975). Zeatin metabolites were separated by a 2D TLC procedure using plates with adjoining layers of cellulose and silica gel (see Fig. 1). A strip (width 4.0 cm, thickness 0.4 mm) of Serva cellulose was first spread along one edge of a 20 × 20 cm plate. This strip served for chromatography in the first dimension (solvent C followed by A) after which the rest of the plate was spread with a silica gel slurry (0.3 mm thick) which flowed to meet the very hard cellulose layer. In the second dimension (solvent A), compounds moved from the cellulose into the silica gel layer for further separation. This 2D TLC procedure does not adequately separate zeatin from dihydrozeatin and 9RZ from dihydrozeatin riboside. To achieve these separations, the 2D TLC eluates were rechromatographed by reversed phase TLC. With silicon oil-impregnated silica gel and solvent G, the R_f values for dihydrozeatin, zeatin, dihydro-9RZ, and 9RZ were 0.21, 0.26, 0.34, and 0.39, respectively. On paraffin-impregnated silica gel (solvent H) the corresponding R_f values were: 0.22, 0.28, 0.31, and 0.36.

When the above 2D TLC procedures were applied to radish extracts, appropriate synthetic metabolites of BAP or zeatin were added as markers. Rechromatography of the metabolite-containing 2D TLC eluates on cellulose (solvent A for BAP metabolites, solvents B and F for zeatin metabolites) confirmed their radiochemical purity. In the case of 3G-BAP, a minor metabolite, identity

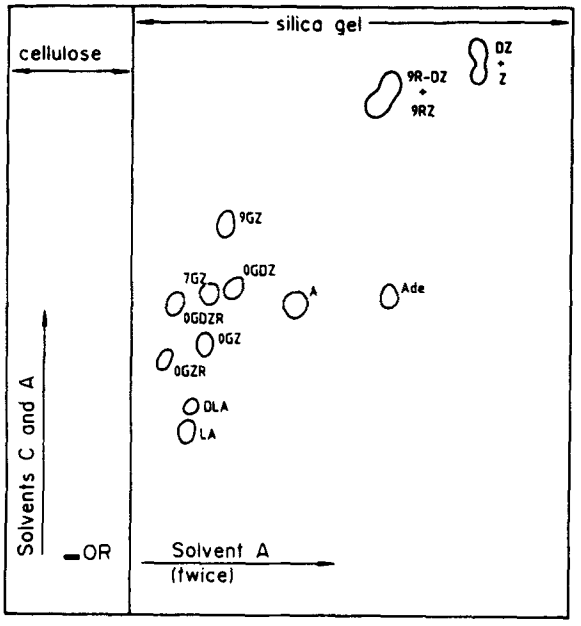

Fig. 1. A two-dimensional thin-layer chromatogram of zeatin metabolites. Ade, adenine; A, adenosine; Z, zeatin; 9RZ, zeatin riboside; DZ, dihydrozeatin; 9R-DZ, dihydrozeatin riboside; 9GZ, 9-β-D-glucopyranosylzeatin; 7GZ, 7-β-D-glucopyranosylzeatin; OGZ and OGDZ, O-β-D-glucopyranosylzeatin and -dihydrozeatin, respectively; OGZR and OGDZR, 9-ribosides of OGZ and OGDZ; LA, lupinic acid; DLA, dihydrolupinic acid; OR denotes the origin.

was further established by paper electrophoresis (Letham et al. 1975), and by degradation to BAP by both acid hydrolysis (Letham et al. 1975) and periodate oxidation. In the latter procedure, an aliquot of the eluate was evaporated and dissolved in water (70 μl) containing $NaIO_4$ (250 μg) and formic acid (2 μl). The solution was left at 30°C for 18 h and then evaporated. The residue was dissolved in a 20% (w/v) aqueous solution of cyclohexylamine (50 μl); after 4 h at 35°C, the solution was cochromatographed with BAP.

To determine the radioactivity due to BAP nucleotide, i.e., BAP riboside 5′-monophosphate, the extract was chromatographed on paper (solvent A). The small peak of radioactivity which cochromatographed with the nucleotide was eluted, hydrolyzed with alkaline phosphatase, and then rechromatographed on silica gel (solvent A). The radioactivity cochromatographing with BAP riboside (9R-BAP) was determined. To determine radioactivity due to 9RZ phosphates ("zeatin nucleotides") and adenosine phosphates, the extracts were chromatographed on silica gel (solvent A) and the fraction eluted from the zone of R_f 0.00 to 0.06 was hydrolyzed with alkaline phosphatase. The hydrolysate was then subjected to TLC on silica gel (solvent A) and the radioactivity which cochromatographed with 9RZ and adenosine was determined. The compounds responsible for the remainder of the radioactivity in the hydrolysate (nearly all of this ^3H was located at the TLC origin) were termed "unidentified polar metabolites."

