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Cytokinin Biochemistry in Relation to Leaf Senescence I. The Metabolism of 6-Benzylaminopurine and Zeatin in Oat Leaf Segments

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Abstract. The metabolism of zeatin and that of 6-benzylaminopurine (BAP) have been compared in oat leaf segments in relation to the markedly differing ability of these cytokinins to retard senescence of such segments. Free BAP and a highly active senescence-retarding metabolite of BAP were detected in oat leaf segments supplied with BAP. The metabolite was identified by mass spectrometry and chromatography as 3-β-D-glucopyranosyl-BAP. The major metabolite of BAP was the 9-glucoside, but this lacked significant senescence-retarding activity. In contrast, in leaf segments supplied with zeatin, no free zeatin and no senescence-retarding metabolite of zeatin were detectable. The major metabolites of zeatin were adenosine, adenine nucleotides, the 9-glucoside, and unidentified polar metabolites. The differing activities of zeatin and BAP in the oat-leaf senescence bioassay appear to be, at least partially, a consequence of their differing metabolism and are not attributable to differences in uptake.

In almost all cytokinin bioassays based on promotion of plant growth, the natural cytokinin zeatin is much more effective than kinetin. Similarly, when zeatin is compared with the synthetic cytokinin 6-benzylaminopurine (BAP) in such bioassays, the former exhibits much greater activity than the latter (see ref. in Letham 1978). This marked difference in activity between zeatin and the synthetic cytokinins BAP and kinetin has been observed in the well-known tobacco callus, carrot phloem, and radish cotyledon cytokinin bioassays. In

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contrast, in cytokinin bioassays dependent on the retardation of senescence of diverse leaves, BAP and kinetin are usually markedly more effective than zeatin and 6-(Δ^2-isopentenylamino)purine (Dumbroft and Walker 1979, Kaminek and Lustinec 1978, Kuhnle et al. 1977, Letham 1967, Varga and Bruinsma 1973). Thus, in the oat leaf senescence bioassay, the lowest detectable concentration of zeatin is 10 μM, whereas that of BAP is 0.01 μM (Varga and Bruinsma 1973). This marked difference in activity may reflect differing cytokinin uptake, differing metabolism of the two cytokinins, or differing intrinsic activity at the functional site. To assess the first two possibilities, the uptake and metabolism of [^{3}H]BAP and [^{3}H]zeatin have been compared in oat leaf segments.

Materials and Methods

Chemicals

The following cytokinins were synthesized by procedures detailed in the cited references: [^{3}H]zeatin 132 mCi/mmol (Letham and Young 1971), [G-^{3}H]BAP 20 mCi/mmol (Wilson et al. 1974), 3-, 7-, and 9-β-D-glucopyranosides of BAP (Letham et al. 1975, Cowley et al. 1978), and glucosides of zeatin and zeatin riboside (Cowley et al. 1978, Duke et al. 1978, 1979). BAP riboside 5′-monophosphate was synthesized by heating a methanol solution of 6-chloropurine riboside 5′-monophosphate and benzylamine at 90°C for 4 h in a sealed tube. The product was purified by chromatography on Dowex 1 (formate form; eluting solvent 4 N formic acid) and then by HPLC, using a radial compression C_8 cartridge column eluted with a concave gradient (0–60%) of MeOH in water containing constant (0.2 N) acetic acid. The purified product was characterized by mass spectra of the tetra-TMS derivative. Electron-impact spectrum (70 eV); *m/z* 725 (M^+, 12), 710 (5), 484 (7), 411 (4), 382 (21), 371 (10), 355 (16), 340 (6), 315 (100, HOP[OTMS]$_3$), 299 (35), 297 (36), 296 (67), 282 (9), 258 (33), 243 (24), 230 (59), 225 (21), 224 (38), 211 (16); chemical ionization spectrum (CH_4 at 1 torr); *m/z* 726 (MH^+, 100), 710 (50).

Uptake of ^{3}H-labeled Cytokinins by Leaf Segments and Tissue Extraction

Oat seedlings (*Avena sativa* cv. Victory I; source of seed: General Swedish Seed Co., Svalov, Sweden) were grown in trays containing sterile potting mixture under the following conditions: 16-h photoperiod of 100 $\mu Em^{-2}s^{-1}$ at 20°C; 8 h of darkness at 15°C. A segment (length 1 cm) was cut 1 cm from the top of the first leaf of each 8-day-old seedling; about 25 segments were placed with the abaxial surface downward in each Petri dish (diameter 9 cm), which contained one circle of Whatman No. 1 filter paper wetted with ^{3}H-cytokinin solution (3.0 ml; 5 or 20 μM). The dishes were wrapped in foil and left at 22°C, usually for 4 days.

The segments were washed sequentially with distilled water, a 5 μM solution of unlabeled cytokinin, and again with distilled water. they were then blotted, weighed, and dropped into methanol/water/acetic acid (70:30:2 v/v/v, 10 ml/g

of tissue) chilled to −20°C. After 2–3 days at −20°C with occasional shaking, the segments and solvent were homogenized, left overnight at 2°C, stirred, and finally centrifuged. The resulting pellets were re-extracted with methanol/water (70:30 by vol) for 12 h at 2°C; the two extracts were combined and evaporated under reduced pressure (bath temp 35°C). The residue was dissolved in 50% ethanol for TLC.

