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Inhibitors of Cytokinin Metabolism III. The Inhibition of Cytokinin *N*-Glucosylation in Radish Cotyledons[1]

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Abstract. Cytokinins are deactivated in radish cotyledons by conversion to 7- and 9-glucosides. In a search for inhibitors of this metabolism, the following compounds were found to be effective: (a) 6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine; (b) 3-isobutyl-1-methylxanthine; (c) papaverine; (d) theophylline; (e) caffeine; and (f) theobromine. The order of effectiveness was: (a) > (b) = (c) > (d) = (e) = (f). While the methylxanthines (b) and (d) inhibited formation of both 7- and 9-glucosides of 6-benzylaminopurine (BAP) approximately equally, compounds (a) and (c) preferentially inhibited formation of BAP 9-glucoside. Inhibition of BAP glucoside formation by (a) at 1.3 mM elevated the level of free BAP and BAP nucleotide 23- and 94-fold, respectively. While abscisic acid (ABA) suppressed conversion of zeatin riboside to zeatin 7-glucoside in radish cotyledons, it did not inhibit conversion of BAP to glucosides. Hence, ABA probably does not inhibit the glucosylating enzymes directly but rather reduces the availability of free zeatin when zeatin riboside is supplied. Auxins and nutrient supply did not affect conversion of zeatin riboside to zeatin 7-glucoside. Relative to cotyledons developed in light, those developed in darkness had a reduced capacity to convert zeatin riboside to zeatin 7-glucoside. The results presented have identified types of chemical structures which could be developed to provide more effective inhibitors of cytokinin *N*-glucosylation.

Cytokinins are deactivated by two types of *N*-conjugation, namely, 7- and 9-glucosylation, and alanine conjugate formation (Letham and Palni 1983). In previous studies, inhibitors of the purified enzymes involved were developed (Parker et al. 1986). A diaminopurine, 6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine (I), was found to be an effective inhibitor of the principal glucosylating enzyme from radish cotyledons (cytokinin 7-glucosyl transferase, EC 2.4.1.118), while certain auxins and urea derivatives inhibited β-(9-cytokinin)alanine synthase (EC 4.2.99.13). In excised radish cotyledons, 6-benzylaminopurine (BAP) is *N*-glucosylated to give the 3-, 7-, and 9-glucosides, termed [3G]BAP, [7G]BAP, and [9G]BAP, respectively. In this tissue [3G]BAP may be converted back to BAP, so it cannot be considered a deactivation product (Letham and Gollnow 1985). Zeatin (Z) and zeatin derived from exogenous zeatin riboside ([9R]Z) yields only one *N*-glucoside in appreciable amounts, 7-glucopyranosylzeatin ([7G]Z). In this paper, the ability of I to inhibit 7- and 9-glucosylation of cytokinins in excised radish cotyledons is assessed. A number of other heterocyclic compounds and plant hormones were also assessed for inhibitory activity. Our aim was to recognize new types of inhibitors of cytokinin *N*-glucosylation to provide a basis for synthesis of more potent inhibitory compounds. Because our ultimate objective was to inhibit cytokinin inactivation in plant tissue and thus influence growth and senescence, the potential inhibitors were tested using excised cotyledons rather than with the purified glucosylating enzyme.

[1] For part II in the series "Inhibitors of cytokinin metabolism" see Zhang et al. (1989).

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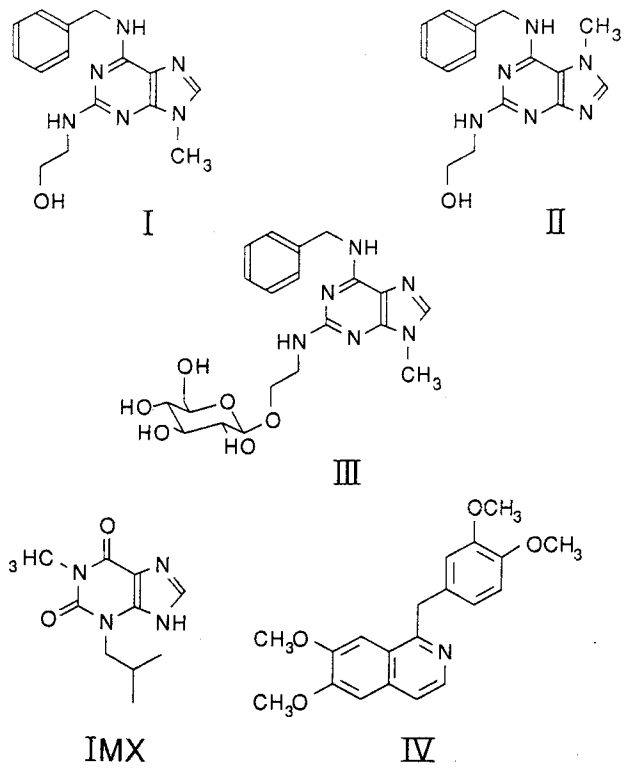

