Growth and Gibberellin A₁ Metabolism in Excised Lettuce Hypocotyls[1]

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ABSTRACT

Excised lettuce (*Lactuca sativa* L. cv. Arctic) hypocotyls retain the ability to elongate in response to exogenously supplied gibberellic acid and gibberellin A₁ (GA₁). We have studied the relationship between metabolism of GA₁ and elongation in this tissue. In 24 hours at 28 C, hypocotyls treated with 3 μM GA₁ double in length while controls elongate less than 45%. After an exogenous hormone supply is removed, hypocotyls continue to grow faster than untreated controls, although as the hormone application time is decreased, the GA₁ concentration required to effect a given length change increases. [³H]GA₁ was used to determine rates of hormone uptake, efflux, and metabolism. In the presence of [³H]GA₁, hypocotyls accumulate and metabolize label for at least 24 hours. When the exogenous label is removed, the amount of acidic GA in the hypocotyl declines rapidly to a constant level while ethyl acetate-insoluble metabolites increase rapidly to a constant level. Lable accumulation and metabolism at any time are proportional to the external GA₁ concentration below 50 μM GA₁. Chromatographic analysis of radioactive compounds present in tissue extracts suggests that unaltered GA₁ is the major component of the acidic ethyl acetate-soluble fraction, and gibberellin A₈ is a minor component. The ethyl acetate-insoluble fraction appears to contain an unidentified GA₁ metabolite with chromatographic properties similar to those of GA₁. The strong retention of accumulated GA₁ confirms the possibility of a continuing requirement for GA₁ during the sustained response to a GA₁ "pulse" but raises the question of accessibility of the stored hormone for growth promotion.

There has been much interest in the role of GA metabolism in controlling the levels and activity of GAs in higher plants (2, 12–16, 19, 22). Most investigations have been conducted with whole plants or seeds or with tissue pieces which do not show a marked response to the applied hormone (6, 7). We have investigated the metabolism of [³H]GA₁[3] in excised lettuce hypocotyls which previously were shown to respond to exogenously supplied GA₃ (21). Although excision can change metabolism occurring in the intact seedling (17), the excised tissue has the advantage that the endogenous hormone supply can be removed. The effects of added GA can be attributed to the exogenous supply, and the response of the tissue to GA or its metabo-

lites can be established. In this paper, we determine the elongation response of excised lettuce hypocotyls to exogenously supplied GA₁ and study the relationship between elongation and hormone metabolism.

MATERIALS AND METHODS

Plant Material. Lettuce (*Lactuca sativa* L. cv. Arctic) seeds were obtained from Samuel Dobie and Son Ltd., Llangollen, Wales, U.K. Hypocotyls were excised from seedlings sown and germinated as described by Silk and Jones (21).

Incubation and Growth Measurement. Lots of 15 hypocotyls were incubated in the light at 28 C in plastic Petri dishes (35 × 10 mm) in 1 ml of medium containing GA₁ as indicated and 10 mM KCl. To terminate a GA treatment ("pulse"), hypocotyls were rinsed with distilled H₂O over cheesecloth and swirled for 15 min in 750 ml distilled H₂O. The rinse and wash were repeated, leading to a 10¹⁰ dilution of the amount of hormone likely to be carried from the pulsing solution by the tissue sample. For the "chase" period, washed sections were incubated in 1 or 2 ml of 10 mM KCl. Growth data were obtained from analysis of microphotographs (21).

Radiochemical Methods. Sections were incubated in [³H]GA₁ (6.02 × 10⁵ cpm/μl; 50 Ci/mmol in ethyl acetate-methanol, 10:1) obtained from L. Rappaport, University of California, Davis, for various times in the presence or absence of carrier GA₁. To terminate a GA pulse in an efflux experiment, sections were rinsed twice as described above, followed by a single 2-min wash in 750 ml distilled H₂O, which should yield a 10⁵ dilution of residual label. The chase was as described above.

Radioactivity was determined for aliquots of the incubation medium. Label in tissue was determined by an adaptation of the methods of Kende (8). Sections were homogenized in methanol (1 ml/15 hypocotyls) in a glass TenBroeck homogenizer. The homogenate was stored for at least 3 hr at 4 C then centrifuged for 10 min at 2000g. The pellet was resuspended in 0.5 ml methanol and recentrifuged. Supernatants were evaporated at 60 C under a stream of filtered air and either resuspended in water for direct chromatographic analysis or resuspended in 0.25 M phosphate buffer (pH 8.5) for fractionation. The buffer was extracted with ethyl acetate, yielding an organic phase designated the basic ethyl acetate-soluble fraction or basic fraction. The buffer was adjusted to pH 2.5 with 2 N HCl and extracted again with ethyl acetate to give an organic phase designated the acidic ethyl acetate-soluble or acidic fraction, expected to contain GA₁ and other acidic GAs. The remaining aqueous phase was designated the ethyl acetate-insoluble fraction, assumed to contain polar GA metabolites, especially GA-glucosides. All fractions were reduced in volume and either counted directly or chromatographed.

