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METABOLISM OF GIBBERELLIN A_1 IN GERMINATING BEAN SEEDS

RONN NADEAU and LAWRENCE RAPPAPORT

Department of Vegetable Crops, University of California Davis, California, 95616, U.S.A.

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Abstract—Tritium labeled gibberellin A_1 ([^{3}H]GA_1) was synthesized by selective hydrogenation of GA_3 and a method was developed for its isolation from two other radioactive side-products. Seeds of *Phaseolus vulgaris*, cv. Kentucky Wonder, were imbibed for 30 hr in an aqueous solution containing [^{3}H]GA_1. Extraction with methanol and solvent fractionation was followed by a thorough search for radioactive metabolic products. The primary conversion product of [^{3}H]GA_1, detected by GLC, was [^{3}H]GA_8-glucoside along with traces of [^{3}H]GA_8. The absence of radioactive GA_3 and GA_3-glucoside was indicated by GLC.

INTRODUCTION

IT HAS long been known that levels of gibberellin (GA) activity vary dramatically during various stages of growth in the life cycle of plants.[1,2] Our objective in this and future investigations is to trace the chemical steps and controlling mechanisms responsible for these variations and to study their relationships to developmental changes. However, this is a most complex problem. There are now 36 known GAs, isolated from the fungus, *G. fujikuroi*, and from many plant species. Therefore, we have chosen to investigate the behaviour of one of them, GA_1, which occurs naturally in *Phaseolus vulgaris* and which can be prepared as [^{3}H]GA_1 in high specific activity by methods outlined by Kende[3] and purified according to procedures described under Experimental. Recently, Pitel and Vining[4] published an improved method for preparation of the compound.

Seeds of French bean, *Phaseolus vulgaris*, were chosen for this study because in previous investigations they were shown to have high levels of GAs (including GA_1) during early development, little or no GA in the mature dry state and increasing levels as the seeds germinate.[5–8] These GAs have been extracted into neutral ethyl acetate, acidic ethyl acetate and water, or acidic butanol soluble fractions (see Lang[2] for review). However, there is limited information concerning their exact chemical identities, variations during developmental processes, and role of their metabolites. Sembdner *et al.*[5] reported that maturing pods of *Phaseolus coccineus* converted [^{14}C]GA_3 to [^{14}C]GA_3-glucoside and lesser amounts of [^{14}C]GA_8-glucoside. The latter compound conceivably arose via a [^{14}C]GA_1 intermediate and so it was of interest to see if [^{3}H]GA_1 converted to [^{3}H]GA_8 and its glucoside.

[1] L. G. PALEG, *Ann. Rev. Plant Physiol.* **16,** 291 (1965).
[2] A. LANG, *Ann. Rev. Plant Physiol.* **21,** 537 (1970).
[3] H. KENDE, *Plant Physiol.* **42,** 1612 (1967).
[4] D. W. PITEL and L. C. VINING, *Can. J. Biochem.* **48,** 259 (1970).
[5] G. SEMBDNER, J. WEILAND, O. AURICH and K. SCHREIBER, in: *Plant Growth Regulators*, SCI Monogr. Vol. 31, p. 70, (1968).
[6] T. HASHIMOTO and L. RAPPAPORT, *Plant Physiol.* **41,** 626, 629 (1966).
[7] G. W. M. BARENDSE, H. KENDE and A. LANG, *Plant Physiol.* **43,** 815 (1968).
[8] A. HSU, Fate of labeled gibberellins A_1 and A_3 in developing and germinating seeds of *Phaseolus vulgaris* L., Ph.D. Thesis, Univ. of Calif., Davis, pp. 19–36 (1969).

In view of reports of conversion in *G. fujikuroi* (see Lang[2]), we also sought evidence for conversion of [^{3}H]GA^1 to [^{3}H]GA^3 and its glucoside.

RESULTS

The incubation and extraction procedures are detailed under Experimental and summarized in Fig. 1; the results from analysis of the fractions are given in Fig. 2. Nine kg of 'Kentucky Wonder' bean seeds were germinated under white light at 25° in water containing $3{\cdot}2 \times 10^8$ cpm of II (specific activity = 5C/m-mol, $5{\cdot}6 \times 10^6$ cpm/μg), and 1 mg/l. of cold GA_1. The imbibed seeds were blended in and extracted twice with 75% methanol. This extract contained $1{\cdot}6 \times 10^8$ cpm (50%) and $1{\cdot}2 \times 10^8$ cpm (38%) was found in the unabsorbed germination medium. The balance of the radioactivity (12%) remained in the extracted seed residue.

