Metabolism of Tritiated Gibberellin A₉ by Shoots of Dark-grown Dwarf Pea, cv. Meteor

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ABSTRACT

Tritium-labeled gibberellin A₉ (3H-GA₉) was metabolized by etiolated shoots of dwarf pea (Pisum sativum cv. Meteor) to GA₃₇, GA₂₃, 2,3-dihydro-GA₃₇, and a number of highly polar, acidic GA-like substances. Identifications were made by gas-liquid radiochromatography and combined gas chromatography-mass spectrometry. Kinetic studies showed that GA₃₇ and 2,3-dihydro-GA₃₇ were produced within 5 hours following 3H-GA₉ application to pea shoots. The polar GA-like substances were produced between 5 and 10 hours after 3H-GA₉ application. Levels of GA₃₇ increased with time, and since no GA₃₇ was produced during the purification procedures, GA₃₇ was, in all probability, produced from 3H-GA₉ within the plant tissue. The radioactive interconversion products produced by pea from 3H-GA₉ have chromatographic properties similar to biologically active GA-like substances present in etiolated shoots of dwarf pea. Large scale applications of 3H-GA₉ with very low specific activity to etiolated pea shoots showed that the radioactivity of the interconversion products was correlated exactly with biological activity as assayed by dwarf rice (Oryza sativa cv. Tan-ginbozu).

Dwarf peas contain two main fractions exhibiting gibberellin (GA)-like activity (15, 17) and, in seeds of the dwarf cultivar Progress No. 9, these two GAs have been identified as GA₃₇ and GA₃₉ (10). Jones (14) detected the presence of two more zones of GA-like activity in buffer extracts from a tall variety of pea, cv. Alaska. One zone appeared more polar than GA₃₇ while the second was less polar than GA₃₉. Railton and Reid (unpublished data) have confirmed the findings of Jones (14) and furthermore have detected the presence of at least six zones of GA-like activity in extracts of chloroplasts and leaves of tall peas, cv. Alaska (25, 26). One of these zones has similar chromatographic properties to those of GA₉.

Studies have been carried out previously on the metabolism of radioactive GAs in dwarf pea (16, 21). 3H-GA₉ was metabolized to a more polar compound, but its identity was not established (15). Similarly, 3H-GA₉ was converted to a more polar

compound with thin layer chromatographic properties similar to those of GA₃₇ (21). Re-examination of the above cited work using superior analytical techniques, has shown that radioactive GA₉ was metabolized by dwarf pea to GA₉ (Railton, Durley, and Pharis, unpublished data) and that radioactive GA₉ was converted to GA₉ and another chromatographically similar compound (7). The metabolism of 3H-GA₉, a precursor of gibberellins produced by the fungus, Gibberella fujikuroi, has been studied in seedlings of dwarf pea cv. Meteor (8). 3H-GA₉ was converted to GA₉, GA₃₇, GA₂₃, GA₂₉, and GA₃₉ and the biosynthetic sequence GA₃₇ → GA₂₃ → GA₂₉ → GA₂₉ → GA₉ was strongly indicated.

We now report the metabolism of 3H-GA₉ in shoots of etiolated seedlings of dwarf pea, cv. Meteor. Gibberellin biosynthesis is believed to proceed in the order of increasing hydroxylation (4, 11, 27) and GA₉, a nonhydroxylated GA could therefore serve as an efficient precursor to other GAs. This, in conjunction with the fact that a compound chromatographically similar to GA₉ occurs in shoots of etiolated dwarf pea seedlings (Railton, Durley, and Pharis, unpublished data), prompted such a study.

MATERIALS AND METHODS

Synthesis of 3H-GA₉. Synthesis was carried out in a manner similar to that described by Cross et al. (4). GA₉ nor-ketone was converted to 17-3H-GA₉ via a Wittig reaction using triphenylphosphine and 3H-methyl iodide. The product was crystallized from acetone-light petroleum (b.p. 60–80°C) and stored at −20°C in absolute ethanol. 17-3H-GA₉ had a specific activity of 20 mc/m mole.

Gas-Liquid Chromatography and Gas Liquid Radiochromatography. Preparation of methyl esters and trimethylsilyl ether derivatives of the methyl esters and conditions for GLC (3) and GLRC (7, 8, 23) were similar to those described previously.