Chromatography. Thin layer chromatography was performed

[3] Abbreviations: GA₁, GA₈: gibberellins A₁ and A₈; GC-MS: gas chromatography-mass spectrometry; GC-RC: gas chromatography-radiochromatography.

on prepared adsorbent layers of ChromAR 1000 (Mallinckrodt Chemical Works, St. Louis, Mo.) or Polygram Sil-G (Merck and Co. Ltd., Darmstadt, GFR). Ascending chromatograms were developed with a mixture of either isopropyl alcohol-ammonium hydroxide-water (8:1:1, v/v/v) or isopropyl alcohol-5 N ammonium hydroxide (5:1, v/v). Dried chromatograms were cut into R_F zones and placed directly into scintillation vials.

Fractions for GC were prepared using the partitioning procedures described above. Samples consisting of at least 200 sections in a total volume of 2 ml incubate containing 1.5 μCi[^{3}H]GA$_1$ were harvested at 22 hr. All fractions were evaporated at 60 C in a stream of filtered air and methylated by the addition of diazomethane in acetone. After removal of residual acetone, the methyl esters were silylated with a mixture of hexamethyldisilazane and trimethylchlorosilane in dry pyridine. The resulting solution was used directly for injection.

GC-MS and GC-RC were performed using a Pye 104 gas chromatographer coupled to an AEI MS-30 mass spectrometer for GC-MS. The gas chromatograph was fitted with a glass column (150 cm × 4 mm) packed with 2% OV-1 on a Gaschrom Q support. Carrier helium flow was maintained at 40 ml/min, and each run was temperature-programmed between 180 and 280 C at a rate of 4 C/min. Trapping for GC-RC was performed manually, and the sample containers were changed at 1-min intervals. To identify radioactive components, 10 μg of silylated GA$_1$ and 16 μg of silylated GA$_8$ were added to provide marker peaks on the flame ionization detector trace. Gas emerging from the column was split 12:1 in favor of the sample trap. Trapped radioactivity was eluted with a toluene-based scintillation fluid and counted at 38% efficiency in a Packard liquid scintillation spectrometer.

RESULTS

Elongation in Response to GA$_1$. Exogenously supplied GA$_1$ increases the elongation rate of excised hypocotyls (Fig. 1). In the presence of 3 μM GA$_1$, hypocotyls elongate 98% ± 24% in 24 hr at 28 C. With 0.3 μM GA$_1$, hypocotyls elongate 76% ± 18% while controls elongate 34% ± 6% in the same period. If an exogenous GA$_1$ supply is removed (i.e. if hypocotyls are "pulsed"), hypocotyls continue to elongate at an elevated rate. As the pulse time is decreased, the GA concentration required
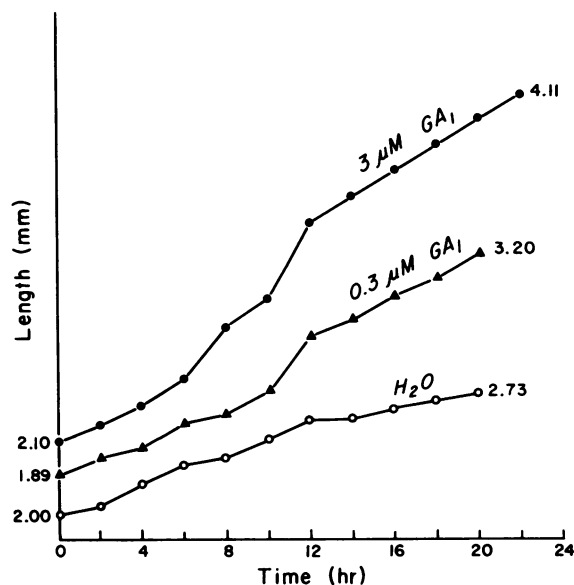
to effect a given length change is increased; that is, the dose response curve is shifted to higher doses for shorter hormone application times (Fig. 2). This general relationship has been observed for GA$_3$ effects on intact seedlings (20) and on excised *Avena* segments (11).