Characterization of Chromatographic Fractions

Radioactivity in chromatographic zones was determined by liquid scintillation counting according to Gordon et al. (1974). TLC zones were eluted with meth-

Table 1. Radioactivity due to BAP and BAP glucosides in maturing radish cotyledon laminae during a pulse-chase experiment. The hypocotyl bases of derooted seedlings were placed in [^{3}H]BAP (15 μM) for 45 min and then transferred to unlabeled BAP (135 μM) for 24 h. Cotyledon laminae were excised at various times for extraction and TLC.

Time (h)	DPM/g tissue × 10^{-3}			
	BAP	7G-BAP	9G-BAP	3G-BAP
0.75	92.5	5.3	2.25	0.45
1.75	159.5	24.2	12.1	3.0
2.75	216.0	33.8	17.0	3.8
4.75	346.0	94.0	42.0	12.6
7.75	392.0	191.0	92.4	22.4
24.75	328.0	247.0	111.0	25.2

anol/water (1:1) to characterize the metabolites present by rechromatography or by enzymic degradation. Hydrolysis of nucleotides to nucleosides with alkaline phosphatase (Parker et al. 1978) and of cytokinin O-glucosides to aglycones with almond β-glucosidase (Letham et al. 1975) was performed according to the cited references.

Trimethylsilyl derivatives for mass spectrometry were prepared according to MacLeod et al. (1976).

Results

Kinetics of Glucoside Formation and Glucoside Stability

In a pulse-chase experiment, [^{3}H]BAP (15 μM; 600 mCi/mmol) and then unlabeled BAP (135 μM) were supplied to maturing radish cotyledons via the hypocotyl base. At the end of the 45-min period during which [^{3}H]BAP was taken up, all three glucosides of BAP were present in the cotyledon laminae (Table 1), the ratio 3G-BAP/9G-BAP/7G-BAP being 0.085:0.42:1.0. One hour later, the ratio had become 0.12:0.50:1.0, and thereafter remained essentially the same. The fact that the ratio remained constant during the chase with unlabeled BAP is consistent with the view that the three glucosides were each formed directly from BAP and that one glucoside did not arise from another by rearrangement or by transglucosylation reactions involving transfer of a glucosyl moiety from a BAP glucoside to free BAP.

^{3}H-labeled 3G-BAP, 7G-BAP, and 9G-BAP (80 μM) were each supplied to maturing radish cotyledons for 5 h. The hypocotyl bases were then placed in water for 20 h after which the cotyledon laminae were extracted. In the cases of cotyledons supplied with 7G-BAP and 9G-BAP, 98% and 99% respectively of the extracted ^{3}H was due to the unmetabolized glucoside and no free [^{3}H]BAP was detected. However, in extracts of cotyledons supplied with 3G-BAP, only 19% of the ^{3}H was attributable to unmetabolized glucoside; the remainder was due to BAP, 7G-BAP, and 9G-BAP (2.9, 47, and 32% respectively of the extracted ^{3}H). The presence of free [^{3}H]BAP was confirmed by elution of the BAP marker spot and rechromatography of the eluate on cellulose (solvent A) and on borate-impregnated silica gel (solvent D). These results

indicated that 3G-BAP slowly released free BAP, which was then probably converted into the stable 7- and 9-glucosides.

The metabolic stability of 7GZ was also assessed in radish cotyledons and seedlings. The following were placed on filter paper wetted with [^{3}H]7GZ (4.5 μM): (a) whole surface sterilized radish seeds which were then allowed to imbibe 7GZ for 24 h; (b) halved surface sterilized radish seeds which imbibed 7GZ for 48 h (the cut surface contacted the filter paper); (c) sterile germinated radish seeds (these had imbibed water for 24 h) which were then allowed to take up the 7GZ for 48 h; (d) cotyledons excised from sterile 2.5-day-old seedlings which then took up the 7GZ for 48 h. Extracts of the above seeds and cotyledons were subjected to TLC. In each case, unmetabolized 7GZ accounted for 90–95% of the extracted ^{3}H, while no other metabolites were identified. Zeatin and 9RZ, if present at all, each accounted for less than 0.2% of the extracted radioactivity. The above experiments were repeated and unlabeled zeatin (8 μM) was added to the [^{3}H]7GZ in each petri dish to act as a "cold trap." However, very similar results were obtained and no free zeatin or 9RZ were detected.

