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Metabolism of Tritiated Gibberellins in *d-5* Dwarf Maize

II. [^{3}H]GIBBERELLIN A_1, [^{3}H]GIBBERELLIN A_3, AND RELATED COMPOUNDS[1]

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ABSTRACT

After 30 minutes of incubation of young leaf sections of *d-5* maize (*Zea mays* L.) in [^{3}H]gibberellin A_1 ([^{3}H]GA_1), the metabolite [^{3}H]GA_8 was present in significant amounts, with a second metabolite, [^{3}H]GA_8-glucose ([^{3}H]GA_8-glu), appearing soon after. A third [^{3}H]GA_1 metabolite, the polar uncharacterized conjugate [^{3}H]GA_1-X, took more than 1 hour to appear. The protein synthesis inhibitor cycloheximide inhibited the production of all [^{3}H]GA_1 metabolites, indicating a possible protein synthesis requirement for [^{3}H]GA_1 metabolism.

By preincubating leaf sections in unlabeled GA_1 before exposure to [^{3}H]GA_1 or by reducing the specific radioactivity of the [^{3}H]GA_1 supplied, it was possible to reduce greatly the conversion of radioactive GA_1 to [^{3}H]GA_8-glu, without affecting conversion to [^{3}H]GA_1-X. Increasing the molar concentration of the [^{3}H]GA_1 fed greatly increased the molar yield of [^{3}H]GA_1-X, whereas the molar yields of [^{3}H]GA_8 and [^{3}H]GA_8-glu were much less affected.

The principal metabolite of [^{3}H]GA_3 was a very polar compound having chromatographic properties similar to those of the conjugate [^{3}H]GA_1-X produced from [^{3}H]GA_1. The naturally occurring GAs [^{3}H]GA_1, [^{3}H]GA_3, and [^{3}H]tetrahydroGA_3 were metabolized to a much greater extent than were the artificial derivatives [^{3}H]ketoGA_1, [^{3}H]GA_1-methyl ester, and [^{3}H]pseudoGA_1. Only [^{3}H]GA_1 and [^{3}H]GA_3, with their identical D ring structures, were converted to [^{3}H]GA_1-X type compounds; [^{3}H]ketoGA_1 and [^{3}H]tetrahydroGA_3, with modified D rings, were not converted to this type of conjugate.

Gibberellin metabolism in plants involves conversion of a GA to other GAs (4–7, 11, 17–20), or to biologically active or inactive conjugated derivatives such as GA-glucosides (1, 4, 13, 21, 23, 24). Since metabolism results in modification of the types and levels of GAs, it could be a major factor controlling plant growth and development. However, it remains to be demonstrated that GA metabolism is a precisely controlled process of primary importance for the regulation of GA status and plant growth. Approaches toward an understanding of this relationship have included identification of the metabolites produced, structure-function analysis using inactive derivatives, and efforts to characterize the enzyme systems involved in metabolism.

Feeding studies with tritiated GA_1 have indicated that the enzymes of GA_1 metabolism in barley aleurone layers are specific for natural GAs, as minor chemical modification of the GA_1 molecule fed, affected its metabolism considerably (10, 26). In the same vein, a cell-free system from bean seeds specifically catalyzed the conversion of [^{3}H]GA_1 to [^{3}H]GA_8 (15, 16).

Several factors have been reported to influence the metabolism of tritiated GAs, including abscisic acid in barley aleurone layers (14, 26), exogenously supplied glucose in *Pharbitis nil* cotyledons (1), and leaf age in maize (4). Day length had a considerable effect on [^{3}H]GA_5 metabolism and flowering in *Silene armeria* (26), although [^{3}H]GA_1 metabolism was not influenced by day length in *Solanum andigena* (17). Red light enhanced the conversion of [^{3}H]GA_9 into other GAs in homogenates of isolated barley leaves (22). It is conceivable that endogenous chemical as well as environmental factors might influence plant growth by way of direct effects on the enzymes of GA metabolism.

In an earlier paper (4), we described the metabolism of GA_1 in sections and intact plants of *d-5* maize. The experiments described in the present paper were aimed at investigating the nature of the *in vivo* enzyme systems metabolizing [^{3}H]GA_1 and related compounds in *d-5* maize.