Materials and Methods

Growth of Radish Cotyledons and Assay of Glucosylation Inhibitors

Radish (*Raphanus sativus*, cv. Long Scarlet) seeds were washed in running tap water overnight, surface sterilized with sodium hypochlorite solution (2 g/L available chlorine) for 5 min, rinsed with sterile water, and then allowed to germinate on sterile agar (10 g/L) in petri dishes for 24 h. Uniformly germinated seeds were then selected and placed on sterile media (Murashige and Skoog 1962) modified by replacement of NH_4NO_3 and KNO_3 with *L*-asparagine (300 mg/L). The seedlings were then allowed to develop for 6–7 days (day length 16 h, 300 $\mu Em^{-2}\ s^{-1}$; day/night, 25°/20°C); the cotyledons were then excised and placed in petri dishes (diameter 9 cm), containing one circle of Whatman No. 1 paper and 3 ml of solution. For testing of potential inhibitors of glucosylation, solutions of the chemicals were prepared in 2 mM potassium phosphate, pH 5.9; for the studies concerning abscisic acid (ABA) and other hormones, as well as the effect of light on [9R]Z metabolism, all solutions contained the modified Murashige and Skoog nutrients mentioned above. Unless stated otherwise, excised cotyledons were incubated under continuous weak light (7 $\mu Em^{-2}\ s^{-1}$) at 22°C for 4 days.

The specific activity of radioactive cytokinins used were as follows: zeatin riboside, [8-^{3}H][9R]Z, 250 mCi/mmol; and 6-benzylaminopurine, [G-^{3}H]BAP, 20 mCi/mmol.

Preparation of Extracts and Chromatography

Extracts of cotyledons were prepared by the extraction method detailed previously (Tao et al. 1983). Metabolites of BAP, the riboside ([9R]BAP) and BAP glucosides, were separated by two-dimensional thin-layer chromatography (2D TLC) on silica gel as described previously (Tao et al. 1983). Chromatogram zones containing added unlabeled markers were eluted with water for liquid scintillation counting (Gordon et al. 1974). To determine radioactivity due to BAP nucleotides, the zone near the origin and at the location of [9R]BAP 5′-monophosphate after chromatography in the first dimension (*n*-butanol/14 N ammonia/water, 6:1:2 by volume, upper phase, solvent A) was eluted with 50% methanol and the eluted fraction was treated with alkaline phosphatase (Parker et al. 1978). The hydrolysate was chromatographed on silica gel (solvent A) and the ^{3}H cochromatographing with [9R]BAP was determined.

Confirmation of the identity of radioactivity attributed to free BAP, [9R]BAP, and [9R]BAP derived from BAP nucleotides, was provided by rechromatography in additional systems. Furthermore, [^{3}H][9R]BAP was converted by acid hydrolysis (0.5 N HCl, 95°C for 2 h) to a compound which cochromatographed with authentic BAP. Free [^{3}H]BAP was converted to an allyl derivative which cochromatographed with authentic 3-allyl-BAP (Tao et al. 1983).