Uptake of ^{3}H-labeled cytokinins was determined as detailed below. Oat leaf segments supplied with zeatin solution (5 μM and 15 μM) or BAP solution (5 μM) were washed as before, heated with 80% ethanol (10 ml/g tissue) at 90°C for 5 min, and then left at 23°C for 2 h. The plant tissue was then re-extracted with HCl (6 N) for 3 h at 70°C and left at 23°C overnight. The extraction with HCl was then repeated. Aliquots of the three extracts were taken for determination of radioactivity to calculate uptake. The second HCl extract contained less than 4% of the total radioactivity extracted, and no further radioactivity could be detected in the tissue residue.

Chlorophyll Retention Bioassay

Leaf segments, as described above, were placed in Petri dishes (diameter 5 cm; 15 segments per dish) containing one circle of filter paper and the test solution (1.0 ml). The Petri dishes wrapped in foil were left at 22°C for 4 days. To extract the chlorophyll from the segments, they were dropped into boiling 80% ethanol and held at this temperature for 7 min. The absorbance of the cooled solutions was measured at 665 nm after adjusting volumes.

Chromatographic Methods

Layers for TLC were spread with the following materials: silica gel 60 PF_{254} and silanized silica gel 60 HF_{254} (E. Merck), Serva cellulose with added fluorescent indicator (Parker et al. 1978), and borate-impregnated silica gel (spread wtih 0.05 M $Na_2B_4O_7 \cdot 10H_2O$). Solvents for TLC were as follows (proportions by volume): A—1-butanol/14 N NH_4OH/H_2O (6:1:2, upper phase); B—1-butanol/acetic acid/H_2O (12:3:5); C—ethyl methyl ketone/acetic acid/H_2O (16:1:4); D—ethyl methyl ketone saturated with H_2O; E—chloroform/MeOH (9:1); F—H_2O/MeOH (4:1); G—H_2O/MeOH (1:1); H—H_2O/1-propanol (3:1); I—H_2O/EtOH (1:2) saturated with $Na_2B_4O_7 \cdot 10\ H_2O$; J—1-butanol/water upper phase saturated with $Na_2B_4O_7 \cdot 10H_2O$.

TLC zones were eluted by one of the following methods: with water in capped scintillation vials (23°C for 18 h), for determination of radioactivity according to Gordon et al. (1974); with H_2O/MeOH/acetic acid (usually 20:20:1 v/v/v) in a column, for recovery of fractions for rechromatography. When the radioactivity level in silica gel TLC eluates was low, the ^{3}H compounds present were purified by retention on, and elution from, a small cellulose phosphate column (4–10 ml) prior to further TLC (Letham 1973, Summons et al. 1980). Evaporated TLC eluates and other fractions for HPLC were dissolved in water, adjusted to pH 3 with acetic acid, and centrifuged through a filter (0.2 μm pore

size) before injection into the HPLC system. All columns were obtained from Waters Associates (Milform, Massachusetts, USA).

Characterization of Chromatographic Fractions and Metabolites

Hydrolyses with *E. coli* alkaline phosphatase (Parker et al. 1978) and β-glucosidase (Letham et al. 1975) were performed according to the cited references. Adenosine and BAP glucosides were hydrolyzed to bases by heating with 0.5 N HCl at 95–100°C for 1 h.

The permethyl derivative of 9GZ was prepared according to Martin et al. (1981). Trimethylsilyl (TMS) derivatives of purified cytokinin metabolites were prepared according to MacLeod et al. (1976). Electron-impact mass specta were taken at 70 eV, samples being introduced on the direct inlet probe. Silylation reagents were evaporated using the foreline pump before heating the probe. The 3-allyl derivative of BAP was prepared by the following method: BAP (300 μg), or the evaporated eluate of a BAP marker spot from TLC, was dissolved in dry N,N-dimethylformamide (50 μl). Allyl bromide (13 μl) was added, and the mixture was left for 18 h at 37°C and then evaporated *in vacuo*. The product was purified by TLC on silica gel (solvent B) and eluted with ethanol. UV spectrum: λmax 293.5 nm in EtOH, 286 nm in 0.1 N HCl. Mass spectrum: *m/z* 265 (M^+, 100), 264 (22), 224 (53, $M^+ - CH_2 = CH-CH_2\cdot$), 188 (4), 160 (10), 119 (14), 106 (52), 91 (53).