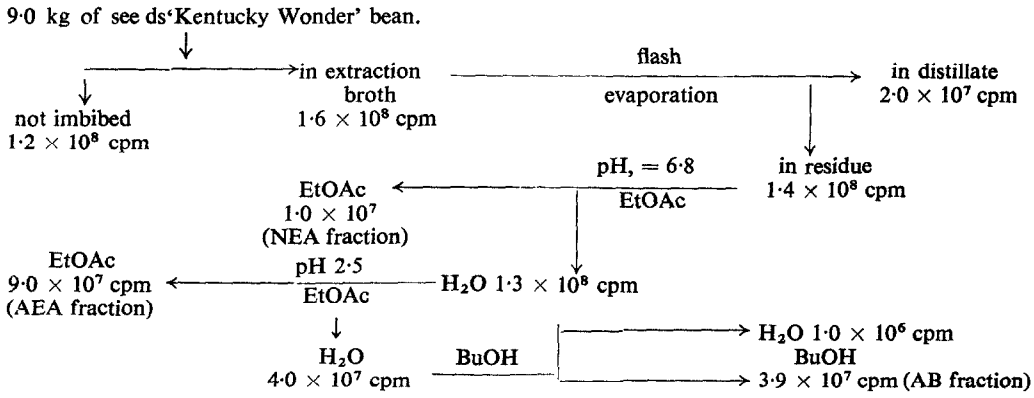

FIG. 1. SUMMARY OF THE EXTRACTION SCHEME AND DISTRIBUTION OF THE RADIOACTIVITY. NINE kg OF 'KENTUCKY WONDER' BEAN SEEDS WERE GERMINATED 30 hr IN AN AQUEOUS SOLUTION CONTAINING $3{\cdot}2 \times 10^8$ cpm OF 1,2-[^{3}H]GA_1.

Flash evaporation of the methanol extract yielded $1{\cdot}4 \times 10^8$ cpm in the concentrate and, unexpectedly, about $2{\cdot}0 \times 10^7$ cpm in the methanol : water distillate. A triple-distilled sample of this distillate had a constant specific activity of 65 $\pm$ 3 cpm. The concentrate was fractionated into three parts by; (1) extracting with ethyl acetate at pH 6·8 to give a neutral ethyl acetate fraction (NEA), (2) adjusting the pH to 2·5 with 0·5 M H_3PO_4 and extracting with ethyl acetate to give an acidic ethyl acetate fraction (AEA), and then (3) extracting with *n*-butanol to give an acidic butanol fraction (AB).

A portion of the AEA fraction was purified by TLC to increase the specific activity and this was methylated and silylated in preparation for GLC. The derivative was co-injected with GA_1, GA_3 and GA_8 methyl ester-trimethylsilyl ether (Me–TMS) standards on two different columns. An effluent splitter was used and mass peaks were detected by flame ionization while RA peaks were detected by collecting effluent at 0·5 min intervals and then analysing each sample by scintillation counting. Although many mass peaks were detected on both columns, only two RA peaks emerged from each, corresponding to GA_1- and GA_8-standards. In this way it was ascertained that 98·5% of the RA in the AEA fraction corresponded to II while 1·5% was due to III. The NEA fraction was similarly analysed and results were identical.