Extracts of cotyledons supplied with [^{3}H][9R]Z were mixed with authentic unlabeled markers and subjected to 2D TLC on silica gel (*n*-butanol/acetic acid/water 12:3:5 by volume, solvent B, for first dimension; solvent A for second) to determine the percentage of total radioactivity due to Z, [9R]Z, [7G]Z plus *O*-glucosyl zeatin [(OG)Z], adenine (Ade), and adenosine (Ados). (OG)Z and [7G]Z cochromatographed in this 2D TLC system but were resolved by elution of the mixed marker spot with 50% ethanol and rechromatography on cellulose (solvent A). The presence of (OG)Z was confirmed by β-glucosidase hydrolysis and rechromatography. When the cotyledon extracts were subjected to chromatography using a bilayer 2D TLC system (Letham and Gollnow 1985), the percentages of radioactivity found for the various metabolites were similar to those determined by the procedure outlined above. Eluates of the 2D TLC Z and [9R]Z spots, derived from extracts of cotyledons supplied with [^{3}H][9R]Z in the presence of some inhibitors, were rechromatographed using TLC systems (Parker et al. 1978) which separate Z from dihydrozeatin (DZ) and [9R]Z from dihydrozeatin riboside [9R]DZ. This indicated that the dihydro compounds did make some contribution (about 30%) to the eluted radioactivity.

Purification of the Glucoside Metabolite (III) Derived from I

Radish cotyledons were supplied with I (1 mM) for 3–4 days under the conditions used for assay of glucosylation inhibitors. When the cotyledon extract was subjected to 2D TLC on silica gel (solvent A followed by solvent B), two components which were not normally present in cotyledon extracts were readily visualized under UV light. The component of high R_f cochromatographed with authentic I and was eluted from the 2D chromatograms with 50% ethanol for mass spectrometry. To purify the polar compound (III, R_f values in solvents A and B, 0.27 and 0.39, respectively) from the extract, the following three preparative TLC purifications were performed sequentially: (1) TLC on silica gel, solvent B; (2) TLC on silica gel, solvent A; and (3) reverse-phase TLC on Merck HPTLC RP8 plates (solvent 40% acetonitrile), the compound (III, R_f 0.26) being eluted with 80% acetonitrile which was also used to wash the layers before use.

Table 1. Radioactivity attributable to Z and its metabolites in extracts of radish cotyledons supplied with [^{3}H][9R]Z in the presence and absence of inhibitors of metabolism.

Inhibitor	Radioactivity due to metabolites (% of total extracted)					
	[7G]Z	(OG)Z	Z + DZ	[9R]Z + [9R]DZ	Ade	Ados
None	33.6	1.2	0.2	1.0	1.1	1.8
IMX, 5.0 mM	6.9	2.4	4.1	3.2	2.0	2.9
IMX, 1.0 mM	16.1	7.1	0.8	2.4	2.2	4.6
I, 1.0 mM	19.7	1.5	1.7	2.7	3.1	6.5
Caffeine, 5 mM	19.4	12.5	2.2	2.4	8.3	5.5
Theophylline, 5 mM	22.8	3.2	1.8	2.2	4.3	6.3
Theobromine, 5 mM	19.8	6.5	1.0	1.7	3.5	4.0

[^{3}H][9R]Z was supplied at 5 μM for 4 days.

Purification of 7-Glucosyladenine ([7G]Ade)

Excised radish cotyledons (11 g) were incubated in petri dishes with adenine (5 mM for 4 days). The water-soluble fraction of the cotyledon extract was purified on a column of cellulose phosphate (Badenoch-Jones et al. 1984) and the eluted fraction was subjected to TLC on cellulose (solvent B). The zone with intense UV absorption at R_f 0.22 was eluted; crystallization from water-isopropanol yielded [7G]Ade identical to 7-β-D-glucopyranosyladenine prepared according to Cowley et al. (1978). Identity was established by TLC, GC-MS of the trimethylsilyl (TMS) derivative, and the CI mass spectrum (CI-MS). Mass spectral characteristics are given below (principal ions, relative intensity in parentheses). The electron-impact mass spectrum (EI-MS) of the penta-TMS derivative (taken during GC): *m/z* 657 (M^+, 4%), 642 (6%), 450 (39%), 361 (67%), 280 (49%), 271 (54%), 217 (100%); CI-MS: positive ion *m/z* 298 (MH^+), negative ion *m/z* 296 ($[M-H]^-$).

The [7G]Ade was used for cocrystallization with an unknown metabolite of [9R]Z.