Purification of Senescence-retarding Metabolite of BAP

Oat leaf segments (10 g), which had taken up BAP (20 μM) for 4 days, were extracted as before. An aqueous solution (10 ml, pH 3.1) of the evaporated extract was passed through a column of cellulose phosphate (5 g, NH_4^+ form, equilibrated to pH 3.1), which was washed with 0.03 N acetic acid (150 ml, 3 column volumes) followed by water (50 ml), and then eluted with 2N NH_4OH (100 ml). The evaporated eluate was dissolved in water (10 ml, pH to 7.5) and extracted with three 10-ml volumes in 1-butanol. The residue obtained by evaporation of the butanol extracts was dissolved in 5% methanol and subjected to HPLC on a C_8 radial compression column (100 mm × 8 mm diameter) that was eluted over 20 min at 3.0 ml min^{-1} with a linear gradient of methanol in H_2O (5–60%) and constant acetic acid (0.2 N). After the 20-min gradient, elution was continued with 60% methanol. Evaporation of the UV-absorbing fraction with a retention time of 25.8 min yielded a residue that was rechromatographed isocratically on the same column, using as solvent (3.0 ml min^{-1}) 40% methanol containing acetic acid (0.2 N). The UV-absorbing component with a retention time of 19.8 min was collected for mass spectrometry.

Results

Oat leaf segments were placed in contact with 5 μM aqueous solutions of ^3H-labeled zeatin and BAP. After 4 days in darkness, the BAP-treated segments

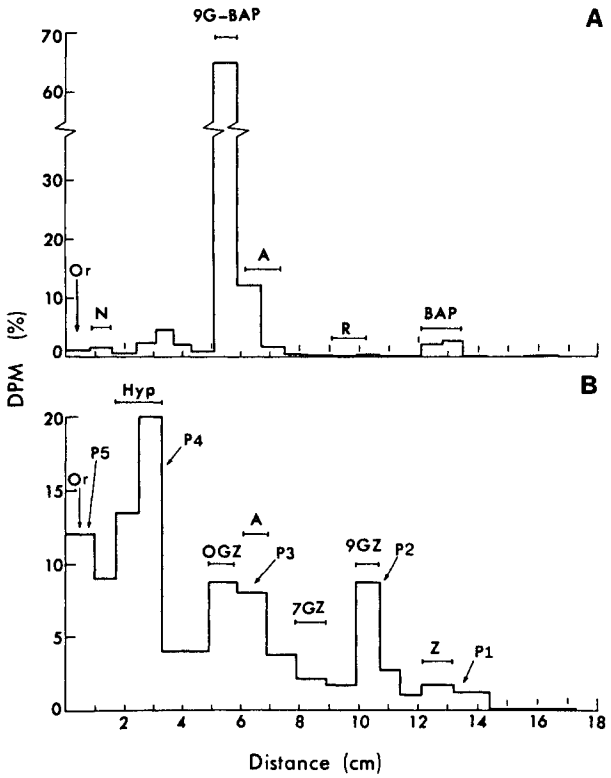

Fig. 1. The distributions of radioactivity over thin-layer chromatograms of extracts of oat leaf segments and the positions of cochromatographed markers (denoted by the barred lines). Or denotes the origin on each chromatogram. *1A*. Segments supplied with [^{3}H]BAP. The layer was silica gel developed once with solvent A. The markers were: N, BAP nucleotide (BAP riboside 5′-monophosphate); 9G-BAP, 9-glucosyl-BAP; A, adenosine; R, BAP riboside; and BAP. *1B*. Segments supplied wtih [^{3}H]zeatin. The layer was cellulose developed twice with solvent A. The markers were Hyp, hypoxanthine; OGZ, O-glucosylzeatin; A, adenosine; 7GZ, 7-glucosylzeatin; 9GZ, 9-glucosylzeatin; Z, zeatin. Peaks of radioactivity eluted for characterization of metabolites are designated P1 to P5.

were dark green, while those supplied with zeatin were yellow in color. The distributions of ^{3}H over chromatograms of segment extracts (Fig. 1) indicated that zeatin was converted into a complex of metabolites which differed considerably in R_f, while the metabolism of BAP was relatively simple and 65% of the ^{3}H was located in one TLC zone which cochromatographed with 9-β-D-glucopyranosyl-BAP. The metabolism of BAP and zeatin in the nonsterile segments normally used throughout this study was compared with the metabolism in segments excised from leaves that had been surface-sterilized with sodium hypochlorite. No difference was found in TLC studies.

The uptake of BAP by the segments exceeded that of zeatin. Thus, after 2 days, the uptake values for 5 μM zeatin, 15 μM zeatin, and 5 μM BAP were 5.2, 22.7, and 8.2 nano moles/g fresh weight, respectively. However, after 4 days, equivalent segments in contact with 15 μM zeatin were yellow, while

