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# Gibberellin Metabolism in Cell-Free Extracts from Spinach Leaves in Relation to Photoperiod[1]

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## ABSTRACT

**Cell-free extracts capable of converting [$^{14}$C]-labeled gibberellins (GAs) were prepared from spinach (*Spinacia oleracea* L.) leaves. [$^{14}$C]-labeled GAs, prepared enzymically from [$^{14}$C]mevalonic acid, were incubated with these extracts, and products were identified by gas chromatography-mass spectrometry. The following pathway was found to operate in extracts from spinach leaves grown under long day (LD) conditions: $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$. The pH optima for the enzymic conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$ and [$^{14}$C]$GA_{19}$ were approximately 7.0, 8.0, and 6.5, respectively. These three enzyme activities required $Fe^{2+}$, $\alpha$-ketoglutarate and $O_2$ for activity, and ascorbate stimulated the conversion of [$^{14}$C]$GA_{53}$ and [$^{14}$C]$GA_{19}$. Extracts from plants given LD or short days (SD) were examined, and enzymic activities were measured as a function of exposure to LD, as well as to darkness following 8 LD. The results indicate that the activities of the enzymes oxidizing $GA_{53}$ and $GA_{19}$ are increased in LD and decreased in SD or darkness, but that the enzyme activity oxidizing $GA_{44}$ remains high irrespective of light or dark treatment. This photoperiodic control of enzyme activity is not due to the presence of an inhibitor in plants grown in SD. These observations offer an explanation for the higher $GA_{20}$ content of spinach plants in LD than in SD.**

Spinach (*Spinacia oleracea* L.) is a LD rosette plant in which stem growth in LD is mediated by GAs[2] (19, 20). Six 13-hydroxylated GAs have been identified in spinach leaves (14). Of these, $GA_{53}$, $GA_{44}$, $GA_{19}$, $GA_{20}$, and $GA_{29}$ are related metabolically as shown in Figure 1. Although all the steps of this pathway have not been unequivocally demonstrated in spinach, the evidence strongly suggests that the pathway does operate (5, 16).

When spinach plants grown in SD are transferred to LD the level of endogenous $GA_{19}$ decreases, while levels of $GA_{20}$ and $GA_{29}$ increase (15). This suggests that the photoperiod regulates the conversion of $GA_{19}$ to $GA_{20}$, which is essential for stem growth. Further evidence for this hypothesis was put forward by Gianfagna *et al.* (5) who found that [$^2$H]$GA_{53}$ applied to spinach leaves was converted to [$^2$H]$GA_{20}$ in LD, but only to [$^2$H]$GA_{44}$ and [$^2$H]$GA_{19}$ in SD.

GA metabolic pathways have been extensively studied using cell-free enzyme preparations from *Cucurbita maxima* endosperm (6, 8, 9), *Pisum sativum* embryos (11), and *Phaseolus coccineus* embryos (18). Cell-free extracts from spinach leaves were therefore prepared to study the pathway of GA metabolism and its photoperiodic control in this LD plant.

## MATERIALS AND METHODS

**Preparation of [$^{14}$C]GAs.** [$^{14}$C]$GA_{12}$ and [$^{14}$C]$GA_{12}$-aldehyde were prepared from *R*-[2-$^{14}$C]mevalonic acid (Amersham, 1.96 GBq mmol$^{-1}$), using a cell-free system from *Cucurbita maxima* endosperm (18). [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, [$^{14}$C]$GA_{19}$, [$^{14}$C]$GA_{20}$, and [$^{14}$C]$GA_{29}$ were prepared from [$^{14}$C]$GA_{12}$ using a cell-free system from *Pisum sativum* embryos (11). The specific radioactivities of the [$^{14}$C]GAs, which were determined by GC-MS (3), were as follows: [$^{14}$C]$GA_{12}$-aldehyde, 2.58 GBq.mmol$^{-1}$; [$^{14}$C]-$GA_{12}$, 6.06 GBq.mmol$^{-1}$; [$^{14}$C]$GA_{53}$, 4.50 GBq.mmol$^{-1}$ or 7.14 GBq.mmol$^{-1}$; [$^{14}$C]$GA_{44}$, 4.88 GBq.mmol$^{-1}$; [$^{14}$C]$GA_{19}$, 3.66 GBq.mmol$^{-1}$ or 4.59 GBq.mmol$^{-1}$. The specific radioactivities of the [$^{14}$C]$GA_{20}$ and [$^{14}$C]$GA_{29}$ were not determined. The radiochemical purity of all [$^{14}$C]GAs was checked by HPLC (10) with on-line radiocounting.

**Plant Material.** Spinach (*Spinacia oleracea* L., cv Savoy Hybrid 612; Harris Seed Co., Rochester, NY) was grown as described previously (19). Plants were maintained in SD in which an 8 h high intensity photoperiod was followed by 16 h darkness. Plants approximately 6 weeks old were transferred to LD in which an 8 h high intensity photoperiod was followed by 16 h of low light intensity from incandescent lamps (19).