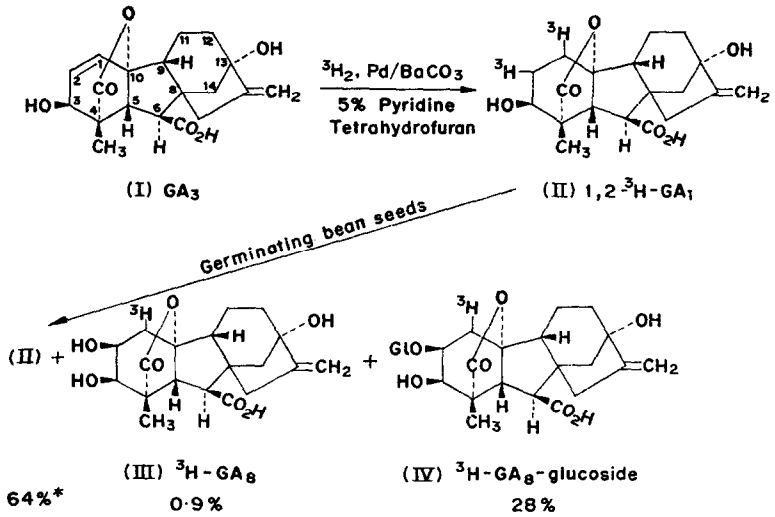

FIG. 2. SCHEME SHOWING THE SYNTHESIS OF 1,2-[^{3}H]GA$_1$ (II) FROM GA$_3$ AND THE CONVERSION OF II TO [^{3}H]GA$_8$ (III) AND [^{3}H]GA$_8$-GLUCOSIDE (IV) BY GERMINATING BEAN SEEDS. PERCENTAGES SHOWN ARE BASED ON RADIOACTIVITY EXTRACTED FROM THE SEEDS.

The AB fraction contained the major metabolic product, identified as IV, [^{3}H]GA$_8$-glucoside, (Fig. 2) in the following ways. The product, as detected by radiochromatogram scanning, registered a single peak on TLC and on electrophoresis with a pH 6·0 pyridine: acetic acid buffer. The Me–TMS derivative of the radioactive material in the AB fraction had a single retention time on GLC (QF-1 and SE-30) columns which corresponded to an authentic GA$_8$-glucoside–Me–TMS standard. Specifically, on QF-1 IV–Me–TMS gave three discernible mass peaks, A, B and C (Fig. 3). Only peak C was radioactive and coinjection of the sample with authentic GA$_8$-glucoside–Me–TMS enhanced peak C. The fact that IV–Me–TMS gave a sizable mass peak by itself indicated that a substantial amount of endogenous GA$_8$-glucoside (not quantitized in this experiment) was present in the imbibed seeds.

Because published work[9] has shown that acid hydrolysis of GA$_8$ and GA$_8$-glucoside yielded one product (V-a, Fig. 4) in which the glucose moiety was cleaved and the D-ring rearranged, we subjected authentic GA$_8$ (III-a) and IV to such treatment in order to further substantiate the identity of IV. The products from GA$_8$ and from IV were identical by TLC and GLC, indicating that they were V-a and V-b, respectively. Additionally, to test for the characteristic *cis*-glycol structure in ring A of GA$_8$ type compounds, V-a and V-b were converted to their methyl ester derivatives (VI-a and VI-b) and then each sample was let stand in 1 ml of acetone containing 10 μl of 50% perchloric acid for 6 hr at room temp.[10] In each case a transformation occurred (acetonides, VII-a and VII-b) and the products were identical by TLC (R_f 0·77 in solvent E). Since the hydroxyl in ring-A of [^{3}H]GA$_1$ is β and since acetonides can form only from *cis*-glycols,[11] these results indicate that IV is indeed a GA$_8$ derivative. Also, IV was enzymatically hydrolysed[9] and the aglycone was

[9] K. SCHREIBER, I. WEILAND and G. SEMBDNER, *Phytochem.* **9**, 189 (1970).
[10] R. NADEAU, Enzymatic and Non-Enzymatic Cyclizations of Terpenoid Terminal Epoxides, Ph.D. Thesis, Stanford Univ., p. 87 (1968).
[11] J. F. W. KEANA, in *Steroid Reactions* (edited by C. DJERASSI), p. 67, Holden–Day, New York (1963).