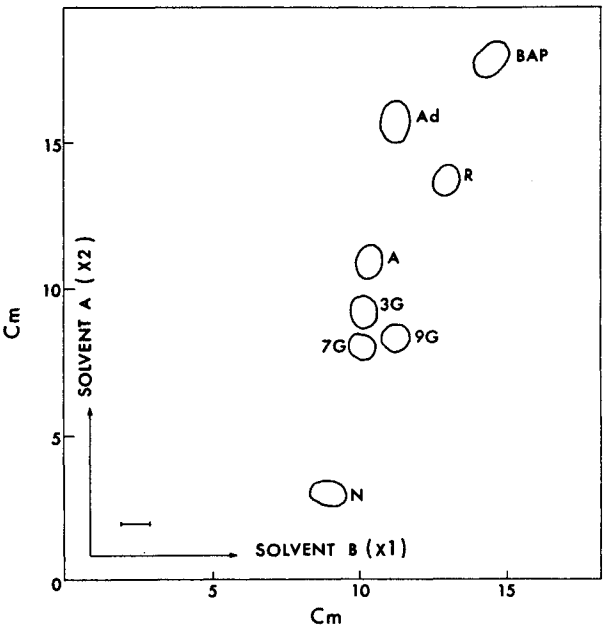

Fig. 2. Two dimensional thin-layer chromatogram of oat leaf extract showing the positions of cochromatographed markers, namely, BAP, BAP riboside 5′-monophosphate (N), 3-, 7-, and 9-glucosyl-BAP (3G, 7G, and 9G, respectively), adenosine (A), BAP riboside (R), and adenine (Ad). The layer was silica gel and was developed twice in the first dimension with solvent A. The origin (width 5 mm) was centered on the barred line.

those on 5 μM BAP were still dark green. Hence the differing senescence-retarding (SR) activities of BAP and zeatin were not simply a consequence of differing uptake.

TLC Studies of BAP Metabolites

In a two-dimensional TLC study on silica gel, the extract of segments treated with [^{3}H]BAP (5 μM) for 4 days was cochromatographed with the following markers: BAP, BAP 9-riboside (9R-BAP); BAP 9-riboside 5′-monophosphate (9R-5′P-BAP); 3-, 7-, and 9-β-D-glycopyranosyl-BAP (3G-BAP, 7G-BAP, and 9G-BAP, respectively); adenine, and adenosine. The positions of these compounds on a typical chromatogram are shown in Fig. 2. Zones that included these marker spots were ruled across the plate in the direction of the second dimension. When the radioactivity in zone segments was determined, peaks of radioactivity were associated clearly with the BAP, 3G-BAP, and 9G-BAP markers; these marker zones accounted for 3.0, 12.7, and 63.3%, respectively, of the total ^{3}H. The only other areas of the two-dimensional chromatogram with appreciable radioactivity contained the ^{3}H-labeled compounds derived from the minor peak of radioactivity centered between 9G-BAP and 9R-5′P-BAP in the first dimension (see Fig. 1, upper histogram). The zones containing the 7G-BAP, 9R-BAP, 9R-5′P-BAP, adenine, and adenosine contained only 1.6, 0.2, 0.6, 0.6, and 0.7%, respectively, of the total ^{3}H in the extract.

The radioactivity in the eluates of the above zones containing the BAP, 3G-BAP, and 9G-BAP markers was examined critically by further chromatography. The ^{3}H in the BAP zone cochromatographed with BAP during further TLC on

cellulose (solvent A) and during reverse-phase TLC on silanized silica gel (solvents G and H). Furthermore, after the eluate had been reacted wtih allyl bromide, the radioactivity cochromatographed with 3-allyl-BAP on silica gel (solvent B). Hence, unmetabolized BAP, which accounted for 3% of the total extracted ^{3}H, was tentatively identified in oat leaf segments supplied with BAP for 4 days. The radioactivity eluted from the 9G-BAP zone of the two-dimensional TLC plate cochromatographed with 9G-BAP during further TLC on silica gel (solvents C and E; the latter separates the α- and β-isomers of 9G-BAP), on cellulose (solvent B), and on silanized silica gel (solvent G). Similarly, the radioactivity in the eluate of the 3G-BAP zone cochromatographed with 3G-BAP during further TLC in all these same systems and also during TLC on borate-impregnated silica gel (solvents I and J). Furthermore, after the 3G-BAP and 9G-BAP eluates had been hydrolyzed with acid, all the ^{3}H cochromatographed with BAP on silica gel (solvents A, B, and D). Hence, the principal ^{3}H-labeled compounds in extracts of oat leaf segments supplied with BAP for 4 days were concluded to be BAP, 3G-BAP, and 9G-BAP, and this conclusion was confirmed by HPLC and mass spectrometric studies reported later in this paper. The contribution of these compounds and of 9R-BAP to the radioactivity of extracts of oat leaf segments that had taken up BAP for 1–3 days is defined in Fig. 3. At all times, 9G-BAP was the major metabolite. While the % DPM due to 3G-BAP and 9G-BAP increased considerably between 1 and 2 days, that due to BAP and 9R-BAP declined markedly. Similarly, the % DPM attributable to 9R-5′P-BAP declined from 2.6% at day 1 to 0.7% and 0.3% at days 2 and 3, respectively.

TLC Studies of Zeatin Metabolites

Oat leaf segments that had taken up [^{3}H]zeatin (5 μM) for 4 days were extracted, and the extract was subjected to TLC. The resulting one-dimensional chromatogram (Fig. 1B) exhibited 5 peaks of radioactivity. One peak, designated P1, cochromatographed approximately with zeatin, while a second (P5) was located at the origin; the remaining peaks cochromatographed with hypoxanthine (P4), O-β-D-glucopyranosylzeatin + adenosine (P3), and 9-β-D-glucopyranosyl-zeatin, 9GZ (P2). The radioactivity in each of these five peaks was examined critically by further TLC.