MATERIALS AND METHODS

Radioactive GAs. The [1,2-^{3}H]gibberellin A_1 ([^{3}H]GA_1), [^{3}H]gibberellin A_3 ([^{3}H]GA_3), [1,2,16,17-^{3}H]tetrahydrogibberellin A_3 ([^{3}H]tetrahydroGA_3), and *ent*-[1,2-^{3}H]-3α,10-dihydroxy-13-methyl-16-oxo-17,20-bisnor-13β gibberellane-7,19-dioic acid 19,10-lactone[3] ([^{3}H]ketoGA_1) used in this study were prepared as described previously (12). Unless otherwise stated, the specific radioactivities of these compounds were [^{3}H]GA_3, 13 Ci/mmole; [^{3}H]GA_1, 14 Ci/mmole; [^{3}H]tetrahydroGA_3, 73 Ci/mmole; and [^{3}H]ketoGA_1, 43 Ci/mmole. Equatorial 3-α-[1,2-^{3}H]hydroxygibberellin A_1 ([1,2-^{3}H]pseudogibberellin A_1), 43 Ci/mmole, was prepared as described previously (26). [1,2-^{3}H]Gibberellin A_1-methyl ester ([^{3}H]GA_1-Me, 43 Ci/mmole) was prepared by methylation of [^{3}H]GA_1, using ethereal diazomethane. The [^{3}H]GA_1-Me was then separated from [^{3}H]GA_1 by TLC on 5- × 20-cm strips of ChromAR sheets which were developed in ethyl acetate-chloroform (3:1, v/v). In this system, [^{3}H]GA_1-Me had an R_F of 0.59, whereas, [^{3}H]GA_1 remained at the origin.

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[3] Also: 2,β,4aα-Dihydroxy-1β,7β-dimethyl-8-keto-4bβ-gibbane-1,10-carboxylic acid 1 → 4a lactone.

Plant Material. The *d-5* maize plants (*Zea mays* L.) were grown in vermiculite in a greenhouse for 7 days, or under artificial lighting for 9 days, after an initial 48-hr germination period, as described previously (4). At that time, the seedlings had three leaves, the first or oldest leaf fully expanded, the second nearly fully expanded, and the third with less than 3 cm of lamina protruding from the leaf sheaths of the older leaves. The *d-5* maize mutants segregate in a ratio of 3:1 tall to dwarf plants. Unless otherwise specified, only tall plants were used as a source of leaf tissue. In most experiments, the tops of the seedlings were severed from the roots, and the third leaf was carefully removed. The lower 2 cm of the third leaf in normal plants, or lower 1 cm in dwarf plants, was then cut off and used in incubation experiments. These leaf sections consisted of tightly rolled leaf tissue undergoing rapid expansion growth. In one experiment, 8-mm diameter leaf discs were punched from the lamina of the first, or oldest, leaf on the seedlings and used in an incubation study.

Incubation Experiments. Leaf tissues were incubated in 2 ml of medium containing 0.05 M potassium phosphate buffer (pH 6.2), 2% sucrose, $MgSO_4 \cdot 7\ H_2O$, (144 mg/l), $Ca(NO_3)_2 \cdot 2\ H_2O$, (288 mg/l) in addition to the tritiated GAs. Twenty-five leaf sections (300 mg total fresh weight) or 12 leaf discs (100–150 mg fresh weight) were incubated per 25-ml Erlenmeyer flask. Incubation conditions and procedures were as described previously (4), except that in some experiments aseptic conditions were not used, and chloramphenicol at 50 μg/ml was added to the medium. Experiments involving incubation of leaf sections for 15 hr indicated that [^{3}H]GA_1 metabolism under aseptic conditions was the same as under nonsterile conditions.

Extraction and Chromatography. All plant tissues were boiled for 1 min in 80% ethanol before homogenization and extraction as described previously (4). TLC of the extracts was done using strips of ChromAR sheet which were developed first in isopropanol-ammonia-water (8:1:1, v/v/v) to a distance of 12 cm, and then in benzene-acetic acid (4:1, v/v) for 65 min.

RESULTS

Short Term Metabolism of [^{3}H]GA_1. Rapidly growing leaf sections from normal plants were incubated in [^{3}H]GA_1 for periods up to 2 hr. Extraction, followed by TLC and radioscanning, indicated the occurrence of [^{3}H]GA_8, [^{3}H]GA_8-glu, and [^{3}H]GA_1-X, compounds tentatively identified previously in extracts of *d-5* maize (4). [^{3}H]GA_8, the first metabolite to appear, was present in significant amounts after 0.5 hr of incubation (Fig. 1). [^{3}H]GA_8-glu was detected shortly thereafter, and [^{3}H]GA_1-X appeared after more than 1 hr. For a control, [^{3}H]GA_1 was added to leaf sections which were immediately extracted without incubation. Only [^{3}H]GA_1 was detected in the extracts of the controls; thus the products shown in Figure 6 were not artifacts of extraction.