Accumulation and Metabolism of [^{3}H]GA$_1$. In the presence of [^{3}H]GA$_1$, hypocotyls accumulate radioactivity for at least 24 hr (Fig. 3), and the rate of uptake is unaffected by the addition of carrier GA$_1$ to the medium (Table I). A time course for the accumulation of acidic gibberellins (acidic ethyl acetate-soluble compounds) and formation of presumed GA$_1$ metabolites (basic ethyl acetate-soluble and ethyl acetate-insoluble compounds) is shown in Figure 3. The three fractions increase during the 24-hr period of incubation with [^{3}H]GA$_1$. Ethyl acetate-insoluble compounds represent less than 20% of total label after a 2-hr incubation and more than 50% of total label after a 24-hr incubation period.

When [^{3}H]GA$_1$ is removed from the incubation medium, efflux and metabolism of accumulated acidic gibberellins are observed. The distribution of label in the acidic, basic, and insoluble fractions of sections pulsed with [^{3}H]GA$_1$ for 2 hr and incubated in the absence of label is shown in Figure 4. Label in the acidic fraction declines rapidly during the first 4 hr of incubation and thereafter remains apparently constant. Label in the ethyl acetate-insoluble fraction and in the chase medium increases during the same 4-hr incubation period and also remains at a constant level for the duration of the incubation period. Radioactivity in the basic fraction represents a minor portion, about 10% of the total label (Fig. 4B). From Figure 4C it can be seen that 21 hr after withdrawal of exogenous GA$_1$, hypocotyls retain 59% of the label accumulated during the 2-hr pulse
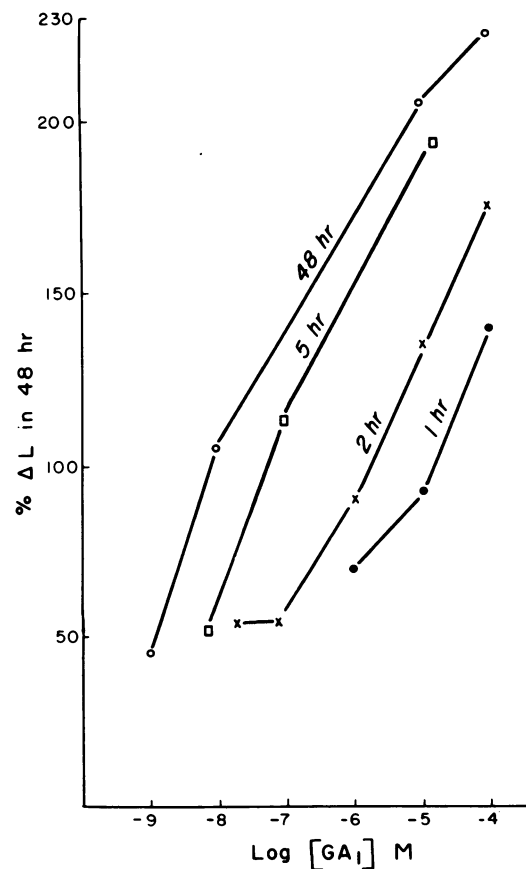

FIG. 1. Effect of GA$_1$ on growth of lettuce hypocotyls incubated with 10 mM KCl; y axis plots displaced for vertical clarity; points are average length of 10 hypocotyls.

FIG. 2. Effect of GA$_1$ concentration on growth of lettuce hypocotyl sections after incubation for 1, 2, 5, and 48 hr in GA$_1$ followed by incubation in 10 mM KCl for the remainder of the incubation period.
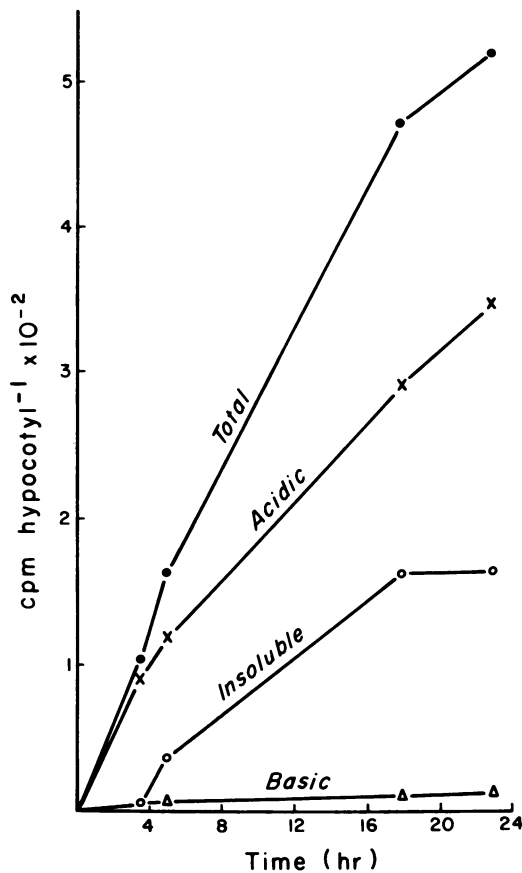
period. Of the label remaining after 21 hr, 31% is in the acidic GA fraction. About 22% of the acidic GA present at the end of the 2-hr pulse is retained for 21 hr.

