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Effect of Photoperiod on Metabolism of [^{3}H]Gibberellins A_1, 3-*epi*-A_1, and A_{20} in *Agrostemma githago* L.[1]

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ABSTRACT

To determine whether daylength influences the rate of metabolism of gibberellins (GAs) in the long-day (LD) rosette plant *Agrostemma githago* L., [^{3}H]GA_{20} and [^{3}H]GA_1 were applied under short day (SD) and LD. Both were metabolized faster under LD than under SD. [^{3}H]GA_{20} was metabolized to a compound chromatographically identical to 3-*epi*-GA_1. [^{3}H]GA_1 was metabolized to two acidic compounds, the major metabolite having chromatographic properties similar to, but not identical with GA_8. [^{3}H]3-*epi*-GA_1 applied to plants under LD was metabolized much more slowly than was [^{3}H]GA_1, and formed a very polar metabolite which did not partition into ethyl acetate at pH 2.5. Very polar metabolites were also formed after the feeds of [^{3}H]GA_{20} and [^{3}H]GA_1. It was not possible to characterize these very polar compounds further because of their apparent instability. The results obtained suggest that in *Agrostemma* GA_{20} is the precursor of 3-*epi*-GA_1, but there is at present no evidence indicating the precursor of GA_1.

In the LD[4] rosette plant *Agrostemma githago*, GAs play an important role in the mediation of the photoperiodic control of stem elongation (5). Although the levels of endogenous GAs fluctuate during the induction of elongation, there is no clear correlation between GA level and stage of developmental response (5, 6). To investigate the possibility that length of photoperiod influences the rate of turnover of GAs, treatment with AMO-1618 (an inhibitor of GA biosynthesis) was used to obtain information about the metabolism of GAs under SD and LD (6). The results (6) indicated that the rate of metabolism of several of the endogenous GAs was greater under LD than under SD. We have now investigated this situation more directly by applications of tritiated forms of three of the native GAs of *A. githago* to plants under SD and LD. Our results also provide information on possible interrelationships between some of the GAs found in *A. githago*.

MATERIALS AND METHODS

Plant Material and Treatments. Plants of *Agrostemma githago* were raised as described previously (5). Treatments were begun when the plants were 7 to 8 weeks old. Each plant received a single application of 100 μl of an aqueous solution containing 0.05% Tween 20 (Sigma), 5% ethanol, and one of the following radioactive GAs: (a) 6×10^6 cpm [2,3-^{3}H]GA_{20} (2.1 Ci/mmol, synthesized by Dr. N. Murofushi, provided by Dr. R. P. Pharis); (b) 7.5×10^5 cpm [1,2-^{3}H]3-*epi*-GA_1 (0.25 Ci/mmol, synthesized by Dr. R. C. Durley, provided by Dr. R. P. Pharis); and (c) 6×10^6 cpm [1,2-^{3}H]GA_1 (25 Ci/mmol, New England Nuclear).

Solutions were applied as drops to the apex and youngest leaves of the plants. In the case of [^{3}H]GA_{20}, applications were made to the appropriate plants after they had been exposed to 8 LD, when the level of GA_{20} is highest (6), while for [^{3}H]GA_1 and [^{3}H]3-*epi*-GA_1 applications were made after 12 LD, that is, at the time when these GAs reach their peak (6). All three GAs were also applied to plants under SD. Following treatment, the plants were grown under the same photoperiod as before until harvested after 1 to 6 d. As a control (0 d), plants were frozen immediately after application of GAs to ensure that the radioactive products did not originate during the extraction and purification procedures, but rather were the result of metabolism. Three plants were used for each of the treatments with [^{3}H]GA_{20} and [^{3}H]GA_1, and two plants for each treatment with [^{3}H]3-*epi*-GA_1.

Analysis of [^{3}H]GAs and Their Metabolites. The aerial parts of treated plants were harvested, frozen in liquid N_2, and freeze-dried. Lyophilized material was extracted twice with methanol (50 ml/g dry weight), and phosphate buffer (0.1 M, pH 7.3) was added to the combined extracts at a rate of 10 ml/100 ml extract. This solution was then evaporated to give an aqueous phase which was adjusted to pH 8.5 and partitioned five times against equal volumes of petroleum ether (B. R. 35–60°C). The ether fractions contained little radioactivity and were discarded.