Labeled compounds that could be present in radioactivity peak P1 were zeatin, zeatin riboside, and their dihydro derivatives. However, sequential chromatography on cellulose (solvent B) and silica gel (solvent A) failed to detect any of these compounds. If present at all in the leaf extract, they would in combination account for less than 0.22% of the total ^{3}H. The corresponding value for extract prepared after uptake of zeatin for only 2 days, when the leaf segments were still green, was less than 0.15%.

Rechromatography of P2 on cellulose (solvent B) yielded a ^{3}H-labeled metabolite (60% of ^{3}H in P2), which cochromatographed with 9GZ in all systems tested, namely, on silica gel (solvents A and B), silanized silica gel (solvent F), cellulose (solvents A and B), and borate-impregnated silica gel (solvent I). Furthermore, when permethylated, the metabolite cochromatographed with

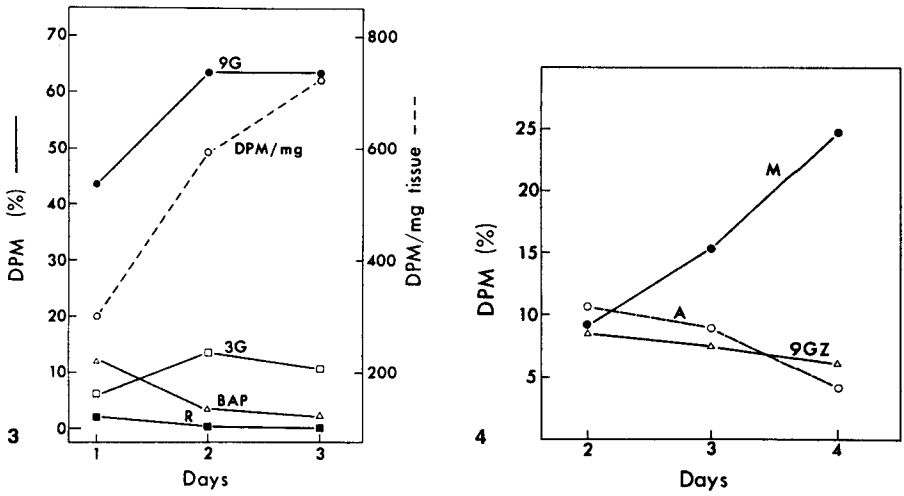

Fig. 3. The radioactivity due to BAP, BAP riboside (R), and 3- and 9-glucosyl BAP (3G and 9G) in extracts of oat leaf segments after uptake of BAP for different times. DPM/mg denotes the total radioactivity extracted per mg fresh weight of tissue. **Fig. 4.** The radioactivity due to 9-glucosylzeatin (9GZ), adenosine (A), and unknown polar metabolites (M) in extracts of oat leaf segments after uptake of zeatin for different times.

permethyl 9GZ on silanized silica gel. Accordingly, the metabolite was identified as 9GZ. When P3 was rechromatographed on cellulose (solvent B), 25% of the ^3H was found to be due to a metabolite that was identified as adenosine, based on the following evidence: cochromatography with adenosine on silica gel and cellulose (solvents A and B); acid hydrolysis to a derivative that cochromatographed with adenine on silica gel (solvent A), and silanized silica gel (solvents F and H). No other ^3H-labeled compounds in P3 were identified, and O-β-D-glucosylzeatin and its riboside did not contribute significantly to the radioactivity.

The radioactivity peak termed P4 was due to unidentified metabolites. These metabolites were unresolved when the cellulose TLC plate was developed in the second dimension (solvent B), and they migrated as a zone with an R_f relative to hypoxanthine of about 0.72. However, HPLC studies indicated that at least two metabolites were present. As the period of zeatin uptake was extended, the contribution of these metabolites to the total radioactivity in the extract increased markedly, while those of adenosine and 9GZ declined gradually (Fig. 4). The eluate of P5 was hydrolysed with alkaline phosphatase and the hydrolysate was subjected to TLC on silica gel (solvents A and B). This indicated that 37% of the radioactivity in the hydrolysate was due to adenosine, which was further characterized by acid hydrolysis to [^3H]adenine; no zeatin riboside was present. Hence, 4.4% of the ^3H in the leaf extract was due to adenosine phosphates.