The influence of environmental factors and chemical stimuli on metabolism of 7GZ was also assessed. Maturing cotyledons which had taken up [^{3}H]zeatin (8 μM) for 12 h, and then water for 3–4 days, were subjected to the following treatments: (a) gibberellic acid (5 mg/liter) supplied via the transpiration stream for 24 h; (b) ABA (0.5 mg/liter) supplied via the transpiration stream for 24 h; (c) heat stress (44°C for 1 h); (d) exposure to a warm air current (applied for 8 h) which induced wilting. None of these treatments resulted in a detectable increase in radioactivity due to zeatin and 9RZ, both of which accounted for less than 1.5% of the extracted ^{3}H; no decrease in radioactivity due to 7GZ was found.

The possible effect of light on metabolism of 7GZ was also assessed. Roots were excised from radish seedlings (6-day-old) grown in complete darkness and [^{3}H]zeatin (40 μM) was supplied to the cotyledons via the transpiration stream for 18 h (all manipulations were done under a green safe light). During the next 3 days, the cotyledons were supplied with water only and were then exposed to red light for 5 min or to white light for 60 min. 7GZ was formed in the dark-grown cotyledons but the light treatments did not promote conversion of 7GZ to zeatin or to other metabolites.

Unlike 7GZ, OGZ was rapidly metabolized in radish cotyledons. When [^{3}H]OGZ (60 μM) was supplied to immature cotyledons for 3 days, 24% of the extracted ^{3}H cochromatographed with 7GZ, 30% with OGZ (after hydrolysis with β-glucosidase, this ^{3}H cochromatographed with zeatin), and 0.5% with zeatin. The identity of the last-mentioned radioactivity was confirmed by reversed phase TLC (solvents G and H). Formation of 7GZ was confirmed by studies in which pentadeuterium-OGZ labeled in the isoprenoid sidechain, O-β-D-glucopyranosyl-[*methyl*,4′,4′-$^2H_5$]zeatin, was supplied to immature cotyledons at 80 μM together with tracer amounts of [^{3}H]OGZ. The metabolite which cochromatographed with 7GZ was purified essentially by the method used in the original isolation of 7GZ (Parker and Letham 1973); however, the final step of paper chromatography was replaced by cellulose TLC (solvent A). The trimethylsilyl derivative of the UV-absorbing product exhibited the

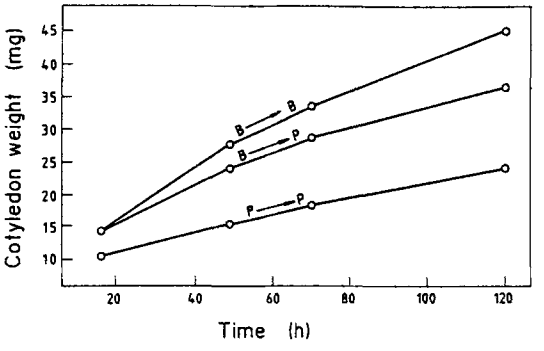

Fig. 2. The mean weight of immature radish cotyledons cultured on the various solutions listed below: P → P, phosphate buffer for 18 h and then transferred to fresh phosphate solution; B → B, [^{3}H]BAP (13 μM, in phosphate buffer) for 18 h and then transferred to fresh [^{3}H]BAP in buffer solution; B → P, [^{3}H]BAP (13 μM) for 18 h and then transferred to phosphate buffer.

following electron-impact mass spectrum, which was scanned over the *m/z* ranges 600 to 760 and 400 to 460: 746 (M^+, 28%), 731 (18%), 657 (90%), 656 (19%), 641 (100%), 610 (54%), 450 (22%), and 411 (15%). This partial spectrum is in accord with that of [$^2H_5$]7GZ and contains ions which are diagnostic for this compound. Accordingly, the major metabolite of OGZ was identified as 7GZ.

Cytokinin Metabolism in Relation to Cotyledon Expansion

Possible relationships between cytokinin metabolism and radish cotyledon expansion were considered in studies of differences in metabolite levels caused by: (1) transfer from BAP-containing to BAP-free media; (2) culture on cytokinin solutions of greatly differing concentrations.