**Preparation of Cell-Free Extracts from Spinach Leaves.** Young and partially expanded spinach leaf blades (1–9 cm in length) were frozen in liquid $N_2$ and pulverized. All subsequent manipulations were carried out at 4°C. Insoluble PVP and 50 mM Hepes buffer, pH 7.0, containing 10 mM DTT and 5 mM ascorbic acid were added (1:0.5:3, w/w/v, plant material:PVP:buffer). The mixture was homogenized using a pestle and mortar, and the homogenate was squeezed through two layers of cheesecloth. The resulting extract was centrifuged at 2,000*g* for 5 min, and then at 200,000*g* for 1 h. The 200,000*g* supernatant was fractionated using $(NH_4)_2SO_4$ precipitation. The fraction precipitating between 30 and 60% $(NH_4)_2SO_4$ saturation was redissolved in a minimum of the homogenization buffer and dialyzed twice against 100 volumes of 50 mM Hepes buffer (pH 7.0) containing 1 mM DTT, 5 mM ascorbic acid, and 10 mM $MgCl_2$, and once against the same mixture but containing 10 mM DTT. The enzyme preparation was dialyzed for 1 h against each change of buffer. The resulting crude enzyme preparation was frozen in 0.5 ml batches in liquid $N_2$ and stored either at −80°C or under liquid $N_2$. Generally 90 g fresh weight of leaves gave a preparation of approximately 7 ml containing 30 to 40

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[2] Abbreviations: GA(s), gibberellin(s); KG, $\alpha$-ketoglutaric acid

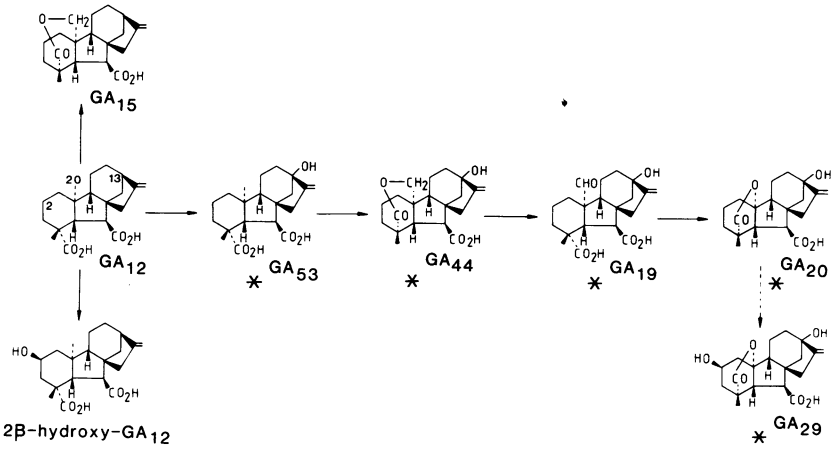


FIG. 1. GA conversions in cell-free systems from spinach leaves grown in LD, except for the step $GA_{20} \rightarrow GA_{29}$ which was not observed. GAs marked with an asterisk are endogenous to spinach (14).

mg/ml protein.

A crude microsomal enzyme fraction was prepared by washing and then resuspending the 200,000*g* pellet in the third dialysis buffer (1 ml buffer per 14 g fresh weight equivalent of tissue).

**Incubations.** The crude enzyme preparation (200 μl), after the addition of $FeSO_4$ and KG (8) to give a final concentration of 0.5 mM and 5 mM, respectively, was incubated with the $^{14}C$-labeled GA (278 Bq, 39–76 pmol) for 1 h (in most cases) at 30°C with gentle shaking (70 rpm). Final substrate concentrations were generally: [$^{14}C$]$GA_{53}$, 303 nM; [$^{14}C$]$GA_{44}$, 279 nM; [$^{14}C$]-$GA_{19}$, 379 nM. After incubation the mixture was adjusted to pH 2.5 with 0.1 N HCl and partitioned against ethyl acetate (3 × 0.5 ml). The organic phase was backwashed with distilled $H_2O$ (1 × 0.5 ml). The resulting organic extract was evaporated to dryness under a stream of $N_2$ and analyzed by HPLC with radiocounting. For GC-MS product identifications, batches of 3.7 to 9.5 kBq of substrate, (333 to 611 nM) were incubated in 2 ml of enzyme.

**HPLC-Radiocounting.** Incubation products were separated by HPLC as described previously (10). In the early phase of this work an analytical column (30 × 0.4 cm i.d.) packed with μBondapak $C_{18}$ (Waters Associates) attached to a $C_{18}$ Guard-PAK precolumn module (Waters Associates) was used. A 30 min gradient of 20 to 90% methanol in 1% aqueous acetic acid at a flow rate of 2.0 ml $min^{-1}$ was run. In later work a 5 μm NOVA-PAK $C_{18}$ column (15 × 0.39 cm i.d.; Waters Associates) was used. A 40 min gradient of 40 to 90% methanol in 1% aqueous acetic acid at a flow rate of 1.0 ml $min^{-1}$ was used. The effluent was passed through a radioactive flow detector (Flo-One model, RadioAnalytic, Inc.) using a liquid scintillator flow cell. A ratio of 1:2, column effluent: scintillation fluid (Flo-Scint II, RadioAnalytic Inc.), was used. Counting efficiency was 85%.