The aqueous extract was adjusted to pH 2.5 and partitioned four times against equal volumes of ethyl acetate. The combined acidic ethyl acetate fractions were chromatographed on the preparative HPLC system described by Jones *et al.* (4). Appropriate fractions from the preparative HPLC were analyzed further on a μBondapak C_{18} column (Waters Associates) as before (4), except that a gradient of 10 to 50% (v/v) methanol in 1% acetic acid was run in 20 min at 2 ml min^{-1}, eluting standard GA_8 in fractions 12 and 13, while GA_1 and 3-*epi*-GA_1 eluted in fractions 18 and 19. To separate [^{3}H]GA_1 from [^{3}H]3-*epi*-GA_1, relevant fractions from the μBondapak C_{18} column were chromatographed on silica thin-layer plates as described previously (6), using hexane:*iso*propanol: acetic acid (60:40:1, v/v/v) as solvent.

To detect very polar metabolites which remained in the buffer phase after partitioning with ethyl acetate at pH 2.5, the residual aqueous phase was placed on a column of 1.5 g charcoal and 3 g Celite. Next, the column was thoroughly washed with water (pH 2.5) and finally eluted with 100 ml acetone. The acetone eluate was chromatographed directly on the μBondapak C_{18} system as above. Radioactivity in aliquots (usually 0.1% of the total) of the

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[4] Abbreviations: LD, long day(s); AMO-1618, 2-*iso*propyl-4-dimethyl-amino-5-methyl-phenyl-1-piperidine-carboxylate-methyl-chloride; SD, short day(s).

various extracts and HPLC fractions was determined by liquid scintillation spectrometry. Counting efficiency was between 45 and 50%.

RESULTS

Metabolism of [^{3}H]GA$_{20}$. In our preparative HPLC system, [^{3}H]GA$_{20}$ was clearly resolved from its metabolites as a single zone of radioactivity in fractions 16 and 17. Although the radioactivity in this zone decreased under both SD and LD, the decline was faster under LD than SD. In LD only, 12% of the [^{3}H]GA$_{20}$ remained after 2 d, compared with 50% in SD (Fig. 1A). Concomitant with the fall in [^{3}H]GA$_{20}$ was the appearance of, and increase in, acidic ethyl acetate soluble metabolites which eluted from the preparative HPLC in fractions 11 to 13. Further analysis on the μBondapak C$_{18}$ column resolved two zones of radioactivity. The very minor zone of activity in fractions 14 and 15 was not investigated further. The major metabolite of [^{3}H]GA$_{20}$, eluting in fractions 18 and 19, co-chromatographed with GA$_1$ and 3-*epi*-GA$_1$. When these fractions were combined and analyzed by TLC, 2% or less of radioactivity was detected at the same R_F as GA$_1$, while the major part co-chromatographed with 3-*epi*-GA$_1$. The level of [^{3}H]3-*epi*-GA$_1$ thus detected in the acidic ethyl acetate fractions increased more rapidly under LD than under SD (Fig. 1B).

The aqueous fractions remaining after partitioning against ethyl acetate were run over columns of charcoal in an attempt to detect metabolites which were too polar to partition appreciably into ethyl acetate, such as highly hydroxylated GAs and GA conjugates. The level of radioactivity in acetone eluates of these charcoal columns on which the residual aqueous fractions had been loaded, increased with time, with a more rapid rise under LD than under SD (Fig. 1C). Attempts to analyze these polar metabolites further were confounded by unaccountable losses of radioactivity during the HPLC process. The very small amounts of activity which were recovered, eluted from the analytical HPLC in a single zone in fractions 5 to 7.

Metabolism of [^{3}H]3-*epi*-GA$_1$. As only a small amount of [^{3}H]3-*epi*-GA$_1$ was available, its metabolism was studied only under LD. Preparative HPLC of the acidic ethyl acetate phases revealed only a single zone of radioactivity in fractions 11 to 13. When these fractions were analyzed further on μBondapak C$_{18}$, again a single zone of activity was found in fractions 18 and 19, corresponding to the elution volumes of both 3-*epi*-GA$_1$ and GA$_1$. Silica TLC of this zone indicated that only [^{3}H]3-*epi*-GA$_1$ was present, and that there had been no conversion to [^{3}H]GA$_1$. The level of radioactivity associated with [^{3}H]3-*epi*-GA$_1$ fell relatively slowly with time, with 56% still present after 4 LD (Fig. 2).