Excised immature radish cotyledons were cultured in petri dishes for 18 h on phosphate buffer or on this buffer containing [^{3}H]BAP (13 μM; 25 mCi/mmol). All cotyledons were then rinsed with distilled water, drained on filter paper, and transferred to fresh solution. For the former cotyledons and one half of the latter, this solution was phosphate buffer; the remaining half of the BAP-treated cotyledons was transferred to fresh [^{3}H]BAP. Transfer from BAP solution to buffer lacking BAP markedly reduced the growth rate of the cotyledons (Fig. 2). Although the amounts of 3G-BAP, 7G-BAP, and 9G-BAP per cotyledon remained constant after this transfer, the levels of free BAP, 9R-BAP, and BAP nucleotide declined rapidly and markedly (Fig. 3). In cotyledons supplied continuously with BAP, the only metabolites which increased in amount per cotyledon after 48 h were 7G-BAP and 9G-BAP (Fig. 3).

A further cotyledon transfer experiment was performed identical to that described above except that zeatin (9 μM) replaced BAP. Radioactivity in 7GZ, zeatin, and 9RZ was monitored. The results obtained were analogous to those found in the experiment with BAP; transfer to zeatin-free media was accompanied by a reduction in growth rate, a rapid decline in radioactivity due to zeatin and 9RZ to essentially zero levels, but the level of 7GZ per cotyledon remained nearly constant.

^{3}H-labeled BAP (25 mCi/mmol) and zeatin were supplied for 45 h to immature cotyledons over a wide range of concentrations. The lowest tested concentrations of BAP and zeatin were 0.3 and 0.04 μM, respectively, while the

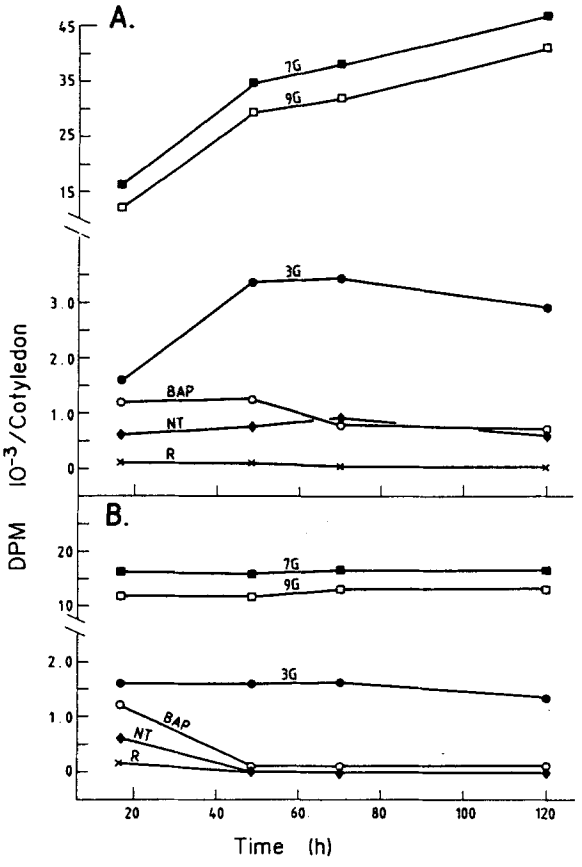

Fig. 3. The radioactivity due to BAP metabolites and free BAP in immature radish cotyledons cultured continuously on [^{3}H]BAP (A), or on [^{3}H]BAP for 18 h and then on the same phosphate solution without [^{3}H]BAP(B). 7G, 7G-BAP; 9G, 9G-BAP; 3G, 3G-BAP; R, 9R-BAP; NT, BAP nucleotide.

respective optimal concentrations for cotyledon growth were 30–120 μM and greater than 100 μM (Figs. 4 and 5). Increase in the concentration of BAP markedly elevated the ^{3}H extracted per unit weight of tissue (Fig. 4), but had no appreciable effect on the contributions of 7G-BAP, 9G-BAP, and 3G-BAP to metabolite radioactivity. At all concentrations tested, free BAP accounted for only 1–2% of the extracted ^{3}H. When the zeatin concentration was increased over the range 0.04 to 4.0 μM, uptake was promoted markedly but the contribution of 7GZ to extracted radioactivity increased only from 20 to 26% (Fig. 5). However, at higher concentrations, and especially at 100 μM, a considerably greater proportion of extracted ^{3}H was due to 7GZ. Relatively little ^{3}H cochromatographed with 9GZ and OGZ at any concentration (Fig. 5), while free zeatin and 9RZ each accounted for less than 1% of the extracted ^{3}H at concentrations of 0.4 μM and above.