**GC-MS.** Analysis by GC-MS was performed as described (18). GAs were identified by retention times and full spectra, which were compared with those of standard compounds. Specific radioactivities were determined by scanning the molecular ion clusters, including strong $[M + 8]^+$ and $[M + 6]^+$ ions due to the incorporation of high specific radioactivity [2-$^{14}C$]mevalonic acid into [$^{14}C$]$GA_{12}$ by the *C. maxima* system (11), and calculating the amount of $^{14}C$ by the method of Bowen *et al.* (3).

**Protein Determination.** Protein was determined according to Bradford (4) using the Bio-Rad protein assay reagent (Bio-Rad Laboratories).

**Reproducibility.** Each experiment was repeated at least once. Although the magnitude of the response obtained with enzyme preparations from different batches of plants varied, the results obtained were consistent.

## RESULTS

**Preliminary Experiments.** The conditions for obtaining an enzyme preparation from spinach leaves grown under LD capable of converting [$^{14}C$]$GA_{19}$ and [$^{14}C$]$GA_{53}$ were first determined. No conversion of either GA was observed in the absence of DTT. Preparations in which the buffer contained 10 mM DTT showed greatly increased enzyme activity over those which contained only 1 mM DTT (Table I). The presence of ascorbic acid in the extraction buffer slightly stimulated enzyme activity (Table I) and reduced browning of the extract.

Extracts made from either petioles or fully mature leaves were much less active than extracts made from young and partially expanded leaves (data not shown). Therefore, enzyme preparations were routinely made from young and partially expanded leaf blades (1–9 cm in length).

Enzyme activity could be precipitated by $(NH_4)_2SO_4$ (Table II). Consequently, $(NH_4)_2SO_4$ precipitation (30–60% $(NH_4)_2SO_4$ saturation), followed by dialysis, was used routinely in enzyme preparations.

**Conversion of GAs by the Enzyme Preparation and Identification of Products.** The GAs thought to be involved in the metabolic pathway in spinach were tested as substrates with the enzyme preparation (Table III). There was no significant conversion of any of these GAs by the 200,000*g* pellet (crude microsomal preparation). No conversion of [$^{14}C$]$GA_{53}$, [$^{14}C$]$GA_{44}$, or [$^{14}C$]-$GA_{19}$ was observed after incubation with the complete incubation mixture either without addition of enzyme or with a boiled enzyme extract.

The identities of the main products of the complete incubation mixture were confirmed by GC-MS, and their specific radioac-

Table I. *Effect of DTT and Ascorbate in Extraction Buffer on Conversion of [$^{14}C$]$GA_{53}$ and [$^{14}C$]$GA_{19}$ by Spinach Cell-Free Extracts*

Enzyme extracts were prepared from plants grown in LD as described in the "Materials and Methods," except that the concentrations of DTT and ascorbate in the buffer were as indicated in the table. Incubations with [$^{14}C$]$GA_{53}$ (303 nM) and [$^{14}C$]$GA_{19}$ (379 nM) were performed for 1 h 40 min.

| Substrate | Additions to Extraction Buffer | Protein Concentration | Conversion to Products |
|---|---|---|---|
| | | *mg/ml* | % |
| [$^{14}C$]$GA_{53}$ | 1 mM DTT + 5 mM ascorbate | 22.8 | 4.9 |
| [$^{14}C$]$GA_{53}$ | 10 mM DTT + 5 mM ascorbate | 30.8 | 27.4 |
| [$^{14}C$]$GA_{53}$ | 10 mM DTT | 30.0 | 18.3 |
| [$^{14}C$]$GA_{19}$ | 1 mM DTT + 5 mM ascorbate | 22.8 | 1.8 |
| [$^{14}C$]$GA_{19}$ | 10 mM DTT + 5 mM ascorbate | 30.8 | 17.0 |
| [$^{14}C$]$GA_{19}$ | 10 mM DTT | 30.0 | 12.3 |

Table II. *Fractionation of Enzyme Preparations from LD Treated Spinach Leaves by $(NH_4)_2SO_4$ Precipitation*

Enzyme extracts were prepared as described in "Materials and Methods," except that $(NH_4)_2SO_4$ fractions were taken as indicated in the table. Incubations with [$^{14}$C]$GA_{53}$ (505 nM), [$^{14}$C]$GA_{44}$ (279 nM) and [$^{14}$C]$GA_{19}$ (approximately 279 nM) were performed for 1 h.

| $(NH_4)_2SO_4$ Saturation | Protein Concentration[a] | | Conversion to Products per Substrate: | | |
|---|---|---|---|---|---|
| | Expt I | Expt II | [$^{14}$C]$GA_{53}$ | [$^{14}$C]$GA_{44}$ | [$^{14}$C]$GA_{19}$[b] |
| % | *mg/ml* | | | | |
| 0–30 | 6.8 | 12.8 | 0 | 0.6 | 0 |
| 30–50 | 37.5 | 50.5 | 96.5 | 62.1 | 5.8 |
| 50–70 | 13.8 | 23.0 | 9.8 | 13.6 | 0.1 |
| 70–90 | ND[c] | 3.1 | ND[c] | 0.9 | 0 |