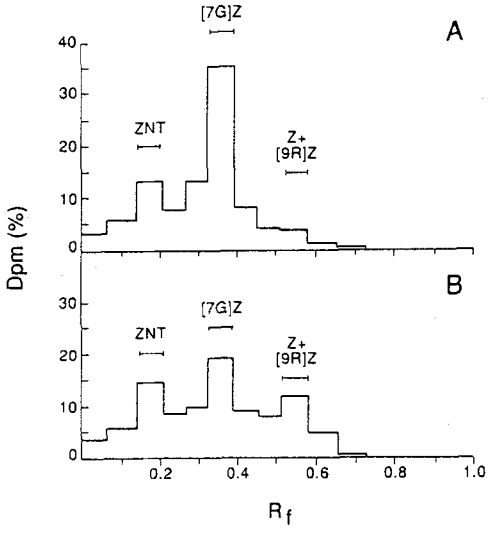

Fig. 1. Chromatography (1D TLC; solvent A) of radioactivity extracted from radish cotyledons incubated for 4 days with [^{3}H][9R]Z. **(A)** Without compound I; **(B)** with compound I (1.0 mM). ZNT denotes zeatin nucleotide, [9R]Z-5′-monophosphate.

Results and Discussion

Inhibition of Glucosylation by Compound I

The ability of I and the 7-methly isomer (II) to inhibit formation of [7G]Z from [^{3}H][9R]Z was examined first. [9R]Z was chosen because it is the principal cytokinin with high activity in radish cotyledons (Tao and Letham, unpublished observations). As in studies with a purified glucosylating enzyme from radish cotyledons (Parker et al. 1986), compound I inhibited 7-glucosylation markedly (Table 1), while II was relatively ineffective as an inhibitor (data not shown). The inhibition of 7-glucoside formation by I was accompanied by an elevation in the proportion of radioactivity due to Z plus DZ, Ade, and Ados. These results (Table 1) are based on 2D TLC and are reflected in the distribution of radioactivity after 1D TLC (Fig. 1). These 1D chromatograms did not suggest that I altered the proportion of low R_f polar metabolites which would include nucleotides. Hence, such metabolites were not considered in the present studies.

Compound I also markedly altered the metabolism of [^{3}H]BAP in radish cotyledons and this effect was clearly evident in the distribution of ^{3}H after 1D TLC (Fig. 2). The proportion of ^{3}H at the R_f of [7G]BAP was reduced markedly by I, while that at the R_f of BAP and the BAP nucleotide were increased greatly. However, only a portion of the latter radioactivity was actually due to the BAP nucleotide as determined by phosphatase hydrolysis and subsequent cochromatography with [9R]BAP. The radioactivity due to particular metabolites following incubation of cotyledons with [^{3}H]BAP in the presence and absence of I is detailed in Table 2. The radioactivity due to all metabolites except the BAP nucleotides was determined by 2D TLC. At

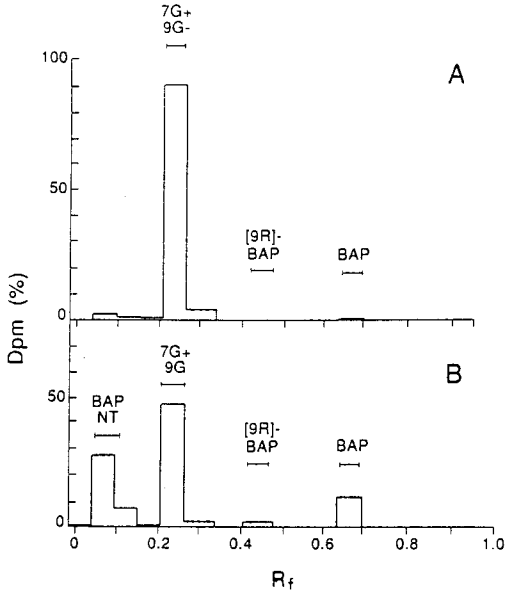

Fig. 2. Chromatography (1D TLC; solvent A) of radioactivity extracted from radish cotyledons incubated for 4 days with [^{3}H]BAP. **(A)** Without compound I; **(B)** with compound I (1.3 mM). BAP NT denotes BAP nucleotide, [9R]BAP-5′-monophosphate. 7G and 9G denote 7- and 9-glucosides of BAP.