Combined Gas Chromatography-Mass Spectrometry. This was carried out with a Varian 1200 GLC connected by a double stage Biemann-Watson type molecular separator to a Varian Mat CHS mass spectrometer. Columns for GLC were 1.8 m x 2 mm i.d. and contained 2% QF-1 on Gaschrom Q (80–100 mesh) at a temperature of 198°C using helium carrier gas at a flow rate of 18 ml/min.

HYDROGENATIONS

GA₈, Methyl Ester. Adams catalyst (5 mg) and glacial acetic acid (2 ml) were shaken under hydrogen for 30 min. GA₈

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4 Abbreviations: GLC: gas liquid chromatography; GLRC: gas liquid radiochromatography; TMSMe: trimethyl silyl ether derivatives of the methyl esters; GC-MS: combined gas chromatography-mass spectrometry.
methyl ester (3 mg), prepared by treating the free acid with ethereal diazomethane, was added, and the mixture was shaken under hydrogen until uptake of hydrogen ceased. The solution was filtered and evaporated, and the residue was crystallized from ether-light petroleum (b.p. 60–80 °C) giving methyl tetrahydro-GA₇₉ as needles, m.p. 208–210 °C, identical (m.p. and infrared spectrum) with authentic material (19). Methyl tetrahydro-GA₇₉ chromatographed as two distinct peaks (C-16 epimers) on GLC, but in a single peak as its TMSMe derivative on the three columns (see Table II). GC-MS of the tetrahydro-GA₇₉ TMSMe derivative gave a scan, M⁺ = 420 (52%), with characteristic ions at m/e 405 (11), 399 (2), 377 (10), 361 (6), 333 (6), 331 (5), 318 (6), 303 (10), 209 (24), 145 (5), 143 (8), 105 (6), 91 (7), 75 (15), and 73 (26).

GA₇₉, Methyl Ester. Adams catalyst (5 mg) and ethanol (2 ml) were shaken under hydrogen for 30 min. GA₇₉ methyl ester, prepared by treating GA₇₉ (30 μg) with ethereal diazomethane, was introduced, and the mixture was shaken under hydrogen for 40 min. The solution was filtered and evaporated, leaving tetrahydro-GA₇₉ as a white powder. The TMSMe derivative of tetrahydro-GA₇₉ chromatographed as two peaks (C-16 epimers) on GLC using the QF-1 and XE-60 columns and as a single peak using the SE-30 column (Table II). The shorter retention time peak (on all columns) was about 10 times the area of the other. GC-MS of the larger peak gave a scan, M⁺ = 420 (24%) with characteristic ions at m/e 405 (25), 389 (12), 388 (12), 373 (87), 360 (9), 345 (42), 330 (32), 313 (45), 298 (40), 286 (56), 285 (100), 270 (49), 243 (54), 225 (63), 183 (65), 93 (46), 75 (71), and 73 (off scale).

Growth and Treatment of Plants. Seeds of dwarf pea (Pisum sativum cv. Meteor) were soaked in running tap water for 20 hr, planted in moist vermiculite, and kept in darkness. Five days after sowing, before elongation of the second internode, ³H-GA₇₉ was applied in 5-μl droplets of ethanol to the plumule hook. In most experiments, 206 μg (13.5 μc) were applied to a total of 40 seedlings.

In preliminary experiments, seedlings were harvested 20 hr after ³H-GA₇₉ application. In later experiments where the kinetics of interconversion were followed, harvests were made 5, 10, 20, 30, and 44 hr after application of ³H-GA₇₉.

Extraction and Purification. After separating the shoots from seeds and roots, the shoots were surface washed by brief agitation in absolute methanol. They were then ground in a precooled mortar with acid-washed sand and ice-cold 80% methanol. After filtering, the methanol was removed in vacuo, and an equal volume of 0.5 M phosphate buffer, pH 9.0, was added to the residual aqueous phase. This was partitioned (6X) against equal volumes of diethyl ether and then against ethyl acetate (6X) at pH 3.0. Finally, the aqueous phase was partitioned against 1-butanol (4X) at pH 3.0. The ethyl acetate-soluble fraction was reduced in vacuo and subjected to TLC on silica Gel H (Merck) irrigated with ethyl acetate-chloroform-formic acid (50:50:1, v/v/v, solvent 1). Radioactive zones were eluted with water-saturated ethyl acetate and analyzed by GLRC. Very polar radioactive zones were further eluted with absolute methanol. Further purification and separation of polar compounds was carried out on TLC with silica Gel H and chloroform-methanol-acetic acid-water (40:12:2:2, v/v/v, solvent 2) as developing solvent.