Similar patterns of label distribution result after a 2-hr pulse whether the pulse is administered early or late in the growth period. Figure 5 shows label in the acidic fraction after a 2-hr pulse administered at zero time and in the same fraction after a 2-hr pulse administered after 15 hr of hormone treatment. After each pulse, radioactivity decayed to a constant level by about 4 hr after removal of labeled hormone.

The distribution of label in the acidic, basic, and insoluble partition fractions and in the incubation medium of sections pulsed with [³H]GA₁ for 2 hr followed by an incubation period of 9 hr in medium without GA₁ is not affected by the presence of carrier GA₁ up to 50 μM (Fig. 6). At 500 μM GA₁ and above, however, the proportion of label in the acidic fraction increases markedly while that in the insoluble fraction decreases.

Chromatographic Analyses of Radioactive Metabolites. Thin layer chromatography of 80% methanolic extracts of lettuce hypocotyl sections incubated continuously in 1.5 μCi[³H]GA₁ for up to 22 hr is shown in Figure 7. Radioactivity is associated exclusively with the known position of GA₁ at R_F 0.5 to 0.61 at incubation times of up to 5 hr in the absence of GA₁. At 22 hr, however, radioactivity is found at R_F 0.09 to 0.26, 0.35 to 0.5,

FIG. 3. Total uptake of [³H]GA₁ by lettuce hypocotyl sections and the distribution of this label among acidic, basic, and ethyl acetate-insoluble fractions.

Table I. Effect of Increasing Carrier GA₁ Concentration on the Accumulation of [³H]GA₁

15 hypocotyls were incubated for 3 hr in [³H]GA₁ plus cold GA₁.

cpm in bathing soln	[GA_external]	cpm in hypocotyls	cpm normalized[1]
830160	10^{-4}	3316	8.9×10^{-5}
556220	10^{-5}	1896	7.6×10^{-5}
980240	10^{-6}	2817	6.4×10^{-5}
1338260	10^{-7}	1581	2.6×10^{-5}
674840	10^{-12}	1457	4.8×10^{-5}

[1]Accumulated label shown in column 3 x (hypocotyl)⁻¹ x hr⁻¹ x (label in bathing solution)⁻¹.
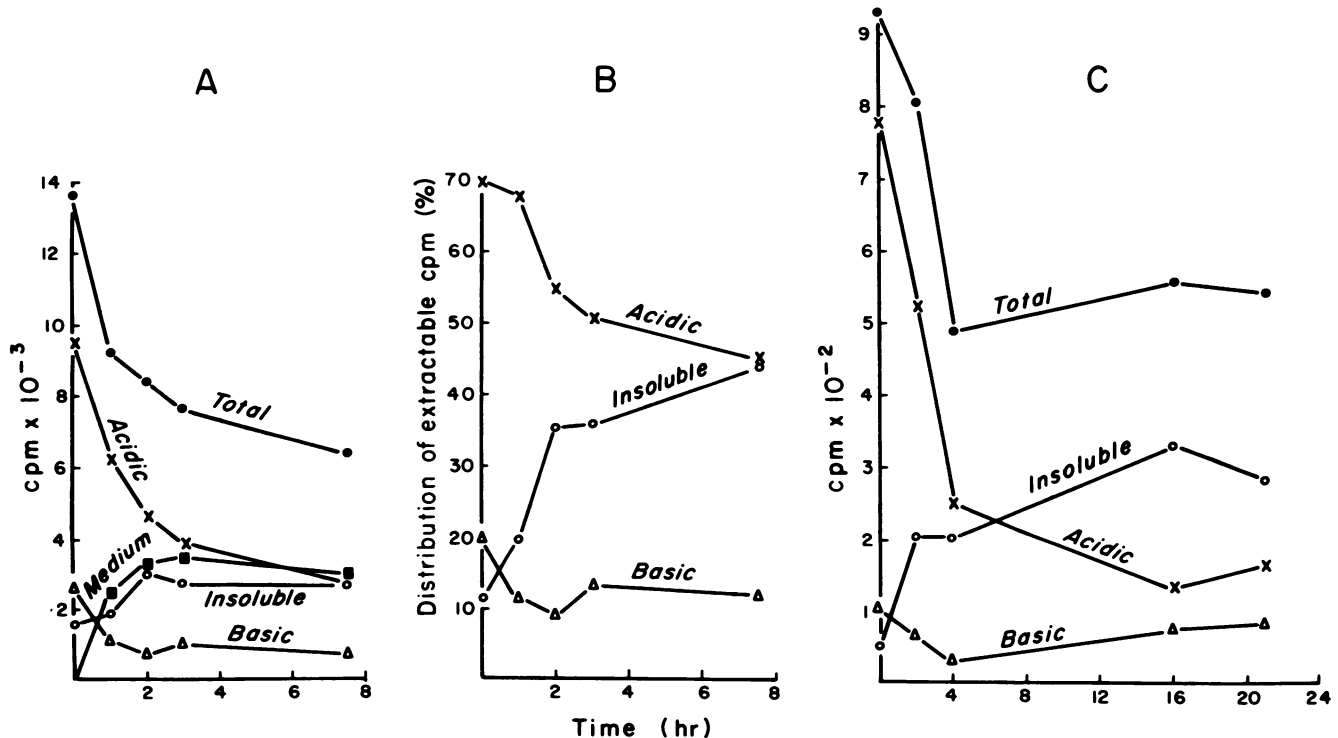