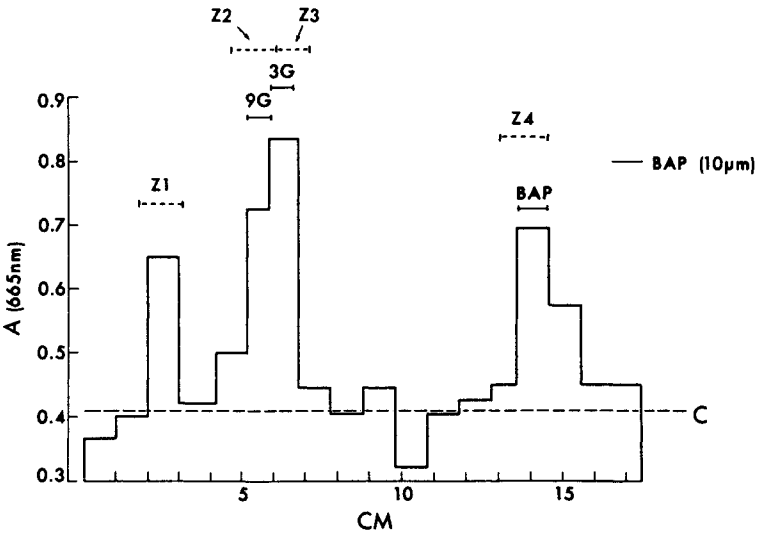

Fig. 5. The distribution of activity in the chlorophyll retention bioassay over a thin-layer chromatogram of extract of oat leaf segments supplied with [^{3}H]BAP (20 μM) for 4 days. The positions of cochromatographed 9-glucosyl-BAP (9G), 3-glucosyl-BAP (3G), and BAP are denoted by barred lines. The TLC plate (silica gel) was developed twice with solvent A. The horizontal line (C) defines the A value for the controls in the bioassay. The zones Z1 to Z4 were eluted for characterization of the senescence-retarding compounds.

Senescence-retarding Metabolites in Leaf Extracts

Oat leaf segments supplied with [^{3}H]zeatin (20 μM) for 4 days were extracted, and the extracts were subjected to TLC on cellulose (solvent A). No SR activity was detectable in the chromatogram zones using the oat leaf chlorophyll-retention bioassay. In contrast, when the corresponding extracts of segments supplied with [^{3}H]BAP were chromatographed (silica gel, solvent A) strong SR activity was detected in the resulting TLC zones (Fig. 5). Zones corresponding to those designated Z1 to Z4 in Fig. 5 were eluted from further TLC plates to characterize the active compounds. The results obtained are summarized below.

Z1. HPLC (C_8 radial compression column; gradient of 0–60% methanol with constant acetic acid, 0.2 N) showed that a complex of ^{3}H-labeled compounds was present in Z1 eluate. However, the SR activity was not associated with a ^{3}H-containing fraction, Since such SR activity was not detected in extracts of segments supplied with zeatin, it is suggested that the activity in Z1 eluate is due to an antisenescence factor which is induced, or is maintained, by BAP.

Z2. The eluate of this zone was subjected to HPLC (C_8 column), and the column effluent was monitored for UV-absorption, radioactivity, and SR activity in the oat leaf bioassay (Fig. 6). After trimethylsilylation, the fraction

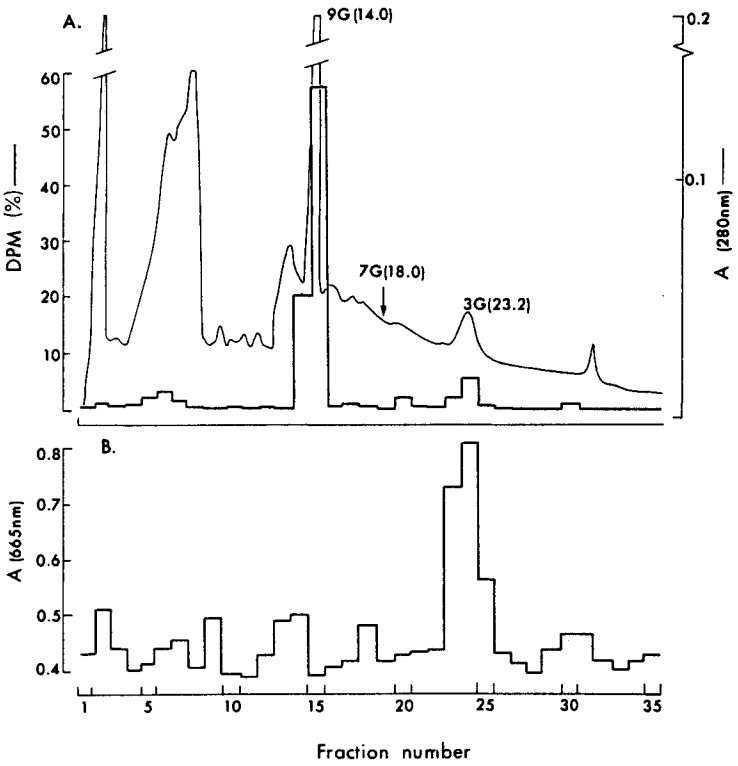

Fig. 6. The distribution of UV absorbance and radioactivity (A) and activity in the chlorophyll retention bioassay (B) during HPLC of the fraction eluted from TLC zone 2 of Fig. 5. The retention times (min) for 9-, 7-, and 3-glucosyl-BAP (9G, 7G, and 3G, respectively) are denoted. A C_8 radial compression column was used and was eluted for 20 min (3 ml min^{-1}) with a linear gradient of 5–60% MeOH and constant acetic acid (0.15 N). Elution with 60% MeOH was then continued for 8 min when the solvent was changed to 100% MeOH for a further 7-min elution. One fraction was collected every minute throughout the HPLC.

containing the major peak of radioactivity and the principal UV-absorbing component (fraction 15) exhibited the mass spectrum presented as Fig. 7. This is in complete accord with the spectrum for (tetra-TMS)9G-BAP. The UV-absorbing, ^{3}H-labeled component in fraction 15 co-eluted with 9G-BAP. Hence, taking into consideration the earlier TLC studies, the major metabolite of BAP was identified unequivocally as 9-β-D-glucopyranosyl-BAP.