Liquid scintillation spectrometry was carried out using a Packard Tri-Carb scintillation spectrometer and a modified Bray’s solution (2). Quenching and counting efficiencies were determined using internal standards.

Sample Preparation for GC-MS. ³H-GA₇₉ (4.1 μc) was added to GA₇₉ (2 mg) in ethanol (2 ml), and the solution was applied to a total of 400 etiolated dwarf pea seedlings in the same manner as described above. Extraction procedures were also identical to those described above. The acidic, ethyl acetate-soluble fraction, in the form of a brown gum, was dissolved in a minimal volume of 0.1 M phosphate buffer, pH 8.0, and purified further on a column of poly-N-vinylpyrrolidone (12). Extracts were then separated on TLC using ethyl acetate-chloroform-formic acid (50:50:1, v/v/v), and radioactive zones were eluted as described above. Estimates of amounts of GAs present in these zones were determined by bioassay. Prior to GC-MS, individual zones of activity were purified further on a silica gel partition column (6, 22), using a modified gradient, chambers 1 to 4 containing respectively, ethyl acetate-hexane, 35:65 (137.5 ml), 50:50 (131.3 ml), 65:35 (125.2 ml), and 100:0 (111 ml).

Bioassays. Dwarf rice (Oryza sativa cv. Tan-ginbozou) was the assay material throughout (5, 20).

RESULTS

When ³H-GA₇₉ was applied to shoots of etiolated seedlings of dwarf pea and the acidic, ethyl acetate-soluble fraction chromatographed on TLC (ethyl acetate-chloroform formic acid [50:50:1, v/v/v] solvent 1) four main zones of radioactivity were found. Approximately equal numbers of counts were observed in zone 1 (Rf 0.0–0.2) and zone 3 (Rf 0.5–0.7). Significantly less radioactivity was associated with zone 2 (Rf 0.2–0.4). Zone 4 (Rf 0.8–0.9) contained high levels of radioactivity and co-chromatographed with ³H-GA₇₉.

The butanol fraction contained relatively high levels of radioactivity, suggesting interconversion of ³H-GA₇₉ to very polar compounds such as GA-glycosides. The distribution of radioactivity between the extraction solvents was: diethyl ether, 1.2 × 10⁶ dpm; ethyl acetate, 57.6 × 10⁵ dpm; butanol, 13.6 × 10⁵ dpm; and residual buffer, 18.4 × 10⁵ dpm.

Each zone of radioactivity associated with the acidic, ethyl acetate fraction was analyzed further by GLRC. Following elution from TLC with water-saturated ethyl acetate, the radioactive zones were reduced to dryness, converted to the TMSMe derivatives, and analyzed by GLRC on three separate liquid stationary phases, 2% QF-1, 2% SE-30, and 1% XE-60 on Gaschrom Q. The retention times of the compounds in the metabolite zones are depicted in Table I. Zone 1 was associated with high levels of impurities which made preliminary analysis by GLRC impossible. Zone 2 contained two compounds, one with identical retention times to those of the TMSMe derivative of GA₅₉, and the second, with similar retention times to those of the TMSMe derivative of GA₂₉, although the low levels of radioactivity associated with the latter compound preceded definite identification. Zone 3 separated into two radioactive compounds on GLRC. One compound had identical retention times to those of the TMSMe derivative of GA₂₉ and the other similar, though not identical retention times to those of the TMSMe derivative of GA₂₉. The close chromatographic similarity of the radioactive metabolite to derivatized GA₂₉ on 2% SE-30 and its shorter retention time on 1% XE-60 suggested a compound structurally similar to GA₂₉, but lacking one double bond. Zone 4 contained only GA₂₉.