Effects of Cycloheximide on [^{3}H]GA_1 Metabolism. Leaf sections were preincubated for 2 hr in medium containing 5 μg/ml of CHI[4], followed by incubation in medium containing both [^{3}H]GA_1 and CHI. As a control, leaf sections were preincubated in medium without CHI, followed by incubation in [^{3}H]GA_1. Incubation in CHI for 2 hr was sufficient to block more than 95% of protein synthesis in maize leaf sections, as measured by inhibition of [^{3}H]leucine incorporation into protein (3). The effect of CHI on [^{3}H]GA_1 metabolism is shown in Table I. In the presence of CHI, most of the radioactivity remained in unmodified [^{3}H]GA_1, and the inhibitory effect of CHI on metabolism was relatively much greater at 8 than at 3 hr. Total uptake of radioactivity was also reduced by CHI. CHI at the concentration used was not completely inhibitory, as in its presence some metabolism still occurred (Table I).

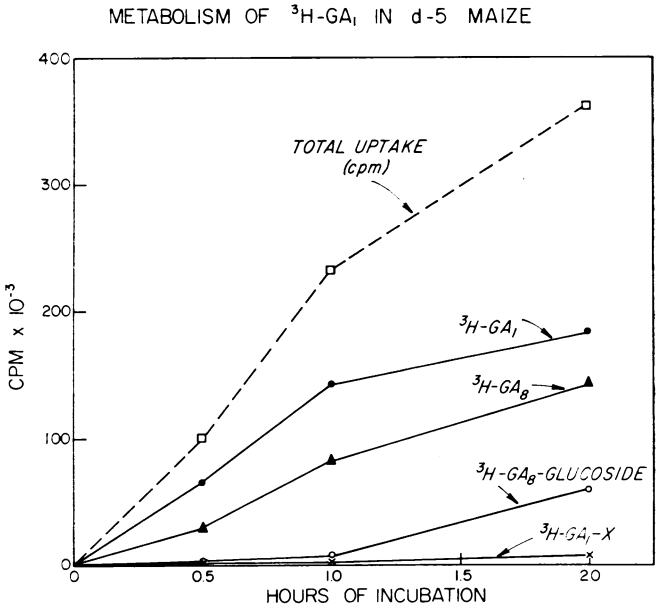

FIG. 1. Short term metabolism of [^{3}H]GA_1 in leaf sections of *d-5* maize. Leaf sections were incubated in 2 ml of medium containing 0.03 μg/ml of [^{3}H]GA_1 (43 Ci/mmole, 1×10^8 cpm/μg). Details of incubation conditions, extraction, and characterization of [^{3}H]GA_1 and its metabolites are described in "Materials and Methods." Data represent radioactivity extracted from 25 leaf sections.

Table I. *Effect of CHI on Metabolism of [^{3}H]GA_1 in Leaf Sections of d-5 Maize*

Leaf sections cut from the rapidly expanding basal end of the emerging third leaf of 11-day-old seedlings were preincubated for 2 hr in 2 ml of medium containing 5 μg/ml CHI, and then transferred to 2 ml of medium containing 0.03 μg/ml [^{3}H]GA_1 (43 Ci/mmole, 1×10^8 cpm/μg) and 5 μg/ml CHI for further incubation. Fresh weight of tissue used was 300 mg (25 leaf sections) per 25-ml Erlenmeyer flask. Control treatments contained no CHI. Details concerning composition of the medium, incubation conditions, and characterization of metabolites are under "Materials and Methods." Data are expressed as radioactivity extracted per 25 leaf sections, and pertain to normal (nondwarf) plants only.

Compound	3 Hr Incubation in [^{3}H]GA_1		8 Hr Incubation in [^{3}H]GA_1	
	Cyclo-heximide	Control	Cyclo-heximide	Control
	cpm			
[^{3}H]GA_1	441,470	170,270	663,050	126,990
[^{3}H]GA_8	27,360	216,180	40,480	222,050
[^{3}H]GA_8-glu	8,800	90,350	17,150	467,630
[^{3}H]GA_1-X	9,470	50,240	19,230	324,940
Total cpm recovered	487,100	527,040	739,910	1,241,620

Effects of GA_1 Pretreatment and Specific Radioactivity on [^{3}H]GA_1 Metabolism. In view of the apparent protein synthesis

[4] Abbreviation: CHI: cycloheximide.