The HPLC column effluent showed only one major peak of SR activity that was coincident with a minor peak of UV absorption (retention time equal to that of 3G-BAP) and with a minor peak of radioactivity (Fig. 6). There was insufficient material in the fraction for mass spectral characterization. However, when this UV-absorbing metabolite was rechromatographed on a HPLC μBondapak Phenyl column, SR activity, radioactivity, and UV absorption were again coincident with a retention time equal to that of 3G-BAP (Fig. 8). The SR metabolite in Z2 was concluded to be this glucoside.

Z3. When eluate of this TLC zone was subjected to HPLC (C_8 column, solvent as used for Z2), a single prominent peak of SR activity coincided with

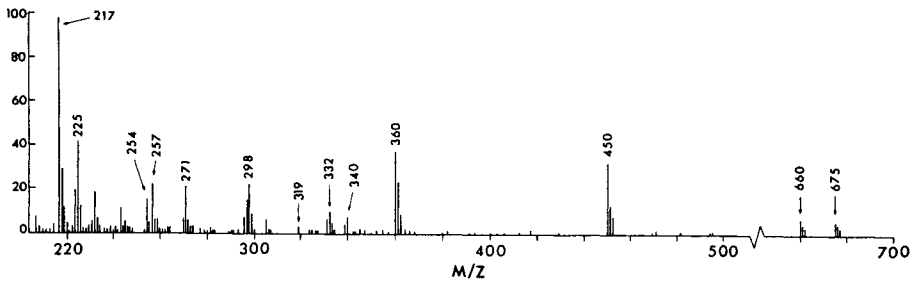

Fig. 7. The electron-impact mass spectrum of the TMS derivative of the UV-absorbing compound in HPLC fraction 15 of Fig. 6.

a peak of ^3H and a peak of UV-absorption (retention time equal to that of 3G-BAP). SR activity again appeared to be due to 3G-BAP.

Z4. During HPLC (C_8 column, 10–70% methanol gradient), the SR compound in this zone co-eluted with a ^3H-labeled, UV-absorbing component having a retention time equal to that of BAP.

The above results indicated that the SR activity in the extracts of the oat leaf segments was due principally to 3G-BAP and to a lesser degree to BAP. To further establish this, the principal SR compound was purified on a larger scale according to the procedure detailed under *Materials and Methods*. The TMS derivative of the resulting product exhibited a mass spectrum (Fig. 9) that was identical to that of (tetra-TMS)3G-BAP except for the presence of ions (*m/z* 221, 263, 264, 280, and 480) attributable to minor impurities. The TLC and HPLC results presented earlier, together with the mass spectral data now mentioned, provide an unambiguous identification of 3G-BAP as a senescence-retarding metabolite of BAP in oat leaf segments.

Discussion

While zeatin is more active than BAP and kinetin in promoting growth in cytokinin bioassays, the converse applies in bioassays based on retardation of leaf senescence. Previously, it has been speculated that this is a consequence of differing mechanisms of cytokinin action in the two types of bioassays, which could therefore exhibit differing cytokinin structure-activity relationships (Letham 1967). An alternative explanation proposes that isoprenoid cytokinins are more readily degraded enzymically than are BAP-type cytokinins in leaf tissue (Kaminek and Lustinec 1978). Unfortunately, our knowledge of the mechanism of cytokinin action has still not advanced sufficiently to critically assess the former possibility. However, the results presented herein do give support to the latter proposal. Thus, when [^3H]BAP was supplied to oat leaf segments for 2 days, free BAP accounted for 3.7% of the total extracted ^3H (Fig. 3). In addition, an active senescence-retarding metabolite of BAP was detected and identified as 3G-BAP. In contrast, when the segments received zeatin, no free zeatin, zeatin riboside, or their dihydro derivatives could be detected in the tissue extracts, nor could any senescence-retarding metabolite of zeatin. While it is known that cytokinin structure influences cytokinin up-