In order to test the possibility that the GA₂₉-like metabolite present in zone 3 might be a dihydro derivative of GA₂₉, a portion of the residue from zone 3 was hydrogenated over Adams catalyst as described under “Materials and Methods.” Samples of authentic GA₂₉ and GA₂₉ were also hydrogenated in an identical manner. The retention times of the TMSMe derivatives of the hydrogenation products were compared on GLRC (Table II). Tetrahydro-GA₂₉ had identical retention times to those of the hydrogenated GA₂₉, metabolite, further...
confirming GA90 as a metabolite of 3H-GA9. Tetrahydro-GA31 had identical retention times to those of the hydrogenated GA90-like metabolite from zone 3. The metabolite was thus identified as dihydro-GA31. The indicated metabolism of 3H-GA9, applied to etiolated shoots of dwarf pea is summarized in Figure 1.

In order to obtain further evidence of the identity of the metabolites of 3H-GA9, from TLC zones 2 and 3 by GC-MS, and to determine the biological activity of these metabolites, the above experiment was carried out on a larger scale (see "Materials and Methods"). The correlation of radioactivity from TLC and biological activity are shown in Figure 2. Four zones of radioactivity were present, corresponding to zone 1, zone 2 containing GA9, and the GA9-like metabolite, confirming GA90 as a metabolite of 3H-GA9. Tetrahydro-GA31 had identical retention times to those of the hydrogenated GA90-like metabolite from zone 3. The metabolite was thus identified as dihydro-GA31. The indicated metabolism of 3H-GA9, applied to etiolated shoots of dwarf pea is summarized in Figure 1.

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zone 3 containing GA<sub>30</sub> and dihydro GA<sub>29</sub>, and zone 4 containing precursor GA<sub>1</sub>. Significant biological activity was associated with all zones except zone 1. The lack of biological activity in zone 1 may be the result of impurities which were markedly inhibitory to GA-induced rice leaf sheath growth. Further purification of zone 1 on columns of charcoal-celite has shown that it does indeed exhibit a low level of biological activity (Railton, Durley, and Pharis, unpublished data).

The remainder of the residues from TLC zones 2, 3, and 4 from the large scale experiment were purified further on a silica gel partition column (6, 22) using a modified gradient (see "Materials and Methods") prior to GC-MS. The residue from TLC zone 2 was eluted as two peaks of radioactivity from the silica gel column. The first was derivatized and examined by GC-MS. A scan of the major peak, which corresponded to the TMSMe derivative of GA<sub>30</sub> (by GLRC), gave a mass spectrum identical with that of the published spectrum of the TMSMe derivative of GA<sub>30</sub> (1). The second peak contained insufficient GA to obtain an identifiable mass spectrum. TLC zone 3 eluted from the silica gel column in fractions 10 to 13 and these were combined and reduced to dryness in vacuo. Half of the sample was converted to the TMSMe derivative, and the other half was esterified with ethereal diazomethane, hydrogenated over Adams catalyst (see "Materials and Methods"), and subsequently converted to the TMSMe derivatives. The nonhydrogenated sample was examined by GC-MS and scans were obtained of the two peaks corresponding to dihydro-GA<sub>30</sub> and GA<sub>30</sub> observed during the earlier GLRC. The first scan gave a mass spectrum identical with that published for the TMSMe derivative of GA<sub>30</sub> (1). The second scan gave the mass spectrum shown in Figure 3. The hydrogenated sample was examined by GC-MS in an identical fashion. Scans were obtained corresponding to the two major radioactive peaks observed during earlier GLRC of the hydrogenated radioactive sample. The first scan gave a mass spectrum identical with that of the TMSMe derivative of tetrahydro-GA<sub>30</sub>. The second scan gave a mass spectrum identical to that of the TMSMe derivative of the major epimer of the tetrahydro-GA<sub>30</sub>. TLC zone 4 contained a single compound which when methylated and examined by GC-MS gave a mass spectrum identical to that of the methyl ester of GA<sub>1</sub> (1).

The new metabolite in TLC zone 3 was therefore identified as dihydro-GA<sub>30</sub>. From the data obtained here, we could not fully ascertain whether the metabolite was 2,3-dihydro-GA<sub>30</sub> or 16,17-dihydro-GA<sub>30</sub> (see Figs. 1 and 4 for structures). However, circumstantial evidence favors 2,3-dihydro-GA<sub>30</sub>, since the metabolite was produced from GA<sub>1</sub> in a similar yield to that of GA<sub>30</sub>, both conversions requiring a one step hydroxylamination. Synthesis of 16,17-dihydro-GA<sub>30</sub> from GA<sub>1</sub> would require several steps, and if produced, would be expected to be in lower yield. The mass spectral fragmentation pattern of the TMSMe derivative of the metabolite (Fig. 3) was similar to that of the TMSMe derivative of GA<sub>30</sub>, many of the major ions of the former being 2 atomic mass units larger than those of the latter.