Zeatin was also supplied for 21 h to maturing cotyledons, via the seedling hypocotyl base, at two widely differing concentrations, 4 and 100 μM. This difference did not appreciably alter the percentage of metabolite radioactivity due to 7GZ (4 μM, 31.0%; 100 μM, 31.9%) in the laminae, but the radioactivity that cochromatographed with OGZ increased markedly, from 7.9% to 14.8%, with the increase in zeatin concentration. The identification of this radioac-

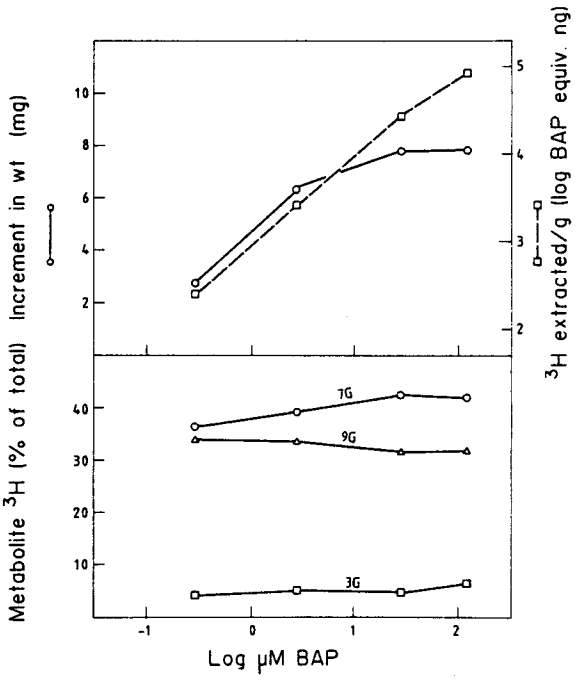

Fig. 4. Radioactivity extracted from immature radish cotyledons supplied with various concentrations of [^{3}H]BAP, the contributions of metabolites to this radioactivity, and the increments in cotyledon weight induced by BAP. 7G, 7G-BAP; 9G, 9G-BAP; and 3G, 3G-BAP.

tivity as ^{3}H-labeled OGZ was confirmed by the following evidence: (a) cochromatography with authentic OGZ on silica gel (solvent B) and borate-impregnated cellulose and silica gel (solvent D); (b) hydrolysis with β-glucosidase to a ^{3}H-labeled compound which cochromatographed with zeatin (reversed phase TLC, solvents G and H; cellulose TLC, solvent F). OGZ has not been reported previously as a metabolite of zeatin in radish cotyledons.

Cytokinin Metabolism in Relation to Cotyledon Senescence

The metabolism of cytokinins (BAP, zeatin, and 9RZ) in senescent cotyledons was compared with that in nonsenescent ("maturing") cotyledons. BAP (4.4 μM) was supplied via the bases of the petioles for 24 h to maturing cotyledons and to senescing cotyledons of two types: "early senescent" (cotyledons light green, slight yellowing) and "fully senescent" (yellow to greenish yellow). While the glucosides of BAP collectively made similar contributions to metabolite radioactivity in senescent and nonsenescent cotyledon laminae, their relative proportions differed considerably between the two types of cotyledons (Table 2). In the nonsenescent cotyledons, 7G-BAP was the dominant glucoside, but in the senescent cotyledons, the 7- and 9-glucosides were present in very similar proportions while 3G-BAP was barely detectable.

The metabolism of zeatin was compared in maturing cotyledons and in cotyledons showing early senescence when the hormone was supplied, via the bases of the petioles, at 4 μM for 24 h. The percentages of metabolite radioactivity due to 7GZ, OGZ, and adenosine in the former cotyledon laminae were 30.1, 7.6, and 3.8%, respectively, while for the latter the corresponding

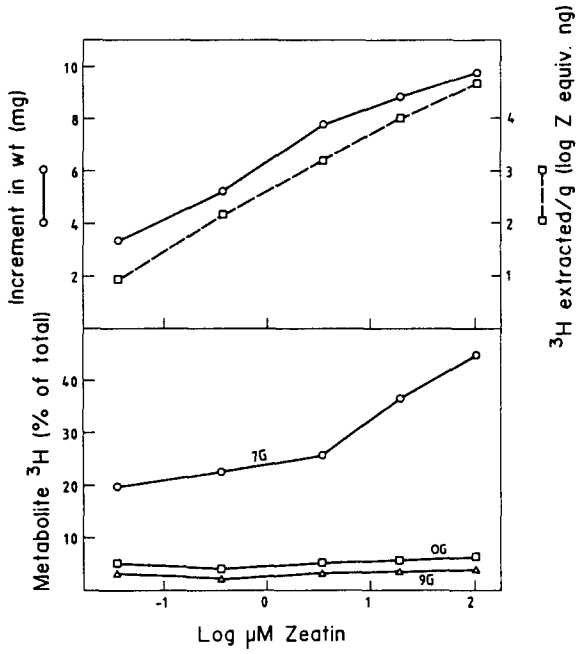

Fig. 5. Radioactivity extracted from immature radish cotyledons supplied with various concentrations of [^{3}H]zeatin, the contributions of metabolites to this radioactivity, and the increments in cotyledon weight induced by zeatin. 7G, 9G, and OG denote 7-, 9-, and O-glucosides of zeatin, respectively.