FIG. 4. Distribution of radioactivity in the medium, total homogenate, and acidic, basic, and ethyl acetate-insoluble fractions of hypocotyl sections. A and B: During 7.5-hr incubation in 10 mM KCl following a 2-hr pulse of [³H]GA₁ expressed as total cpm (A) and % extractable cpm (B); C: during 21-hr incubation in 10 mM KCl following a 2-hr pulse.
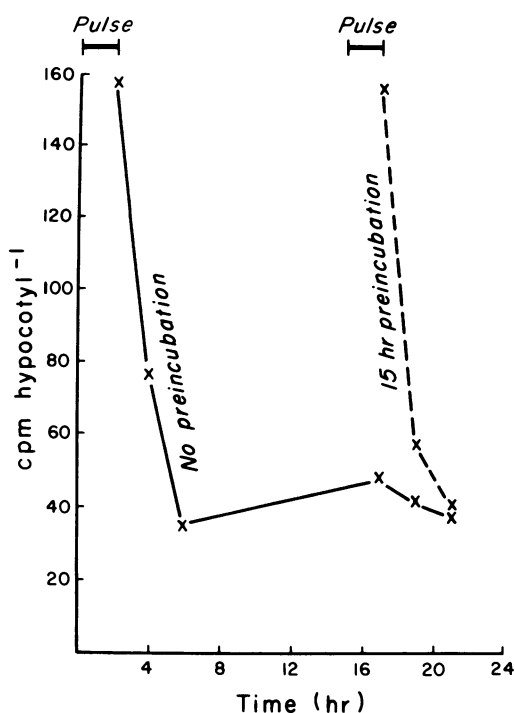
FIG. 5. Radioactivity in the acidic ethyl acetate-soluble fraction of extracted hypocotyl sections pulsed with [³H]GA₁ for 2 hr and incubated in 10 mM KCl with no preincubation and with 15 hr preincubation in 50 μM carrier GA₁.