A small accumulation of radioactivity was detected in the acetone eluates of the charcoal columns (Fig. 2). Once again, much of the activity associated with this very polar fraction disappeared during analytical HPLC. The radioactivity which was

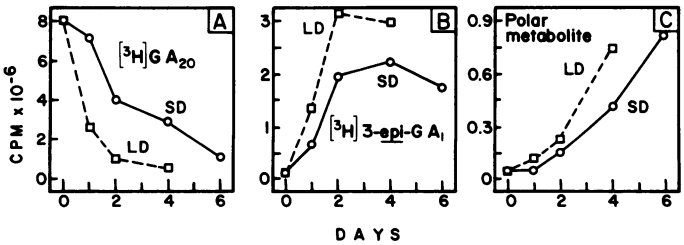

FIG. 1. Metabolism of [^{3}H]GA$_{20}$ under SD and LD. A, Radioactivity associated with [^{3}H]GA$_{20}$; B, activity associated with [^{3}H]3-*epi*-GA$_1$; C, activity associated with a very polar metabolite, not partitioning into ethyl acetate at pH 2.5. Cpm indicates total radioactivity obtained from all three plants in each treatment.

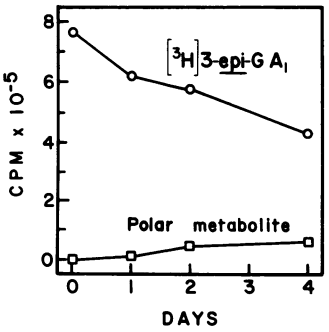

FIG. 2. Metabolism of [^{3}H]3-*epi*-GA$_1$ under LD and corresponding production of a very polar metabolite.

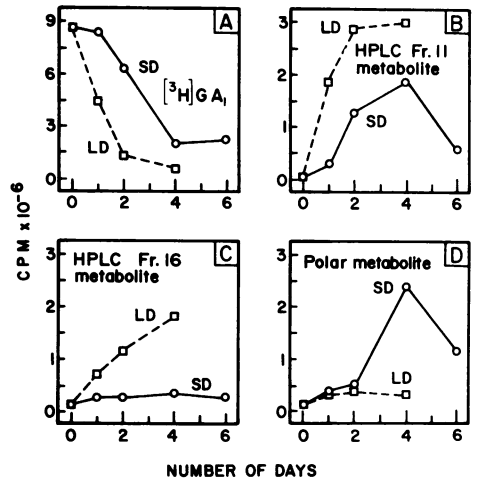

FIG. 3. Metabolism of [^{3}H]GA$_1$ under SD and LD. A, Radioactivity associated with [^{3}H]GA$_1$; B, activity associated with fraction 11 from analytical HPLC; C, activity associated with fraction 16 from analytical HPLC; D, activity associated with a very polar metabolite, not partitioning into ethyl acetate at pH 2.5.

recovered, was eluted in HPLC fractions 5 to 7, and so was chromatographically the same as the very polar metabolite formed after feeding [^{3}H]GA$_{20}$.

Metabolism of [^{3}H]GA$_1$. Preparative HPLC of the control acidic ethyl acetate fraction revealed a single zone of radioactivity in fractions 12 and 13, corresponding to [^{3}H]GA$_1$. There was a steady decline in activity in these fractions with time, and a redistribution of activity into a much broader zone from fractions 9 to 13. Consequently, fractions 8 to 14 from the preparative HPLC were combined for each sample before further analysis. Analytical HPLC of these bulked fractions revealed three zones of radioactivity. The least polar of these, in fractions 18 and 19, corresponded to [^{3}H]GA$_1$. Silica TLC of these fractions provided no evidence for the conversion of [^{3}H]GA$_1$ to [^{3}H]3-*epi*-GA$_1$. As was the case with [^{3}H]GA$_{20}$, the rate of metabolism of [^{3}H]GA$_1$ was greater under LD than under SD, with only 16% remaining after 2 LD compared with 72% after 2 SD (Fig. 3A).

Both of the metabolites of [^{3}H]GA$_1$ detected in the acidic fraction were more polar than GA$_1$. The major metabolite, eluting in fraction 11 of the μBondapak C$_{18}$ had an elution volume slightly less than that of GA$_8$ in this reverse-phase system. Accumulation of this metabolite was faster under LD than under SD (Fig. 3B). The second metabolite eluted in fraction 16 and was chromatographically different from any of the standard GAs which have been run in this HPLC system. Although this metabolite accumulated steadily under LD, it was scarcely present at all under SD (Fig. 3C).

Charcoal column analysis of the aqueous fractions showed very

little highly polar radioactivity from the LD feed of [^{3}H]GA_1 (Fig. 3D). There was, however, a substantial level of radioactivity in this fraction after 2 SD (Fig. 3D). As before, these fractions were run on μBondapak C_{18}, and the radioactivity again disappeared.