Attempts were made to further purify the polar metabolite in zone 1, and these attempts have only been partially successful. The marked polarity of the metabolite suggested that it could be the glucoside of a weakly polar gibberellin which would partition into ethyl acetate in small amounts. The glucoside of GA<sub>1</sub>, for example, partially migrates into ethyl acetate at pH 3.0 (G. Sembdner, personal communication). However, partition studies suggested that the polar metabolite was a free acid, since it was almost wholly removed from 0.5 M phosphate buffer at pH 3.0 by ethyl acetate and could be reextracted again with 0.5 M phosphate buffer at pH 2.0.
compound migrated as a single zone of radioactivity when chromatographed on TLC using solvent 2 and was more polar than GA₃ but less polar than GA₈-glycoside (Fig. 5A). When the metabolite was treated with ethereal diazomethane and rechromatographed in the same solvent system it separated into three main zones of radioactivity (Fig. 5B) suggesting the presence of free carboxyl groups and indicating that the zone was composed of at least three separate radioactive compounds.

In order to obtain information about the sequence of interconversions of ³H-GA₃ into the various metabolites, kinetic studies were carried out. After applying ³H-GA₃ to etiolated pea shoots, harvests were made 5, 10, 20, 30, and 44 hr later. The distribution of radioactivity in the various fractions extracted from the shoot is given in Table III. The acidic, ethyl acetate-soluble fractions were chromatographed on TLC as before using solvent 1, and each Rₐ zone was eluted, derivatized, and examined by GLRC. The distribution of radioactivity on TLC plates at each harvest is shown in Figure 6, and the radioactivity associated with each metabolite is shown in Table III.

Five hours after ³H-GA₃ application, both GA₈ and 2,3-dihydro-GA₃ had been produced in significant amounts but the levels of radioactivity associated with GA₈ and the polar metabolite zone were of a lower order (Fig. 6a). Approximately 10 times more dihydro-GA₃ than GA₈ had been produced from ³H-GA₃ within 5 hr (Table III). Within the next 5 hr, the levels of radioactivity associated with the polar metabolite zone had increased dramatically as had the levels of both dihydro GA₃ and GA₈. Smaller increases were observed in the levels of GA₈ (Fig. 6b). The low levels of radioactivity associated with the butanol fraction at the 10-hr harvest (Table III) relative to those of the polar metabolite zone further suggested that this zone was not glycosidic in nature.

Table III. Kinetics of the Metabolism of ³H-GA₃ by Etiolated Shoots of Dwarf Pea Seedlings

Radioactivity associated with the various fractions and acidic metabolites of ³H-GA₃ at various times (5, 10, 20, 30, 44 hr) following ³H-GA₃ application. The 153 × 10⁴ dpm originally were applied to a total of 200 etiolated dwarf pea seedlings. Forty seedlings were extracted at each harvest time.

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<th>Time</th>
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Fig. 4. Structures of gibberellins.

Fig. 5. Distribution of radioactivity on TLC (chloroform-methanol-acetic acid-water; 40:12:2:2, v/v) of zone 1 from extracts of etiolated dwarf pea seedlings treated with ³H-GA₈. A: Free acid; B: methyl ester.
Twenty hours after \(^{1}H\)-GA\(_{a}\) application, the levels of the polar metabolite zone, 2,3-dihydro-GA\(_{a}\) and GA\(_{a}\), were maximal (Table III). Radioactivity associated with the butanol fraction had also reached a maximum by 20 hr and thereafter decreased to a steady level at the 30- and 44-hr harvests. Levels of radioactivity in the residual aqueous phases inexplicably varied widely from harvest to harvest.

The data suggested that \(^{1}H\)-GA\(_{a}\) was simultaneously hydroxylated at C-12 and C-13 to give 2,3-dihydro GA\(_{a}\) and GA\(_{a}\), respectively. The rapid rise in levels of the polar metabo-

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**LITERATURE CITED**