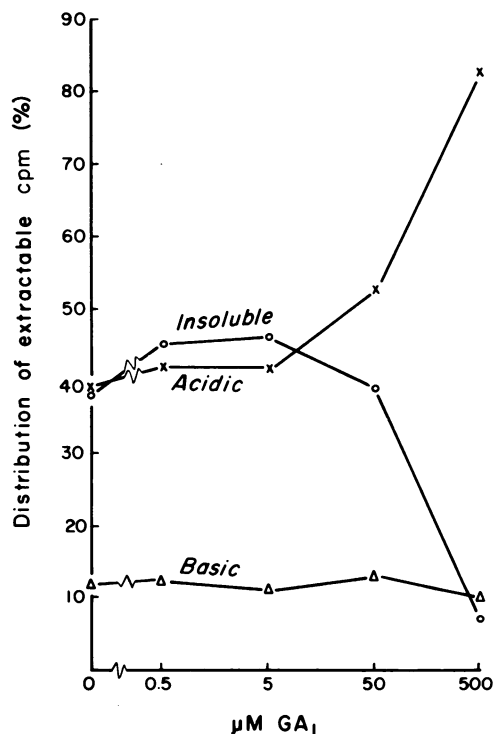FIG. 6. Effect of carrier GA₁ concentration applied during the pulse and subsequent incubation period on the distribution of radioactivity in acidic, basic, and ethyl acetate-insoluble fractions extracted from hypocotyl sections incubated 9 hr in 10 mM KCl after a 2-hr pulse of [³H]GA₁.
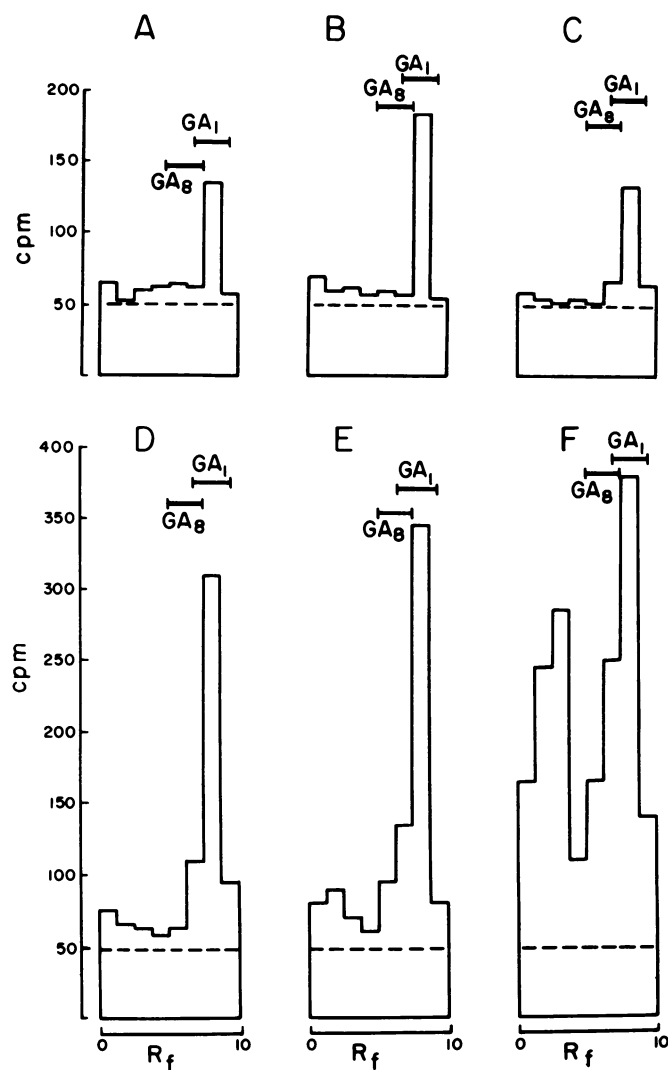

FIG. 7. TLC on Polygram Sil-G of 80% (v/v) methanolic extracts of hypocotyl sections incubated in [³H]GA₁ for 15 min (A), 30 min (B), 1 hr (C), 2.75 hr (D), 5 hr (E), and 22 hr (F). Chromatograms developed with isopropyl alcohol-5 N NH₄OH (5:1, v/v). Background radioactivity indicated by broken lines.

and 0.5 to 0.61 (Fig. 7). When a methanolic extract from sections incubated for 8 hr without GA₁ after an initial pulse of 1 hr with 1.5 μCi[³H]GA₁ is partitioned into acidic, basic, and insoluble fractions and chromatographed, the distribution of

radioactivity is typical of that shown in Figure 8. Label is found at the position of GA₁ in all three fractions, and as indicated by previous experiments, most of the label at this time is distributed about equally between acidic and ethyl acetate-insoluble fractions (Figs. 3, 4, and 6).

GC-RC of an acidic ethyl acetate-soluble fraction from hypocotyl sections incubated with 1.5 μCi[³H]GA₁ for 22 hr shows the major radioactive component at the retention time of GA₁ (Fig. 9). Approximately 14% of the total radioactivity was recovered in compounds with longer retention times, and approximately 33% of these counts were coincident with the GA₈ marker peak. Since formation of [³H]GA₈ by 2-hydroxylation of [³H]GA₁ involves the elimination of ³H at the 2 position, a correction for loss of radioactivity must be applied by multiplying by 1.66. On this basis, GC-RC data suggest a maximum conversion of 7% of [³H]GA₁ to [³H]GA₈ during a 22-hr incubation period. GC-RC analysis also indicated a trace of GA₁ in the basic fraction after partitioning, but attempts to chromatograph the insoluble fraction were unsuccessful.