1.3 mM (a saturated solution), I reduced the percentage of ^{3}H due to total glucosides ([3G]BAP + [7G]BAP + [9G]BAP) from 90.9% to 48.7%, while ^{3}H due to free BAP and the BAP nucleotide were increased 23- and 94-fold, respectively. In the presence of I at 1.3 mM, the ratio of [^{3}H][7G]BAP to [^{3}H][9G]BAP was increased 2.7-fold. Compound I at 0.04 mM did not reduce the percentage of ^{3}H due to [7G]Z, but did lower that attributable to [9G]BAP (Table 2). Thus over the concentration range 0.04–1.3 mM, I preferentially inhibited formation of [9G]BAP (note ratio data in Table 2). This preferential effect can be explained by the observation that radish cotyledons contain two enzymes which glucosylate cytokinins but form 7- and 9-glucosides in markedly different proportions (Entsch and Letham 1979). For one enzyme (A), the ratio of [7G]BAP/[9G]BAP formed is about 10, while the ratio is 1.5 for the other (enzyme B) which was the enzyme studied in detail (Entsch et al. 1979). From studies of substrate binding to the latter enzyme, I was proposed as an inhibitor. The ability of I to cause preferential inhibition of formation of [9G]BAP can be explained by preferential inhibition of enzyme B.

On 2D TLC of extracts of cotyledons supplied with I, two components which were not normal constituents of the cotyledons could be visualized under UV light and these were purified chromatographically for identification. The identity of the less polar compound, which cochromatographed with I on TLC, was confirmed as unmetabolized I by the EI-MS. The molecular weight of the polar compound was determined by the CI-MS to be 460. This substance exhibited R_f values similar to those of [7G]Z during 2D TLC. The EI-MS of the underivatized compound exhibited ions only at *m/z* 298, 280, 279, 267, and 254, which are indicative of an intact I moiety. The EI-MS of the TMS derivative of the polar compound is shown in Fig. 3. The ions *m/z* 297, 281, 280, 267, and 254 are attributable to an intact moiety of I and cleavage within the substituent at the 2-position of the purine ring. The molecular ion, *m/z* 748, and the ions *m/z* 451, 450, 361, 305, 217, 204 are indicative of a silylated hexose residue (MacLeod et al. 1976). The high ratio of fragment ions *m/z* 204:217 suggests that this is a hexopyranoside (see Morris 1977 and references therein). Treatment of the polar compound with β-glucosidase followed by 2D TLC and elution, as above, afforded I which was identified by TLC and EI-MS. Thus, the polar compound was assigned structure III, the *O*-β-D-glucoside of I.

The glucosyl transferase enzyme purified from radish cotyledons (enzyme B, above) did not glucosylate I and formed only a 7-glucoside of the related compound 6-benzylamino-2-(2-hydroxyethylamino)purine which lacks a 9-methyl group. Formation of a glucoside of I is due to an unknown glucosylating enzyme and may result in inactivation of I as an inhibitor of cytokinin *N*-glucosylation.

Inhibition by Methylxanthines and Papaverine

In an endeavor to find additional types of compounds which would inhibit *N*-glucosylation of cytokinins, several substances with a fused bicyclic ring system and a "cytokinin-type" side chain were tested in the radish cotyledon system. As a result, 3-isobutyl-1-methylxanthine (IMX) and papaverine, 1-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline (IV), were recognized as compounds which inhibited 7- and 9-glucoside formation.

IMX at 5 mM markedly inhibited formation of [7G]Z from [9R]Z (Table 1) and of [7G]BAP and [9G]BAP from BAP (Table 2). It was much more effective than the related methylated xanthines, theophylline, caffeine, and theobromine. Inhibition of *N*-glucoside formation from BAP by these methylated xanthines and by IMX resulted in elevated levels of free BAP and enhanced formation of the BAP nucleotide. When [^{3}H][9R]Z was supplied, the methylated xanthines caused increases in the percentages of ^{3}H due to Z and to the metabolites of Z,

Table 2. Radioactivity attributable to BAP and its metabolites in extracts of radish cotyledons supplied with [^{3}H]BAP in the presence and absence of inhibitors of metabolism.