DISCUSSION

Previous studies (5) showed that stem elongation in *A. githago* is under photoperiodic control and that endogenous GAs are involved in some way in the mediation of this response. However, measurements of endogenous GAs (5, 6) indicated that the induction of elongation involves more than either a qualitative change in the endogenous GAs, or a simple quantitative change in their levels. To explain the observed fluctuations in GA levels during induction, it was suggested (5) that stem elongation might be controlled by the rate of turnover of GAs, which in turn is controlled by photoperiod (5). Indirect evidence that at least one component of turnover rate (namely, metabolism) is affected by photoperiod has already been obtained (6). When GAs were measured after treatment with the GA biosynthesis inhibitor AMO-1618, the levels of several GAs fell more rapidly under LD than under SD (6). The results of the present study provide further, more direct, evidence that photoperiodic induction of stem elongation is associated with an altered rate of GA metabolism in *A. githago*. Both [^{3}H]GA_{20} (Fig. 1A) and [^{3}H]GA_1 (Fig. 3A) were metabolized faster under LD than under SD, and production of their major acidic metabolites was correspondingly greater under LD than under SD (Figs. 1 and 3).

All three [^{3}H]GAs used in this study occur naturally in *A. githago* (6) and were of high specific activity. Applications were made to plants at times when the endogenous levels of these GAs would have been at their highest (6). Consequently, the metabolites derived from the [^{3}H]GAs should provide a reasonable indication of the natural pathways of metabolism of these GAs in *A. githago*. [^{3}H]GA_{20} was converted under both LD and SD to a compound which was chromatographically identical to 3-*epi*-GA_1. The levels of activity seen in this metabolite (Fig. 1B) are consistent with the loss of label from carbon-3 as a result of 3α-hydroxylation reaction, particularly when the radioactivity found in the very polar fraction (Fig. 1C) is taken into account. [^{3}H]GA_{20} was also 3α-hydroxylated to 3-*epi*-GA_1 in *Bryophyllum* (3), in addition to being 2β-hydroxylated to GA_{29}. Conversion of GA_{20} to GA_{29} has also been demonstrated in *Pisum* (12), in *Phaseolus* (14), and in spinach (8), but there was no evidence for this reaction occurring in *A. githago*. Furthermore, no evidence was obtained for 3β-hydroxylation of [^{3}H]GA_{20} to [^{3}H]GA_1 in the present study, although GA_1 is native to *A. githago* (6) and such a conversion has been observed in other species (7, 14).

In both *Hordeum* (13) and *Phaseolus* (10), [^{3}H]3-*epi*-GA_1 was not metabolized at all, while in *Zea* (2) it was metabolized much more slowly than [^{3}H]GA_1. A similar situation was seen in this study, with the rate of metabolism of [^{3}H]3-*epi*-GA_1 under LD (Fig. 2) being very much slower than that of [^{3}H]GA_1 under either LD or SD (Fig. 3A). Taken together with the known low biological activity of 3-*epi*-GA_1 (1, 6), this result suggests that in *A. githago*, 3-*epi*-GA_1 could well be a deactivation product of GA_{20}. The low level of radioactivity associated with the very polar metabolite formed from [^{3}H]3-*epi*-GA_1 (Fig. 2) is indicative of metabolic reactions at carbons-1 and -2, leading to substantial loss of label. However, the anomalous loss of activity in these very polar fractions during the HPLC process precludes further speculation about their identity. The lack of conversion of [^{3}H]3-*epi*-GA_1 to [^{3}H]GA_1, and vice versa, suggests that the 3-*epi*-GA_1 and GA_1 found in plants of *A. githago* (6) are not present as the result of a simple chemical equilibrium reaction, but may well have different precursors. The evidence described above suggests that GA_{20} is the precursor of 3-*epi*-GA_1, but there is at present no evidence to indicate the precursor of GA_1.

In several species (2, 9, 11, 14), [^{3}H]GA_1 is 2β-hydroxylated to [^{3}H]GA_8. Although the major metabolite of [^{3}H]GA_1 in *A. githago* had an elution volume from the μBondapak C_{18} HPLC, which is indicative of a trihydroxylated GA, it was not identical with GA_8. In view of the lack of evidence for 2β-hydroxylases in *A. githago* (see above) and the lack of detection of GA_8 as a native GA in *A. githago* (M. G. Jones, unpublished data), this is not surprising. The nature of the major metabolite of [^{3}H]GA_1 in *A. githago* remains unknown at present, although the loss of label resulting from this metabolism (compare Fig. 3B with Fig. 3A) again suggests reactions at carbon-1 or -2. The nature of the other metabolites of [^{3}H]GA_1 likewise remains unknown, although it is interesting to note that there appeared to be a qualitative difference between the minor metabolites formed under LD (Fig. 3C) compared with those formed under SD (Fig. 3D).

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