The occurrence of GA₁ in the acidic ethyl acetate fraction of extracts of hypocotyls incubated for 22 hr in 1.5 μCi[³H]GA₁ was confirmed by GC-MS. Single ion current monitoring at m/e
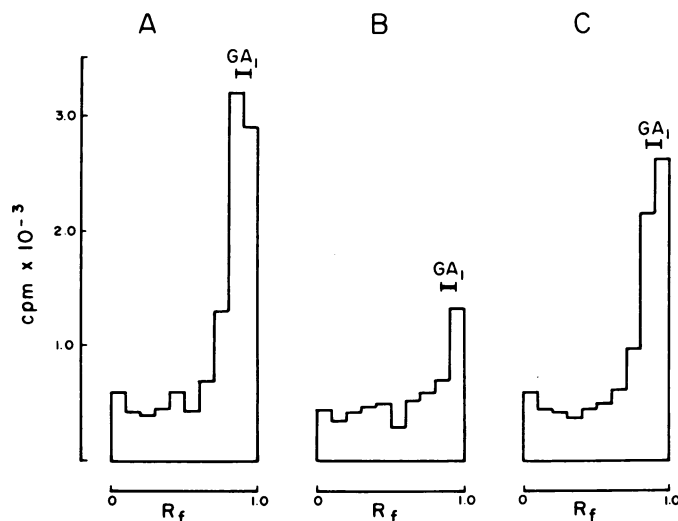
FIG. 8. TLC on Chrom AR 1000 of acidic (A), basic (B), and ethyl acetate-insoluble (C) fractions of extracted hypocotyl sections incubated in 10 mM KCl after a 2-hr pulse of [³H]GA₁. Chromatograms developed with isopropyl alcohol-NH₄OH-water (8:1:1, v/v/v).

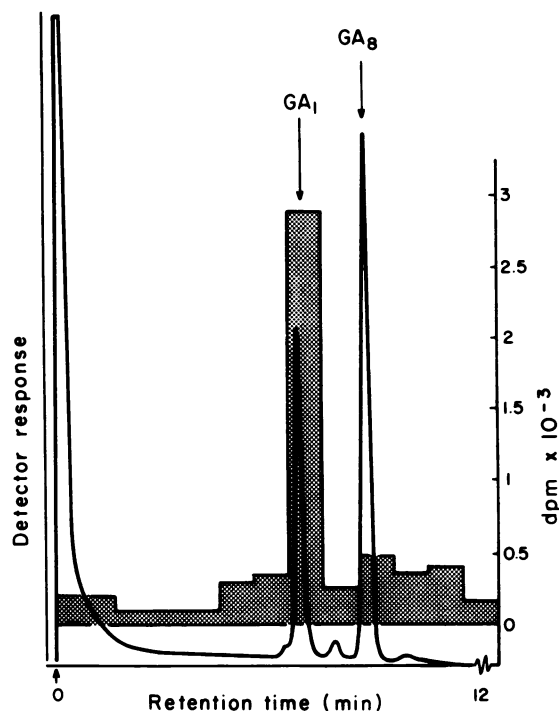FIG. 9. GC-RC of an acidic ethyl acetate-soluble fraction extracted from hypocotyl sections incubated in [³H]GA₁ for 22 hr. Shaded area indicates radioactivity; arrows indicate GA₁ and GA₈ marker peaks.

506 (parent ion for Me-TMS-GA₁) produced a well defined peak with the same retention time as authentic GA₁ and with a relative intensity of 54.6%. No parent ion peaks for GA₈ or other gibberellins were detected.

DISCUSSION

Implications of [³H]GA₁ Metabolism. The uptake of [³H]GA₁ continues for more than 24 hr of incubation and is unaffected by the presence of carrier GA₁ up to 100 μM. Since nonradioactive GA₁ does not compete with [³H]GA₁ for entry into the hypocotyls, it can be concluded that any uptake and retention sites are present at a concentration greater than 100 μM. This conclusion

is consistent with that of Musgrave *et al.* (13) who studied uptake of GA₁ into barley aleurone layers.

Since addition of nonradioactive GA₁ at concentrations less than 50 μM does not change the distribution of label among the soluble and insoluble fractions (Fig. 6), we conclude that the amount of polar metabolites present at a given time is proportional to the exogenous GA₁ concentration. When the incubation medium contains more than 50 μM GA₁ with tracer amounts of [³H]GA₁, the formation of labled metabolites declines. The metabolizing enzymes may be saturated by the endogenous GA₁ concentration resulting from uptake of 50 μM GA₁, or the capacity to metabolize GA₁ may be impaired at high hormone concentrations. GA₁ at 100 μM has been shown to be toxic to pea stem segments (13), and in our system, the growth rate with 100 μM GA₁ was lower than that with 10 μM GA₁. Enzyme saturation would imply that labeled hormone is diluted by the nonradioactive hormone so that the real rate of GA₁ metabolism is constant above 50 μM GA₁. Impairment of GA₁ metabolism would result in a decline in GA₁ metabolism rate above 50 μM GA₁. It is not possible to determine from the data whether the metabolism rate is constant or declines at the high exogenous hormone levels.

Davies and Rappaport (2, 3) have shown that even 1.14 μM carrier GA₁ depresses formation of [³H]GA₈-glycoside in corn leaf sections. We can conclude either that compared to corn leaf, lettuce hypocotyl tissue has a high ratio of metabolizing enzymes to endogenous GA₁ or that the metabolizing system for formation of the ethyl acetate-insoluble compounds is different from the glycosylation system of corn. As discussed below, TLC of the insoluble fraction supports the second possibility.