[a] Expt I refers to the conversion of [$^{14}$C]$GA_{53}$, and Expt II to the conversions of [$^{14}$C]$GA_{44}$ and [$^{14}$C]$GA_{19}$. [b] Specific radioactivity of [$^{14}$C]$GA_{19}$ not determined. [c] Not determined.

tivities were determined (Table IV). [$^{14}$C]$GA_{12}$ was converted to [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{53}$ to [$^{14}$C]$GA_{44}$, [$^{14}$C]$GA_{44}$ to [$^{14}$C]$GA_{19}$, and [$^{14}$C]$GA_{19}$ to [$^{14}$C]$GA_{20}$. [$^{14}$C]$GA_{20}$ was not further metabolized under the conditions employed.

**Cofactor Requirement.** The cofactor requirements for the soluble GA oxidases of *Cucurbita maxima* endosperm (8) were tested in the spinach cell-free preparation. KG, $Fe^{2+}$ (as $FeSO_4$), and $O_2$ (air) were all necessary for enzymic conversion of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$ (Tables V and VI). There was still some enzymic conversion of [$^{14}$C]GAs, even in the absence of $Fe^{2+}$, presumably due to traces of $Fe^{2+}$ remaining after dialysis (Table V). Therefore, EDTA was included to chelate any remaining $Fe^{2+}$, and this showed conclusively the requirement for $Fe^{2+}$ (Table V). Ascorbate, which probably acts by maintaining $Fe^{2+}$ in its reduced form (8), was found to stimulate the conversion of [$^{14}$C]$GA_{53}$ and [$^{14}$C]$GA_{19}$ (Table V). However, in the case of [$^{14}$C]$GA_{44}$, conversion was greater when ascorbate was absent (Table V). None of the three enzymic activities was stimulated by NADPH in the presence of $Fe^{2+}$, KG, and ascorbate (data not shown).

**pH Optima.** The pH optima for the enzymes converting [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$ are shown in Figure 2. In all cases Hepes buffer was used, and the pH was adjusted with HCl or KOH. The pH optimum for the [$^{14}$C]$GA_{53}$ conversion is approximately 7.0, for the [$^{14}$C]$GA_{44}$ conversion approximately 8.0, and for the [$^{14}$C]$GA_{19}$ conversion approximately 6.5.

**Linearity of the Enzyme Assays.** Incubations with [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$ were carried out for different lengths of time at 30°C. The reactions were linear, at least for 1 h (Fig. 3). When an extract was diluted with buffer, activities of the three enzymes oxidizing $GA_{53}$, $GA_{44}$, and $GA_{19}$ were proportional to the protein concentration in the preparations (Fig. 4).

**Kinetic Parameters.** Preliminary kinetic studies with crude enzyme preparations were carried out for the enzymes oxidizing [$^{14}$C]$GA_{53}$ and [$^{14}$C]$GA_{44}$. The values of the apparent $K_m$ for the $GA_{53}$-oxidizing enzyme as derived from Lineweaver-Burk plots in two experiments were 193 and 627 nM. The values obtained for the $GA_{44}$-oxidizing enzyme were 335 and 597 nM. Since no exact values were obtained for the kinetic parameters, and since all activities were measured in the same incubation mixture in spite of different pH optima, the different enzyme activities cannot be compared with each other. However, since enzyme activities are proportional to the protein concentration (Fig. 4), it is valid to compare the relative values obtained for a given substrate. Because of these circumstances, we have preferred to present the results as percent conversion rather than as enzyme specific activities.

**Effect of Daylength on Enzyme Activity.** Plants grown in SD were placed in LD (continuous light) for increasing periods of time. Enzyme extracts were made at different times from leaves of equal fresh weight, and tested for $GA_{53}$, $GA_{44}$, and $GA_{19}$ oxidation. Preparations from plants grown in SD and harvested at the end of the 16-h dark period did not convert [$^{14}$C]$GA_{53}$ or [$^{14}$C]$GA_{19}$, but conversion was measurable after 9 h in the light and further increased thereafter (Fig. 5). In another experiment activity was measurable after as little as 4 h (data not shown). The activity of the $GA_{44}$-oxidizing enzyme remained high over the entire period, indicating that this enzyme is not under photoperiodic control.

Plants that were given 3 LD and then returned to SD for 1 d lost enzyme activity for the $GA_{53}$ and $GA_{19}$ conversions. This is consistent with the observation (19) that stem elongation ceases

Table III. *Substrates Converted by a Crude Enzyme Preparation from Spinach Leaves Grown in LD*

Enzyme extracts were prepared as described in "Materials and Methods." Incubation times were 2 h, and the volume was 2 ml, except for [$^{14}$C]$GA_{12}$-aldehyde, [$^{14}$C]$GA_{20}$ and [$^{14}$C]$GA_{29}$, where the incubation time was 1 h and the volume was 0.2 ml. Tentative product identifications were made on the basis of HPLC retention times.