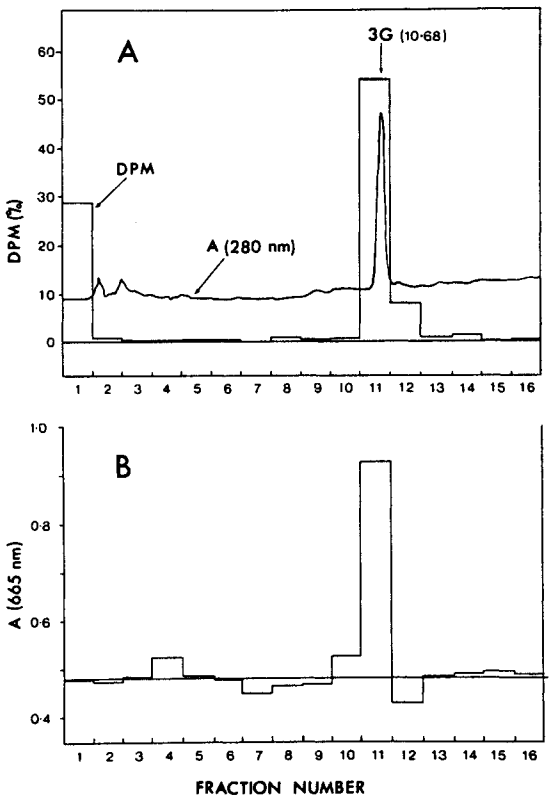

Fig. 8. The distribution of UV absorbance and radioactivity (A) and activity in the chlorophyll-retention bioassay (B) during HPLC of fractions 23 and 24 in Fig. 6. A μBondapak Phenyl column was used with a linear gradient of 0–70% MeOH and constant acetic acid (0.15 N). The retention time (min) of 3-glucosyl-BAP (3G) is shown.

take (Doree and Guern 1973, Laloue et al. 1981), the present study eliminates the possibility that the differing activities of BAP and zeatin in oat leaf segments are due simply to differences in uptake.

In the present study, both 3G-BAP and 9G-BAP were identified as metabolites of BAP. It should be noted that the TMS derivatives of these two glucosides exhibit mass spectra that are virtually identical. This appears to be a consequence of thermal rearrangement of the 3-glucoside derivative into the 9-glucoside derivative (Letham et al. 1975, Eichholzer et al. 1978). Hence chromatographic methods that clearly distinguish between 3G-BAP, 9G-BAP, and also 7G-BAP are particularly important. Such a method is provided by reverse phase HPLC as shown in Fig. 6.

9GZ was the only metabolite of zeatin with an intact isoprenoid sidechain that could be identified in the oat segment extracts, but this metabolite is inactive in the oat leaf senescence bioassay (Letham et al. 1983). The intense metabolism of zeatin described above was observed at 2 days, when the oat leaf segments supplied with zeatin were still green. At this time, no free zeatin or zeatin riboside could be detected in the tissue extracts. Furthermore, when [^{3}H]zeatin was supplied to segments in the presence of a concentration of BAP (10 μM) that retarded senescence strongly, the normal pattern of zeatin metabolism was observed (experiments not detailed in this paper). Therefore, this

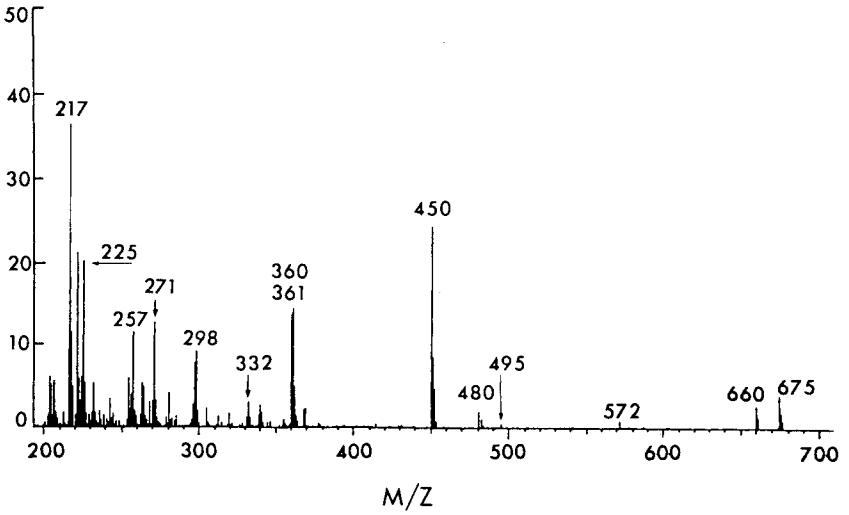

Fig. 9. The electron-impact mass spectrum of the TMS derivative of the principal senescence-retarding metabolite in oat leaf segments supplied with BAP.

metabolism does not appear to be a consequence of the senescence that occurs in the absence of an effective exogenous cytokinin. Unidentified polar metabolites of zeatin were detected in TLC fraction P4, and the associated radioactivity is presented in Fig. 4. When supplied to the oat leaf segments, both ^{3}H-labeled 9GZ and adenosine were metabolized partially to compounds with the chromatographic properties of the P4 metabolites (experiments not detailed herein). Thus the metabolites may be derived from both 9GZ and adenosine.

Although 7- and 9-glucosides of cytokinins have been detected in a number of plant tissues, either as metabolites of exogenous cytokinins or as endogenous compounds (Letham and Palni 1983), a cytokinin 3-glucoside (namely 3G-BAP) has been reported in plant tissues only twice, in radish cotyledons (Letham et al. 1975) and now in oat leaf segments as a senescence-retarding metabolite (the present study). The activity of 3G-BAP is similar to that of BAP in retarding senescence not only of oat leaf segments, but also of radish leaf discs, wheat leaf segments, and Chinese cabbage leaf discs (Letham et al. 1983). Therefore 3G-BAP may be a metabolite of particular significance in BAP-induced senescence retardation.

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