Table 2. The radioactivity due to BAP glucosides in extracts of the laminae of senescent and nonsenescent radish cotyledons supplied with [^{3}H]BAP. The bases of the petioles were placed in [^{3}H]BAP (4.4 μM) for 24 h.

	Radioactivity (% of total due to extracted metabolites)		
Cotyledon type[a]	3G-BAP	7G-BAP	9G-BAP
Nonsenescent (11 days)	7.9	42.4	29.6
Early senescent (21 days)	2.4	38.4	39.2
Fully senescent (27 days)	<1.0	36.5	38.8

[a] Age of seedlings from which excised is given in parentheses.

percentages were 21.8, 8.4, and 13.8%, respectively. Hence senescence was associated with a decline in conversion to 7GZ and an increase in production of adenosine.

When 9RZ (1 μM) was supplied to senescent and to nonsenescent cotyledons via the bases of the hypocotyls for 48 h, these changes in conversion to 7GZ and adenosine were also prominent (Table 3). In addition, appreciably more nucleotide metabolites (phosphates of adenosine and 9RZ) were formed in senescent laminae than in nonsenescent; however, the unidentified polar metabolites (see Methods) contributed similarly to radioactivity in both types of

Table 3. Radioactivity attributable to various metabolites in methanol extracts of laminae of senescent and nonsenescent cotyledons supplied with [^{3}H]9RZ. The riboside (1 μM) was supplied for 48 h via the bases of the hypocotyls.

Cotyledon type	DPM extracted with 80% MeOH		Radioactivity due to metabolites (% of total extracted with MeOH)						
	DPM/mg tissue	% Total extracted[a]	9RZ	Adenosine	7GZ	OGZ	A-P[b]	9RZ-P[b]	Unidentified polar metabolites[c]
Fully senescent	212	68	13.2	9.0	6.9	1.1	5.1	0.9	9.0
Early senescent	307	73	12.2	8.5	12.1	1.4	3.3	0.6	6.2
Non senescent	243	81	10.5	5.5	20.3	2.2	1.2	0.3	6.1

[a] The total radioactivity is the sum of radioactivity extracted by 80% MeOH and then NCS tissue solubilizer.
[b] A-P and 9RZ-P denote phosphates of adenosine and 9RZ, respectively.
[c] See Methods.

laminae. Expressed as a percentage of the total recovered, the radioactivity extracted by NCS solubilizer from senescent laminae exceeded that derived from nonsenescent laminae (Table 3). Metabolites in the petioles of the cotyledons were also examined. Per unit weight of tissue, the amounts of radioactivity extracted from petioles, and laminae with 80% methanol were similar for particular types of cotyledons. However, relative to laminae, petioles yielded much greater radioactivity per unit weight of tissue (5.7, 6.8, and 11.0 fold for fully senescent, early senescent, and nonsenescent cotyledons, respectively) when subjected to subsequent extraction with NCS. For the petioles of fully senescent, early senescent, and nonsenescent cotyledons, the total radioactivity extracted per milligram of tissue and, in parentheses, the percentage of this extracted with NCS were: 840 dpm (67%), 1076 dpm (73%), and 811 dpm (78%), respectively. In nonsenescent petioles, the dominant metabolite of 9RZ extracted with 80% methanol was 7GZ (23% of extracted ^3H), but in early senescent petioles, adenosine phosphates, unidentified polar metabolites, and adenosine (13, 13, and 12%, respectively, of ^3H extracted with methanol) made the major contributions to extract radioactivity.

In similar experiments, 9RZ (6 μM) was supplied to nonsenescent, early senescent, and fully senescent cotyledons for 24 h via the petiole bases. 7GZ was the dominant metabolite only in nonsenescent laminae (19% of metabolite radioactivity), whereas adenosine (17–21% of metabolite ^3H) was the principal metabolite in the two types of senescent laminae.

In these experiments in which 9RZ was supplied to cotyledons, an unidentified metabolite was detected which was more prominent in senescent cotyledons than in nonsenescent. In the former, this unknown metabolite and 7GZ made equal contributions to metabolite ^3H. The unknown metabolite cochromatographed with 7GZ during silica gel TLC (solvents A and B) but exhibited an R_f greater than that of 7GZ during TLC on cellulose with solvent A (R_f relative to 7GZ, 1.21). This is the chromatographic behavior which would be expected of the 7-glucoside of dihydrozeatin.