| Substrate | Substrate Concentration | Protein Concentration | Tentative Product Identifications | Product Retention Time |
|---|---|---|---|---|
| | *nM* | *mg/ml* | | *min*[a] |
| [$^{14}$C]$GA_{12}$-aldehyde | 551 | 38.8 | No significant conversion | |
| [$^{14}$C]$GA_{12}$ | 611 | 45.9 | [$^{14}$C]$GA_{53}$ | 22.9 |
| | | | [$^{14}$C]$GA_{15}$ | 24.1 |
| | | | [$^{14}$C]2$\beta$-hydroxy-$GA_{12}$[b] | 19.1 |
| [$^{14}$C]$GA_{53}$ | 383 | 24.0 | [$^{14}$C]$GA_{44}$ | 15.7 |
| | | | [$^{14}$C]$GA_{19}$ | 16.8 |
| [$^{14}$C]$GA_{44}$ | 333 | 33.2 | [$^{14}$C]$GA_{19}$ | 16.8 |
| | | | [$^{14}$C]$GA_{20}$ | 12.3 |
| [$^{14}$C]$GA_{19}$ | 401 | 31.0 | [$^{14}$C]$GA_{20}$ | 12.3 |
| [$^{14}$C]$GA_{20}$ | Unknown | 38.8 | No conversion | |
| [$^{14}$C]$GA_{29}$ | Unknown | 38.8 | No conversion | |

[a] Retention times were measured on the NOVA-PAK column (see "Materials and Methods"). [b] Identification of 2$\beta$-hydroxy-$GA_{12}$ was made by Prof. J. MacMillan in Bristol, U.K.

Table IV. *Identifications by GC-MS of Products from Incubations of [$^{14}$C]$GA_{12}$, [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$ in Spinach Enzyme Preparations*

Enzyme extracts were prepared from plants in LD as described in "Materials and Methods." Incubations were with 2 ml batches of enzyme, and were carried out for 2 h. Substrate and protein concentrations were: 611 nM [$^{14}$C]$GA_{12}$, 45.9 mg/ml protein; 383 nM [$^{14}$C]$GA_{53}$, 24.0 mg/ml protein; 333 nM [$^{14}$C]$GA_{44}$, 33.2 mg/ml protein; 401 nM [$^{14}$C]$GA_{19}$, 31.0 mg/ml protein.

| Substrate | Specific Activity of Substrate | Product | Specific Activity of Product |
|---|---|---|---|
| | *GBq/mmol* | | *GBq/mmol* |
| [$^{14}$C]$GA_{12}$ | 6.06 | [$^{14}$C]$GA_{53}$ | ND[a] |
| [$^{14}$C]$GA_{53}$ | 7.14 | [$^{14}$C]$GA_{44}$ | 6.48 |
| [$^{14}$C]$GA_{44}$ | 4.88 | [$^{14}$C]$GA_{19}$ | 4.96 |
| [$^{14}$C]$GA_{19}$ | 4.59 | [$^{14}$C]$GA_{20}$ | 4.00 |

[a] Not determined.

Table V. *Cofactor Requirements for the Conversion of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$ and [$^{14}$C]$GA_{19}$ by Spinach Cell-Free Extracts*

Enzyme extracts were prepared from plants in LD as described in "Materials and Methods." In all cases the protein concentration was 34.4 mg/ml. Incubations of [$^{14}$C]$GA_{53}$ (303 nM) and [$^{14}$C]$GA_{19}$ (329 nM) were for 1.5 h, and of [$^{14}$C]$GA_{44}$ (279 nM) for 1 h.

| Cofactor | | | | Conversion to Products per Substrate: | | |
|---|---|---|---|---|---|---|
| $FeSO_4$ (0.5 mM) | KG (5 mM) | Ascorbate (5 mM) | EDTA (2 mM) | [$^{14}$C]$GA_{53}$ | [$^{14}$C]$GA_{44}$ | [$^{14}$C]$GA_{19}$ |
| + | + | + | − | 32.9 | 47.8 | 19.4 |
| − | + | + | − | 16.8 | 17.1 | 14.1 |
| − | + | + | + | 0.5 | 1.2 | 0.1 |
| − | + | − | + | 0 | 1.7 | 0 |
| + | − | + | − | 1.5 | 9.0 | 0.4 |
| + | + | − | − | 8.0 | 64.0 | 7.0 |

Table VI. *Requirement for $O_2$ in the Conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$ by Spinach Cell-Free Extracts*

Enzyme extracts were prepared from plants in LD as described in "Materials and Methods." For the nitrogen treatments the tubes with the enzyme extracts were flushed with $N_2$ for 30 min prior to incubation with the substrates. Incubations of [$^{14}$C]$GA_{53}$ (303 nM), [$^{14}$C]$GA_{44}$ (279 nM), and [$^{14}$C]$GA_{19}$ (379 nM) were for 1 h.

| Substrate | Protein Concentration | Conversion to Products | |
|---|---|---|---|
| | | Air | Nitrogen |
| | *mg/ml* | *%* | |
| [$^{14}$C]$GA_{53}$ | 40.0 | 48.9 | 1.4 |
| [$^{14}$C]$GA_{44}$ | 38.8 | 53.4 | 11.9 |
| [$^{14}$C]$GA_{19}$ | 40.0 | 10.8 | 0.3 |

when plants are transferred back from LD to SD. The activity of the $GA_{44}$ conversion was high enough that the substrate was used up irrespective of photoperiod (Table VII). This experiment was repeated with saturating substrate concentrations (5 μM) to ensure representative results (Table VII).