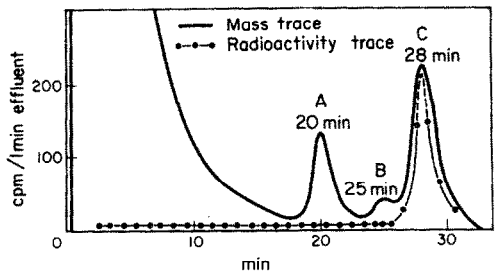

FIG. 3. RADIOACTIVE MATERIAL IN THE AB FRACTION WAS REFINED BY TLC AND ELECTROPHORESIS AND WAS THEN GLC'd AS THE Me–TMS DERIVATIVE. MASS PEAKS (————) WERE DETECTED BY f.i.d. AND RADIOACTIVE PEAKS (—●—●—) BY COLLECTING EFFLUENT. EACH DOT REPRESENTS AN EFFLUENT COLLECTION FOR COUNTING (SEE EXPERIMENTAL FOR DETAILS).

identified as [^{3}H]GA_8 by TLC and GLC. The absence of radioactive GA_3-glucoside in the AB fraction was indicated by failure to detect any derivatized radioactive substance corresponding to the GLC retention time of authentic GA_3-glucoside–Me–TMS.

DISCUSSION

Our GLC and TLC studies on the metabolites produced from [^{3}H]GA_1 by germinating seeds of *Phaseolus vulgaris* indicate that the only products formed are [^{3}H]GA_8 and [^{3}H] GA_8-glucoside; [^{3}H]GA_3 and its glucoside were notably absent. Thus, there appears to be a direct hydroxylation mechanism for the conversion of GA_1 to the observed metabolites, rather than one which would involve GA_3 as an intermediate.

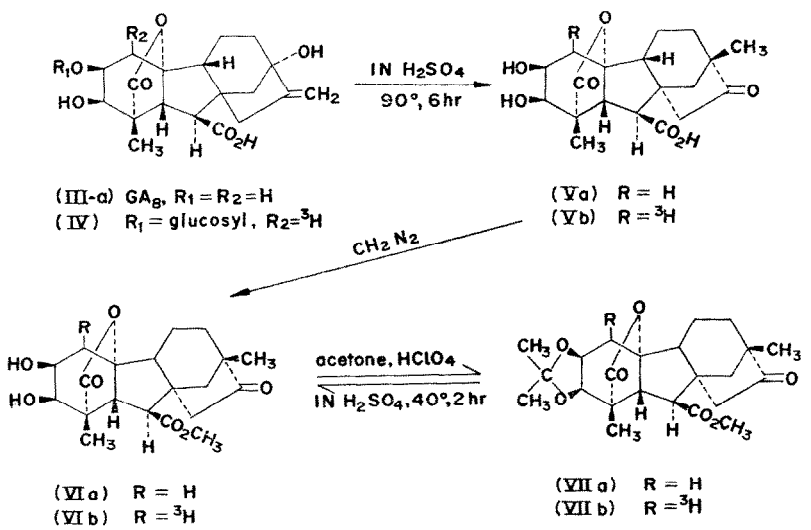

FIG. 4. SUMMARY OF THE CHEMISTRY USED TO PROVE THE IDENTITY OF [^{3}H]GA_8-GLUCOSIDE (IV). TREATMENT OF GA_8 (III-a) AND IV WITH ACID GAVE THE WAGNER–MEERWEIN REARRANGEMENT PRODUCTS (V-a, V-b). METHYLATION GAVE VI-a, VI-b AND ACETONIZATION VII-a, VII-b (SEE TEXT).

Since III comprises 1·5% ($1{\cdot}4 \times 10^6$ cpm) of the radioactivity in the AEA fraction and since all of the activity ($3{\cdot}9 \times 10^7$ cpm) in the AB fraction is due to IV, all but 3·7% of the intermediate III formed is converted by the end of the 30-hr incubation to the glucoside. Either III is rapidly converted as it is formed to IV, or alternatively, it is formed only during

the early hours of germination and then gradually is converted to IV until almost no III remains after 30 hr.

Since GA_1 is absent in dry seeds but present in imbibed seeds,[3,5,6] these results lead to the conclusion that during germination GA_1 is gradually formed but during the same time converted via GA_8 to the relatively inactive glucoside. Hence we suggest that the enzymes causing the transformation of GA_1 to GA_8-glucoside may serve to maintain the active GA_1 at a level commensurate with normal development.

Since neither inhibitor experiments nor GA_8-glucoside feeding experiments were run in the present study, utilization of conversion products was not ascertained. It is nevertheless clear that in *Phaseolus vulgaris*, by far the largest product of [^{3}H]GA_1 supplied during seed germination, is [^{3}H]GA_8-glucoside which appears to remain reasonably stable in the first 30 hr after the beginning of inbibition. Until appropriate feeding experiments are run it will not be possible to assess wholly the role of the glucoside.