When exogenous GA₁ is removed, accumulated label declines rapidly to an apparently constant level (Fig. 4). Decay of the label in the acidic fraction is caused by efflux and metabolism. Since the decay rate after a short pulse does not change during 15 hr of growth, we conclude that the plateau in the decay curve is not caused by a decline in activity of efflux and metabolizing systems. The efflux curves suggest the existence of at least two compartments which accumulate GA₁. One readily releases acidic GA; the second compartment strongly retains accumulated hormone.

Chromatography of unpartitioned methanolic extracts indicates that at incubation times of up to 5 hr in [³H]GA₁, radioactivity is predominantly associated with a compound having the same R_F as GA₁. With increasing time of incubation, however, radioactivity appears in at least two other components differing from GA₁ in R_F value. Co-chromatography of authentic GA₈ indicates that the radioactivity at R_F 0.35 to 0.5 is similar in chromatographic behavior to GA₈; however, the identity of the labeled product at R_F 0.09 to 0.26 remains to be determined. Chromatography of acidic, basic, and insoluble partition fractions also indicates the predominance of label in the GA₁ region of chromatograms. GC-MS and GC-RC have confirmed that the radioactivity in the acidic fraction is associated with GA₁, and GC-RC indicates the presence of trace amounts of GA₈. Although attempts to perform GC on the insoluble and basic fractions have so far been unsuccessful, it is clear that the identity of these compounds should be pursued, particularly since TLC indicates that these compounds probably represent either minor or highly labile modifications of the [³H]GA₁ molecule.

Sustained Response to GA₁. The ability of lettuce hypocotyl sections to respond to a short pulse of GA₁ is of particular interest since it is in marked contrast to the response of excised plant parts to auxin (4, 5). It has been noted that many plants continue to respond to GA for long periods after an exogenous hormone supply is withdrawn. For instance, a single application of GA₃ to eggplants results in the elongation of pistils for more than 60 days (18). GA₃ applied as an ethanolic spray to the leaves of dwarf pea plants elevates the growth rate for at least 7

days, after which the growth rate falls to the initial low value if the original application were dilute (10 nM) or remains high for at least 21 days if the original application were more concentrated (100 μM) (1). This is in contrast to auxin-stimulated elongation response which declines rapidly after removal of the exogenous hormone supply (4, 5).

The lasting response to a short GA treatment can be explained by either of two possibilities. GA may act at the time of treatment (the "pulse time") either to induce a self-sustaining reaction or to catalyze some form of "stored growth." Alternatively, there may be a continuing requirement for GA during the response period and the plant may accumulate and retain enough hormone for use during the chase period. Researchers do not agree on the length of exposure to GA required for particular responses. McComb considered that the stability and movement of GA_3 in dwarf peas sufficiently accounted for the 7-day response (9). On the other hand, he (10) proposed that GA induced a self-sustaining process (the formation of endogenous GA) in the apices of *Centaurium minus*. Nothmann and Koller (18) also suggested that a self-sustaining process was initiated by a single GA application to eggplant. The authors considered that the growth of flowering branches must have diluted the original hormone concentration.

Our investigations of the uptake, efflux, and metabolism of $1,2[^3H]GA_1$ were designed to determine the plausibility of a continuing requirement for GA_1 during rapid growth. The results demonstrate that excised lettuce hypocotyls retain enough active hormone to account for their growth after removal of an exogenous hormone supply. Comparison of Figures 3 and 4C reveals that after 18 hr of growth, sections pulsed for 2 hr contain 3.2% of the acidic GA in sections maintained continuously in the pulse medium. Since accumulated GA is proportional to the concentration of GA in the bathing solution (Fig. 6), we can estimate that a 2-hr pulse with 10 μM GA_1 might provide approximately the same growth increment as a continuous pulse with 100 nM GA_1. Figure 2 shows that the growth after a 2-hr pulse with 10 μM GA_1 is not more than the growth realized during continuous incubation with 100 nM GA_1.

We note that the retention and slow metabolism of accumulated GA_1 in lettuce hypocotyls are in contrast to the rapid loss of auxin from pulsed coleoptiles. The difference in retention pattern could well explain the difference in pulse kinetics. At the same time, the apparent compartmentation of accumulated hormone raises the question of the accessibility of accumulated gibberellin for growth stimulation. If there is a continuing requirement for GA during hormone-stimulated growth in this system, then the acidic gibberellin in the slow compartment and/ or the ethyl acetate-insoluble GA_1 metabolites must be effective in stimulating growth.

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