Discussion

Compared with many tissues, radish cotyledons metabolize cytokinins in a relatively simple manner (Entsch et al. 1980). N-glucosylation to give the 3-, 7-, and 9-glucosides is dominant and isoprenoid sidechain cleavage to give adenine and its derivatives is a minor form of metabolism. The reported kinetic study of BAP glucoside formation in radish cotyledons indicates that each glucoside is formed directly from BAP. Transglucosylation reactions in which glucose is transferred enzymically from one glucoside to free BAP to yield a different glucoside do not appear to occur, although such transfer reactions are important in formation of certain glucosides of other types, e.g., coumarin glucosides (Letham 1978). Similarly, rearrangement of one BAP glucoside to yield a second glucoside was not detected.

While the 7- and 9-glucosides of BAP exhibit high stability in maturing radish cotyledons, the 3-glucoside of BAP is readily converted into the 7- and 9-glucosides, and this probably occurs via free BAP which was identified as a metabolite of 3G-BAP. However, some direct enzymic conversion of 3G-BAP

to 9G-BAP cannot be excluded. Heating to high temperatures can cause conversion of 3-substituted adenines to 9-substituted adenines (Letham et al. 1975), but such rearrangement is unlikely to have contributed to the conversion of 3G-BAP to 9G-BAP in the present study. Release of appreciable amounts of free BAP from 3G-BAP, but not from 7G-BAP and 9G-BAP, probably accounts for the high activity of the 3-glucoside and the very weak activity of the 7- and 9-glucosides in cytokinin bioassays. In early studies (Parker and Letham 1973), 7GZ was found to exhibit great stability in maturing radish cotyledons but no critical attempt was made to detect released free zeatin. In the present study, radish seeds were germinated in the presence of [^{3}H]7GZ, which was also supplied to germinated radish seed and to excised immature cotyledons. Free [^{3}H]zeatin and [^{3}H]9RZ were not detected in any of these experiments and little of the 7GZ supplied was metabolized. This further establishes the metabolic stability of cytokinin 7-glucosides in radish cotyledons and seedlings. However, [^{3}H]OGZ was converted into 7GZ in immature cotyledons. This conversion could proceed by two routes: (1) hydrolysis of OGZ to yield free zeatin which was then converted into 7GZ; (2) conversion of OGZ into its 7-glucoside, the O-glucosyl moiety of which was subsequently hydrolyzed to give 7GZ. Two observations support the former route. First, a minor metabolite of OGZ was detected which exhibited the chromatographic properties of zeatin. Second, OGZ is very active in the radish cotyledon bioassay which utilizes immature radish cotyledons (Letham et al. 1983). This activity can be explained by release of free zeatin from OGZ as proposed under (1), but not by the occurrence of route (2) in which all metabolites are 7-glucosides. OGZ is unlikely to be active *per se* in cytokinin bioassays and 7-glucosides exhibit only feeble cytokinin activity (Letham et al. 1983).

In the present study, OGZ was identified as a metabolite of zeatin in maturing radish cotyledons, but contributed appreciably to metabolite radioactivity only when zeatin was supplied at high concentrations. OGZ was not formed to a similar degree as a metabolite of zeatin in immature cotyledons and appreciable O-glucosylation is, therefore, a form of metabolism associated with cotyledon maturity. OGZ was not identified in our original studies of zeatin metabolism in maturing radish cotyledons which showed that 7GZ was the major metabolite. However, in extracts of cotyledons supplied with zeatin, cytokinin activity was detected after chromatography (see Fig. 6 of Parker and Letham 1973) which cannot be attributed to 7GZ. This glucoside is essentially inactive in the soybean callus bioassay used (Letham et al. 1983). The activity detected in this earlier work was probably partly due to OGZ.