EXPERIMENTAL

TLC methods. ChromAR sheet, which is 70% silica gel—30% glass fiber, was used throughout. $AgNO_3$ impregnated ChromAR sheets were made by dipping them into a solution containing 42 g of $AgNO_3$/l. of solution, then drying them in a 90° oven for 30 min. Developing solvents used were: EtOAc–$CHCl_3$–HOAc, 15:5:1, *A*; EtOAc–$CHCl_3$–HOAc, 15:5:0·1, *B*; *i*-PrOH–3N NH_4OH, 5:1, *C*; Hexane–EtOAc, 9:1, *D*; and EtOAc–$CHCl_3$, 3:1, *E*.

GLC Methods. The methods used were similar to those reported by Cavell *et al.*[12] The stainless steel columns (0·3 cm × 1·8 m) were packed with 3% SE-30 and 3% QF-1 on Gas-Chrom Q. Methyl esters were prepared with diazomethane and TMS ethers with Sil-Prep (hexamethyldisilazane–trimethylchlorosilane–pyridine, 3 : 1 : 9). Radioactive GLC peaks were monitored by using an effluent splitter, collecting fractions at the exit port and analysing each by scintillation counting. Mass peaks were detected by flame ionization.

Purification of 1,2-[^{3}H]GA_1 (II). The New England Nuclear preparation (P) showed a single radioactive peak at R_f 0·65 (0·60–0·70), on ChromAR TLC developed with solvent *A*. However, when analysed as the Me–TMS derivative of QF-1, 205°, P–Me–TMS showed three radioactive peaks: 6·5, 7·7, and 8·2 min in a cpm ratio $\simeq$ 1:1:2. The 7·7 min peak corresponded exactly with the retention time of authentic GA_1–Me–TMS. The other two components have not been identified.

A 2·4 × 10^9 cpm sample of P was streaked onto a 5 × 20 cm strip of 10% $AgNO_3$-ChromAR and developed in solvent *A*. Scanning revealed two radioactive zones P_1, R_f 0·49 (0·43–0·58), 6·8 × 10^8 cpm, 28%; P_2 R_f 0·65 (0·58–0·72), 6·7 × 10^8 cpm, 28%. Recovery of activity from these zones was accomplished by washing the zones with MeOH, adding a few drops of NaCl solution, filtering to remove salts, then evaporating the MeOH. A sample of P_1–Me–TMS on QF-1 at 205° displayed the previously observed 6·5 and 7·7 min radioactive peaks; P_2–Me–TMS displayed the 8·2-min peak.

Separation of the two radioactive components of P_1 was accomplished by using 10% $AgNO_3$-ChromAR and the above procedure but with solvent *B* instead of *A*. Thus P_1, 1·9 × 10^8 cpm, gave P_{1a}, R_f 0·11 (0·06–0·14), 7·5 × 10^7 cpm, 39%; and P_{1b}, R_f 0·18 (0·14–0·23), 7·7 × 10^7 cpm, 41%. GLC of P_{1a}–Me–TMS and P_{1b}–Me–TMS indicated that both were homogeneous, with retention times of 6·5 and 7·7 min, respectively. Thus the pure II was in fraction P_{1b} and this was checked and substantiated by GLC on an SE-30 column. Later attempts to accomplish the purification in one step with solvent *B* met with poor results.

Incubation procedures. Mature 'Kentucky Wonder' bean seeds were purchased from Ferry-Morse Seed Co. 9·0 kg of the seeds were distributed one layer thick over 12 polyethylene lined trays (750 g per tray). The [^{3}H]GA_1 (3·2 × 10^8 cpm) and cold GA_1 (6·0 mg) were dissolved in 6 l. of H_2O and 500 ml added to each tray. When the germinating seeds had imbibed most of the solution (~5 hr), 450 ml more water was added to each tray. After 30 hr of incubation under fluorescent light at 25° the imbibed seeds were collected and washed briefly in MeOH. The MeOH wash was combined with the residual incubation medium (total cpm = 1·2 × 10^8) and the seeds were ground in lots with a total of 20 l. of 75% MeOH with a Waring blendor. The resulting suspension was centrifuged in lots of 1 l. and the supernatants were decanted. Resuspension of the centrifugates in another 20 l. of 75% MeOH and centrifugation gave a second supernatant which was combined with the first. The total supernatant, 1·6 × 10^8 cpm, on flash evaporation yielded 1·4 × 10^8 cpm, 2·8 l. in the concentrate and approximately 2·0 × 10^7 cpm, 30 l. of distillate. A sample of the distillate which contained 65 ± 3 cpm per 100λ was triple-distilled without causing a change in its