Plants exposed to 8 LD (continuous light) were placed in darkness and harvested after various times. Enzyme activities for the [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$ conversions were measured in extracts from equivalent fresh weights of material (Fig. 6). The activities of the $GA_{53}$- and $GA_{19}$-oxidizing enzymes decreased rapidly during the first hours in darkness and then remained low for the duration of the experiment. The activity of

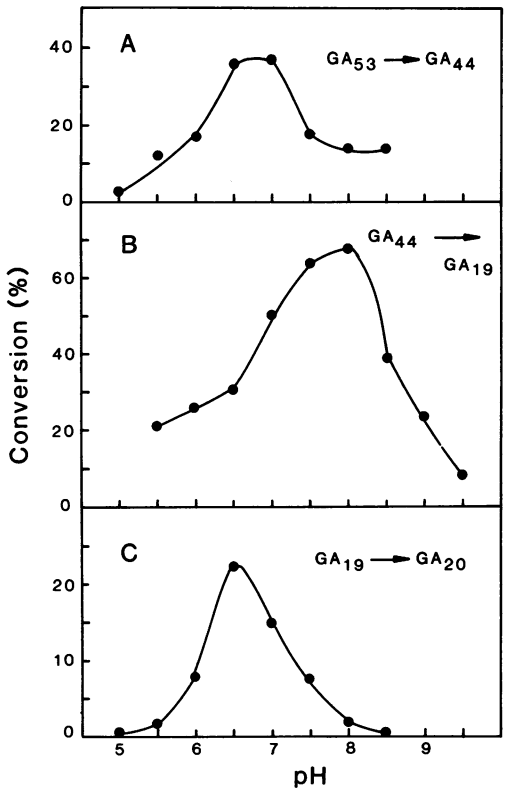


FIG. 2. Effect of pH on conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$ and [$^{14}$C]-$GA_{19}$ by cell-free extracts from spinach grown in LD. A, Conversion of [$^{14}$C]$GA_{53}$ to [$^{14}$C]$GA_{44}$. Protein concentration was 30.8 mg/ml, and incubation of [$^{14}$C]$GA_{53}$ (303 nM) was for 1 h. B, Conversion of [$^{14}$C]-$GA_{44}$ to [$^{14}$C]$GA_{19}$. Protein concentration was 15.6 mg/ml, and incubation of [$^{14}$C]$GA_{44}$ (279 nM) was for 30 min. C, Conversion of [$^{14}$C]$GA_{19}$ to [$^{14}$C]$GA_{20}$. Protein concentration was 30.8 mg/ml, and incubation of [$^{14}$C]$GA_{19}$ (379 nM) was for 1 h.

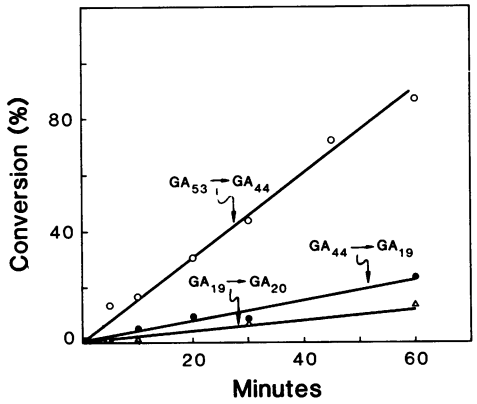


FIG. 3. Linearity of conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$ and [$^{14}$C]-$GA_{19}$ with time by cell-free extracts from spinach grown in LD. Substrate and protein concentrations were: [$^{14}$C]$GA_{53}$, 303 nM, 30.8 mg/ml protein; [$^{14}$C]$GA_{44}$, 279 nM, 38.8 mg/ml protein; [$^{14}$C]$GA_{19}$, 379 nM, 30.8 mg/ml protein.

the $GA_{44}$-oxidizing enzyme again remained high, indicating that the activity of this enzyme is not affected by darkness.

**Mixture of SD and LD Leaves.** The decreased ability of preparations from plants in SD to oxidize [$^{14}$C]$GA_{53}$ and [$^{14}$C]-$GA_{19}$ could be due to the formation of an inhibitor under this photoperiod. Therefore, extracts were made from leaves of plants grown in SD, from leaves of plants given 8 LD, and from a 1:1 mixture of the two. Table VIII shows that a SD enzyme prepa-