Cytokinins are very effective retardants of leaf senescence in diverse species, yet there is only one previous comparative study (Letham et al. 1977) of cytokinin metabolism in senescent and nonsenescent leaves. In senescent leaves of *Populus nigra*, the dominant metabolites of [^{3}H]9RZ were compounds which lacked a ribose moiety, in particular, OGZ and its dihydro derivative. However, in expanded but nonsenescent leaves, the ribosides of these compounds were the principal metabolites. Cytokinin deribosylation is thus a form of metabolism associated with senescence in this *Populus* species. In the present study, senescent and nonsenescent radish cotyledons were found to exhibit differences in cytokinin metabolism. Two differences in BAP metabolism were evident. First, in senescent cotyledons, 3G-BAP, the BAP glucoside

with high cytokinin activity, was not formed in appreciable amounts as a metabolite of BAP, but was a minor metabolite in nonsenescent cotyledons. Second, the ratio of 7G-BAP/9G-BAP for senescent cotyledons (0.94) differed appreciably from the ratio found for nonsenescent cotyledons (1.43). In radish cotyledons, two enzymes from both 7- and 9-glucosides of BAP, but the product ratios differ markedly. One enzyme, which elutes from DEAE-cellulose by low salt concentrations, forms the 7-glucoside predominantly, while the other enzyme, which elutes at higher salt concentrations, produces the two glucosides in similar proportions (Entsch and Letham 1979). In senescent radish cotyledons, the relative activity of the former enzyme may have declined markedly. Differences were also detected between senescent and nonsenescent cotyledons in the metabolism of zeatin and 9RZ. 7GZ was dominant as a metabolite of zeatin and 9RZ only in nonsenescent cotyledons, while adenosine and adenine nucleotides were much more prominent metabolites in senescent cotyledons than in nonsenescent. Hence cotyledon senescence is associated with enhanced isoprenoid sidechain cleavage which irreversibly inactivates cytokinin. A large proportion (over 70%) of the radioactivity extracted from both senescent and nonsenescent petioles, but not from laminae, was derived by treatment with NCS tissue solubilizer. This ^{3}H could be derived from RNA since NCS is strongly basic.

In this contribution, cytokinin metabolism has been studied in radish cotyledons in relation to cytokinin induced cotyledon expansion, in an attempt to provide information regarding the physiological significance of particular metabolites. Although cytokinin metabolism has been studied in numerous tissues which respond to cytokinin, there is only one previous investigation which was designed so that deductions could be made regarding the significance of metabolites. From several studies of cytokinin metabolism in tobacco cell cultures, Laloue and coworkers obtained evidence that 7-glucosides are not detoxification products of supplied cytokinin and that cytokinin bases may be the active form (Gawer et al. 1977; Laloue and Pethe 1982).

A particular cytokinin metabolite could function in one or more of several ways which are listed below and then discussed in relation to cytokinin metabolites formed in radish cotyledons, especially cytokinin glucosides. A metabolite of cytokinin, or of any plant hormone for that matter, could be: (a) a detoxification product formed when the amount of exogenous hormone is so high as to be toxic; (b) an inactivation product, formation of which is coupled with hormone action; (c) a deactivation product formed to lower normal physiological hormone levels; (d) an active form, i.e., a molecular species which binds to a site of action to evoke a growth or physiological response; (e) a storage form which would release free active hormone when required; (f) a translocation form in which the hormone moves from one tissue to another. In radish cotyledons, formation of 3-, 7-, and 9-glucosides occurs when the cytokinin concentration is far below an optimal concentration (see Figs. 4 and 5); indeed 7GZ is still the dominant metabolite of zeatin when the latter is supplied at a thousandth of the optimal concentration. Hence, 3-, 7-, and 9-glucosides are not simply detoxification products of cytokinin in radish cotyledons. In these cotyledons, certain inactive cytokinin analogues are also converted to 7- and 9-glucosides (Letham et al. 1982); one of these, 3,4-dimethoxybenzylaminopurine, does not suppress the activity of BAP in the radish cot-

yledon cytokinin bioassay (Letham et al. 1982) and presumably does not bind to the functional site. Hence cytokinin 7- and 9-glucosides are not inactivation products formed as a consequence of cytokinin action (proposal b above). Furthermore, cytokinin 3-, 7-, and 9-glucosides are not responsible for radish cotyledon expansion evoked by supplied cytokinin, since transfer of cotyledons from BAP solution to a BAP-free medium causes the growth rate to decline markedly, but the levels of the three glucosides of BAP remain essentially constant (Figs. 2 and 3). However, the levels of free BAP, 9R-BAP, and BAP nucleotide decline rapidly to almost zero levels. This suggests that these three compounds, but not BAP glucosides, could be active forms in the sense used under (d) above. No experimental evidence was obtained in the present study which would substantiate or invalidate the remaining three possibilities, i.e., (c), (e), and (f) above. However, when radioactive 7GZ was applied to fully expanded cotyledons of intact radish seedlings, a small proportion was transported to the root (Letham et al. 1982). In intact radish seedlings, 7GZ may be both a translocation and storage form of zeatin. This will be discussed in the next paper of this series which deals with cytokinin metabolism in intact radish seedlings.

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