[12] B. D. Cavell, J. MacMillan, R. J. Pryce and A. C. Sheppard, *Phytochem.* 6, 867 (1967).

specific activity. The solvent fractionation of the extract was conducted according to procedures already described in detail elsewhere,[6] and is summarized in Fig. 1.

Radioactive components in the AEA fraction. A $5{\cdot}0 \times 10^5$ cpm aliquot of the AEA fraction was streaked onto a 20 × 20 cm piece of ChromAR and developed in solvent *A*. Scanning showed only one peak, R_f 0·65, but it had a small shoulder on the origin side. Isolation of the entire active zone gave $4{\cdot}8 \times 10^5$ cpm, 19 mg, and this material was further refined by treatment to produce the Me–TMS derivative which was then chromatographed on ChromAR with solvent *D*. Scanning showed a zone at R_f 0·52–0·59; elution gave AEA–Me–TMS, $4{\cdot}6 \times 10^5$ cpm, 1 mg.

A 23 000 cpm sample of AEA–Me–TMS was coinjected onto SE-30, 195°, with GA_1, GA_3, and GA_8–Me–TMS standards, the latter three showing retention times of 10·9, 11·9, and 17·8 min, respectively. Collection of fractions of the effluent at 0·5 min intervals over a period of 22 min revealed radioactivity above background in only two areas, 1 (7450 cpm) and 2 (113 cpm), corresponding, respectively, to the co-injected GA_1 and GA_8 standards. Thus the amount of activity in area 2 (GA_8) was 1·5% of that in area 1 (GA_1). The above procedure was repeated on a QF-1 system under conditions which more clearly separated GA_1 and GA_3 standards. Results and conclusions were parallel.

[^{3}H]GA_8-glucoside (IV) in the AB fraction. A $1{\cdot}4 \times 10^5$ cpm sample of AB was streaked onto 5 cm of a 10 × 20 cm piece of ChromAR along with GA_1 and 'cold' IV standards on the other 5 cm portion of the sheet. Development for 1·5 hr in solvent *C* yielded a peak at R_f 0·35 (0·25–0·45) containing $1{\cdot}2 \times 10^5$ cpm, 86%; the GA_1 and IV standards had R_fs 0·73 and 0·35, respectively.

Another $1{\cdot}4 \times 10^5$ cpm sample of AB was streaked onto ChromAR along with GA_1 and IV standards. This was subjected to electrophoresis under the following conditions: pH 6·0 pyridine buffer, 1000 *v*, 60–80 mA, 1·5 hr. The single active peak which resulted at 75 mm from the origin (on the anode side) coincided exactly with the IV standard; the GA_1 standard moved 97 mm.

A $1{\cdot}2 \times 10^5$ cpm sample of AB which had first been refined by TLC (above) was converted to its Me–TMS derivative and then TLC'd on ChromAR with solvent *D*. The only radioactive zone was at R_f 0·67 (0·62–0·71), $1{\cdot}0 \times 10^5$ cpm. GLC of a portion of this sample on QF-1, 250°, showed three main mass peaks with retention times, 20·1, 25·0, and 28·0 min, the last one corresponding exactly in retention time to GA_8-glucoside–Me–TMS standard (Fig. 3). By collecting 1·0 min fractions of the effluent, we showed that the only radioactive area corresponded with the 28·0 min mass peak. A GA_8–glucoside–Me–TMS standard had a 30 min retention time. The above results were confirmed on an SE-30 column which gave retention time of 14·2 and 16·5 min for GA_3-and GA_8-glucoside standards (230°).

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Key Word Index—Phaseolus vulgaris; Leguminosae; germinating bean seeds; gibberellin metabolism; gibberellin A_1; gibberellin A_8; gibberellin A_8-glucoside.