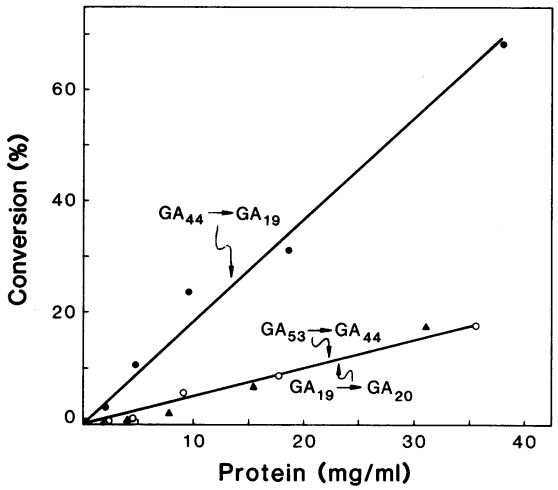


FIG. 4. Linearity of conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]-$GA_{19}$ with protein concentration by cell-free extracts from spinach grown in LD. Incubations of [$^{14}$C]$GA_{53}$ (303 nM), [$^{14}$C]$GA_{44}$ (279 nM), and [$^{14}$C]$GA_{19}$ (379 nM) were for 1 h.

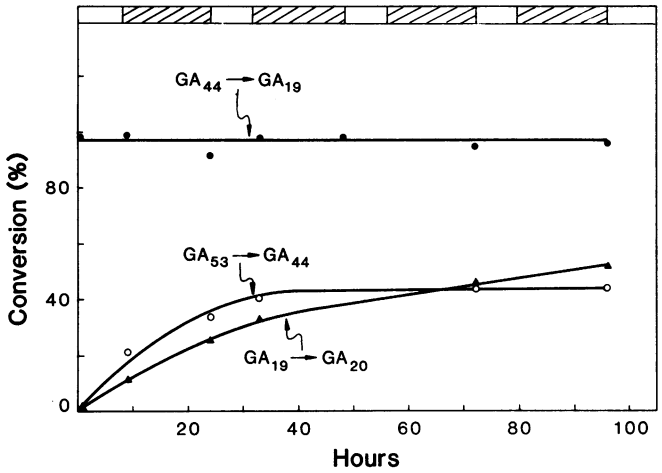


FIG. 5. Time course after transferring plants from SD to LD for conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$. Protein concentrations per time point ranged from 25.3 to 34.8 mg/ml. Incubations of [$^{14}$C]$GA_{53}$ (303 nM) and [$^{14}$C]$GA_{19}$ (379 nM) were for 1 h, and of [$^{14}$C]-$GA_{44}$ (279 nM) was for 1 h 40 min. Hatched bars indicate periods of low light intensity and open bars indicate high light intensity.

ration did not reduce enzyme activity in a LD preparation. This rules out the possibility of an inhibitor being present.

## DISCUSSION

The results show that a cell-free system from spinach leaves converts several native GAs according to the scheme in Figure 1. Although $GA_{12}$ has not been shown to be endogenous to spinach (14), it was converted to $GA_{53}$ (Table IV) and may therefore be an intermediate in GA biosynthesis in spinach. In a number of plants, $GA_{12}$ is known to be an intermediate in the biosynthesis of $C_{19}$-GAs (7). $GA_{12}$-Aldehyde was not converted by the same preparation in the presence of KG, $Fe^{2+}$, and NADPH.

In pea embryos, 13-hydroxylation is associated with the microsomal fraction (11). However, in spinach leaf preparations 13-hydroxylation was found to take place in the soluble fraction and not in the crude microsomal fraction. Even enzyme preparations that had been passed through a 0.45 μm Millipore filter

Table VII. *Effect of Photoperiod on Conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$ in Spinach Cell-Free Extracts*

Enzyme extracts were prepared as described in "Materials and Methods." In experiment I [$^{14}$C]$GA_{53}$ (303 nM) and [$^{14}$C]$GA_{19}$ (379 nM) were incubated for 1 h and [$^{14}$C]$GA_{44}$ (279 nM) for 1 h 40 min. In experiment II all incubations were for 1 h and all substrate concentrations were 5 μM.

| Substrate | Conversion to Products per Light Treatment: SD | | 3 LD | | 3 LD + 1 SD | |
|---|---|---|---|---|---|---|
| | Expt I | Expt II | Expt I | Expt II | Expt I | Expt II |
| | % | | | | | |
| [$^{14}$C]$GA_{53}$ | 0.5 | 4.7 | 44.4 | 14.8 | 6.1 | 9.3 |
| [$^{14}$C]$GA_{44}$ | 98.3 | 94.9 | 94.7 | 95.9 | 97.1 | 94.7 |
| [$^{14}$C]$GA_{19}$ | 0 | 0.2 | 45.5 | 21.1 | 2.4 | 0.1 |
| Protein concentration (mg/ml) | 29.0 | 30.8 | 25.3 | 31.5 | 34.8 | 24.8 |