	Radioactivity due to metabolites (% of total extracted)							
Inhibitor	[7G]BAP	[9G]BAP	[3G]BAP	Total G[a]	[9R]BAP	BAP	Polar metabolites[b]	Ratio 7G/9G
None	49.3	35.2	6.4	90.9	0.6	0.5	2.4 (6%)	1.40
Papaverine, 5 mM	35.4	13.7	5.1	54.2	2.8	13.1	17.4 (46%)	2.58
IMX, 5 mM	32.6	22.1	4.5	59.2	2.0	16.1	16.2 (49%)	1.48
Theophylline, 5 mM	46.0	29.2	4.8	80.0	1.0	7.4	7.2 (42%)	1.58
I, 1.30 mM	32.2	8.6	7.9	48.7	3.3	11.6	34.0 (40%)	3.74
I, 0.60 mM	36.8	11.0	10.4	58.2	ND[c]	3.4	ND	3.35
I, 0.2 mM	46.7	15.2	14.9	76.8	ND	1.0	ND	3.07
I, 0.04 mM	53.8	23.4	10.9	88.1	ND	0.6	ND	2.30

[^{3}H]BAP (5 μM) was supplied for 4 days.

[a] The total radioactivity due to glucosides of BAP.

[b] Metabolites at R_f 0.05–0.15 (silica gel, solvent A). The values in parentheses are the percentages of this radioactivity attributable to BAP nucleotides.

[c] ND, not determined.

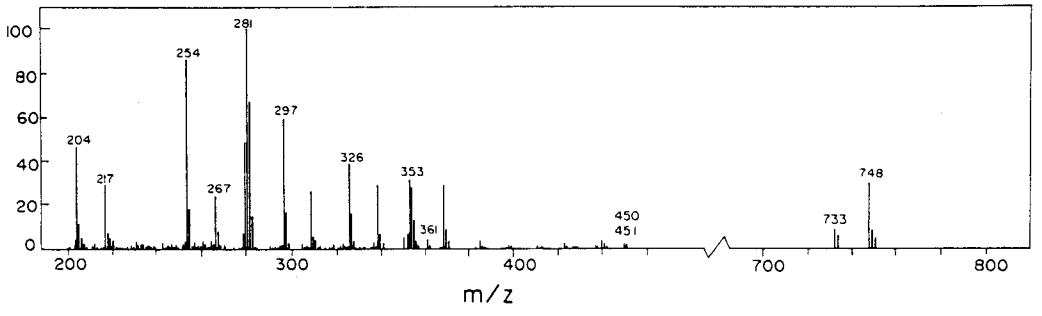

Fig. 3. EI MS of the per-TMS derivative of III.

namely, (OG)Z, Ade, and Ados. In *Amaranthus* cotyledons, theophylline (at 5 mM, as the ethylene diamine complex termed aminophylline) has previously been reported to partially inhibit conversation of BAP to metabolites which may be glucosides (Elliott and Murray 1975). While IMX and the diaminopurine I were about equally effective in inhibiting conversion of [9R]Z to the 7-glucoside (Table 1), I was considerably more effective than IMX in suppressing formation of [7G]BAP and [9G]BAP from supplied BAP. Although I preferentially inhibited formation of the 9-glucoside, IMX inhibited conversion to the two glucosides about equally. The alkaloid papaverine also preferentially inhibited formation of [9G]BAP; in inhibiting formation of total glucosides from BAP, papaverine and IMX were almost equally effective. The basic oxopurines hypoxanthine and xanthine, and also adenine (each at 5 mM), exhibited negligible inhibition of glucoside formation.

2D TLC studies indicated that the methylated xanthines, IMX, theophylline, and theobromine were not converted to glucosides in the cotyledons. Most of the inhibitors tested reduced the uptake of the supplied ^{3}H-labeled [9R]Z and BAP (maximum reduction observed was 45%). However, when the concentration of the supplied cytokinin was reduced to mimic this reduction in uptake caused by inhibitors, the distribution of radioactivity among metabolites was not altered. This is in accord with previous studies. Indeed it was found that a 90% reduction in ^{3}H-labeled cytokinin uptake by radish cotyledons did not appreciably change the percentage of radioactivity present as *N*-glucosides (Letham and Gollnow 1985). Hence, the inhibitor-induced reductions in cytokinin uptake do not affect the validity of the data presented in Tables 1 and 2.