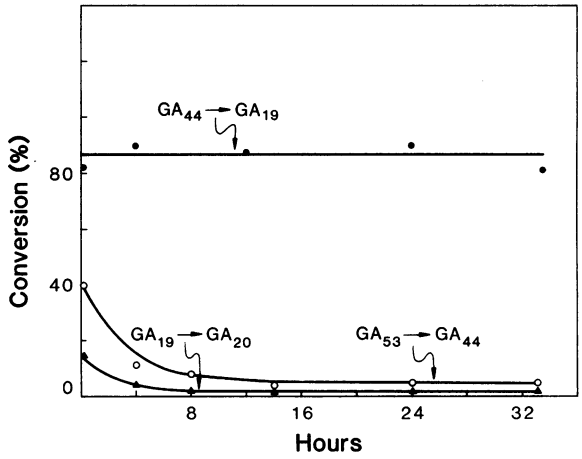


FIG. 6. Time course in darkness following 8 LD for conversions of [$^{14}$C]$GA_{53}$, [$^{14}$C]$GA_{44}$, and [$^{14}$C]$GA_{19}$. Protein concentrations per time point ranged from 11.3 to 21.3 mg/ml. Incubations of [$^{14}$C]$GA_{53}$ (303 nM) and [$^{14}$C]$GA_{19}$ (379 nM) were for 1.5 h, and of [$^{14}$C]$GA_{44}$ (279 nM) was for 1 h 40 min.

Table VIII. *Effect of Mixing Enzyme Preparations from Spinach Plants Growth in SD or LD on the Conversions of [$^{14}$C]$GA_{53}$ and [$^{14}$C]$GA_{19}$*

Enzyme extracts were prepared as described in "Materials and Methods." Incubations of [$^{14}$C]$GA_{53}$ (303 nM) and [$^{14}$C]$GA_{19}$ (379 nM) were for 1 h.

| Substrate | Treatment | Final Protein Concentration | Conversion to Product |
|---|---|---|---|
| | | *mg/ml* | % |
| [$^{14}$C]$GA_{53}$ | SD[a] | 10.3 | 1.1 |
| [$^{14}$C]$GA_{53}$ | LD[a] | 9.7 | 11.3 |
| [$^{14}$C]$GA_{53}$ | SD + LD[b] | 20.0 | 10.5 |
| [$^{14}$C]$GA_{53}$ | SD + LD[c] | 17.0 | 12.4 |
| [$^{14}$C]$GA_{19}$ | SD[a] | 13.9 | 0 |
| [$^{14}$C]$GA_{19}$ | LD[a] | 12.9 | 1.5 |
| [$^{14}$C]$GA_{19}$ | SD + LD[b] | 26.8 | 1.5 |

[a] Diluted: 1:1 with buffer. [b] Extracts were mixed 1:1. [c] Leaves were mixed 1:1 prior to extraction.

to remove any remaining membrane fragments catalyzed 13-hydroxylation of [$^{14}$C]$GA_{12}$ (data not shown). Furthermore, the conversion of [$^{14}$C]$GA_{12}$ to [$^{14}$C]$GA_{53}$ required $Fe^{2+}$ and KG, but not NADPH as in the case of pea embryos (11). Thus, 13-hydroxylation in spinach, as well as oxidations at C-20, are all catalyzed by soluble oxygenases (8).

As a further difference, [$^{14}$C]$GA_{44}$ was converted to [$^{14}$C]$GA_{19}$ in its normal lactonic form in spinach leaf extracts, whereas in pea embryo enzyme preparations only the open lactone form is oxidized to $GA_{19}$ (11). It is possible that spinach leaf preparations contain an enzyme capable of opening the lactone prior to its oxidation.

The conversion of $GA_{20}$ to $GA_{29}$ did not occur in the spinach cell-free system. This may be for technical reasons because $GA_{29}$ is present in relatively high amounts in spinach leaves in LD (15, 16).

Most of the data presented in this paper were obtained with nonsaturating concentrations of substrate, because the availability of substrates was the limiting factor. The data on which major conclusions are based (Table VII) were obtained with saturating (5 $\mu$M) and nonsaturating (279–379 nM) concentrations, and both gave similar results. In addition, it is apparent that even at nonsaturating concentrations, enzyme activity is linearly related to protein concentration (Fig. 4).

The results obtained in this study show that the oxidations of $GA_{53}$ and $GA_{19}$ are under photoperiodic control, whereas the oxidation of $GA_{44}$ is not to any significant extent. Previous work with feeding deuterated $GA_{53}$ (5) suggested that only the conversion of $GA_{19}$ to $GA_{20}$ is under photoperiodic control. The reason for this discrepancy between *in vivo* and *in vitro* conversions is unknown, but it is possible that the longer incubation time with intact plants (48 h) would negate differences in the rate of conversion of $GA_{53}$.

If the enzyme activities measured in crude extracts reflect *in vivo* activities, then the differences in GA metabolism in spinach under SD and LD conditions (15, 16) can be explained. According to the results in Figures 5 and 6, at least the latter part of the GA biosynthetic pathway operates at a very low rate at the end of the 16 h night. Enzyme activities increase during the 8 h light period and then decline again during the subsequent night. Thus, the amount of $GA_{20}$ produced under SD is low. On the other hand, in LD (continuous light) enzyme activities increase to a constant level (Fig. 5), which explains the higher $GA_{20}$ content found in LD than in SD (15). This, in turn, causes stem elongation.

There are many examples in the literature of plant enzymes being activated by light (12). The regulation of several plant genes by light is also well documented (1, 2, 13, 17). At present, it is not known whether the enzymes of the later steps in the GA biosynthetic pathway in spinach are regulated at the level of gene activation or enzyme activity.

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