In a previous study (Palni et al. 1984), the stability of [^{3}H][9R]Z in cultured soybean tissue was increased four-fold in the presence of IMX. However in the present study, the proportion of [^{3}H][9R]Z which remained unmetabolized was not appreciably

increased by IMX. Hence, IMX probably did not suppress conversion of [9R]Z to Z (the metabolic step necessary prior to 7-glucosylation) or reduce degradation to Ados.

[7G]Z was the principal metabolite of [9R]Z in the present studies. However, significant amounts of the total extracted ^3H (6–9%) was in the TLC zone immediately below [7G]Z (see Fig. 1). This metabolite was not detected in earlier studies using excised 3-day-old cotyledons (Letham and Gollnow 1985). It was identified as 7-β-glucopyranosyladenine ([7G]Ade) based on the following evidence: (a) cochromatography with authentic [7G]Ade during TLC with silica gel, borate-impregnated silica gel, and cellulose; (b) hydrolysis (0.5 N HCl at 95°C) to a compound which cochromatographed with Ade during TLC with both silica gel and cellulose; and (c) cocrystallization with [7G]Ade to constant specific activity. The unlabeled [7G]Ade used for cocrystallization was prepared by supplying a high concentration of Ade to excised radish cotyledons (see Materials and Methods). When formation of [7G]Z from [9R]Z was inhibited by xanthine derivatives, conversion to [7G]Ade was similarly reduced.

The Effect of Hormones, Nutrients, and Environmental Factors on N-*Glucosylation*

Cotyledons were incubated with nutrient solution containing abscisic acid (ABA, 10 μg/ml) for 2 days prior to uptake of [^3H][9R]Z (5 μM) for 3 days; cotyledons which received nutrients only for 2 days served as controls. The treatment with ABA reduced the percentage of ^3H in [7G]Ade and [7G]Z by 22 and 35%, respectively. This reduction was nearly balanced by promotion of formation of an unknown polar metabolite of [9R]Z. During TLC on silica gel (solvent B), this exhibited an R_f almost identical to that of [9R]Z-5′-monophosphate. Attempts to identify this metabolite were not successful. I and IMX inhibited conversion of both supplied [9R]Z and BAP to *N*-glucosides and these compounds appear to inhibit the enzyme which transfers glucose to the free cytokinin base (Parker et al. 1986). In contrast, while ABA inhibited formation of [7G]Z from [9R]Z, it did not suppress formation of glucosides from BAP. Hence, ABA does not appear to inhibit the glucosylation reaction directly; it presumably inhibits conversion of [9R]Z to free Z or promotes some form of [9R]Z metabolism so that the availability of Z is reduced.

Ethrel (an ethylene-releasing compound) and the auxins IAA and NAA, did not modify the metabolism of [9R]Z. Similarly, potassium chloride (75 mM), which enhances radish cotyledon expansion (Gordon and Letham 1975), had no effect on [9R]Z metabolism when compared with cotyledons incubated with water alone.

The cotyledons of radish seedlings developed under photoperiod in the usual way metabolized [9R]Z similarly when transferred to continuous light or continuous darkness. However, cotyledons excised from seedlings which had developed in continuous darkness, and cotyledons from seedlings which developed under the usual photoperiod, metabolized [^3H][9R]Z differently when the hormone was supplied in darkness. The percentage of ^3H due to [7G]Ade, [7G]Z, Ados, Ade, [9R]Z, and Z in the former are listed below with values for the latter in parentheses: 9.81 (13.4), 57.7 (38.3), 2.01 (5.83), 1.40 (4.11), 1.32 (2.30), 0.38 (0.69). Hence, development of the cotyledons in continuous darkness instead of normal photoperiods suppresses conversion of [9R]Z to [7G]Z.

This paper describes chemicals and environmental factors which suppress inactivation of exogenous [9R]Z by *N*-glucosylation in radish cotyledons. Extension of this work has led to the synthesis of more potent-related compounds (Hocart et al. 1991). Since [9R]Z is the major cytokinin in radish cotyledons (Tao and Letham, unpublished observations), such compounds may elevate the level of this endogenous cytokinin in the cotyledons and thus influence physiological events. They may also potentiate the activity of exogenously supplied cytokinins such as BAP.

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