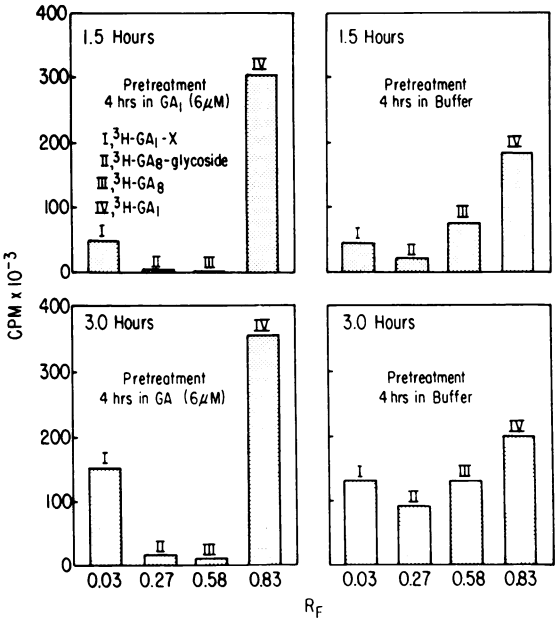

FIG. 2. Effects of pretreating leaf sections with GA_1 on metabolism of [^{3}H]GA_1. Leaf sections were preincubated for 4 hr in buffer containing GA_1 (6 μM) or buffer alone, and then incubated in 0.04 μg/ml [^{3}H]GA_1 (43 Ci/mmole, 1×10^8 cpm/μg). The data represent radioactivity extracted from 25 leaf sections.

requirement for [^{3}H]GA_1 metabolism (Table I), it seemed possible that supplying [^{3}H]GA_1 to *d-5* maize tissues could lead to the induction of enzymes required for GA_1 metabolism. A characteristic of an inducible enzyme system is that metabolism of an inducing substrate can be increased by pretreatment of the tissue with the substrate (28). Accordingly, leaf sections of normal *d-5* maize seedlings were preincubated in dilute, cold GA_1 at 2 μg/ml (6 μM) for 4 hr, and then exposed to [^{3}H]GA_1 to determine if metabolism was affected. Preincubation in GA_1 for 4 hr decreased the conversion of radioactivity in [^{3}H]GA_1 to [^{3}H]GA_8 and [^{3}H]GA_8-glu, whereas conversion of [^{3}H]GA_1 to [^{3}H]GA_1-X was unaffected (Fig. 2).

A drawback of this experiment was that the final specific radioactivity of the metabolites was unknown preventing calculation of the moles of metabolites produced. This information is important because pretreatment in cold GA_1 would almost certainly have lowered the specific radioactivity of the [^{3}H]GA_1 attained later in the tissues. Production of [^{3}H]GA_1-X on a molar basis is therefore likely to have been much greater in the GA_1-pretreated leaf sections, even though, on a radioactivity basis, pretreatment appeared virtually without effect.

Leaf sections were incubated in [^{3}H]GA_1 at four different specific radioactivities to estimate the moles of metabolites produced when different [^{3}H]GA_1 concentrations were supplied. The different specific radioactivities were obtained by keeping the amount of radioactivity supplied as [^{3}H]GA_1 constant, and raising the concentration of cold GA_1 supplied in three 10-fold increases. The effect of feeding different specific radioactivities of [^{3}H]GA_1 is shown in Figure 3, in which data for 7 hr of incubation are presented. Incubation for 3 hr and 12 hr yielded similar results. The data indicate that the system producing [^{3}H]GA_1-X is manifestly different from that producing [^{3}H]GA_8 and [^{3}H]GA_8-glu. In terms of radioactivity, metabolism to [^{3}H]GA_8-glu is reduced by even a single 10-fold increase in the mass of [^{3}H]GA_1 supplied. However, a 100- to 1000-fold increase in the mass of [^{3}H]GA_1 is needed before conversion to [^{3}H]GA_1-X is significantly affected.

The radioactivity data in Figure 3 were used to calculate the molar yield of metabolites at the various concentrations of [^{3}H]GA_1 supplied. Results are shown in Figure 4. These calculations were based on the assumption that any contribution of endogenous GAs to the final specific radioactivity of metabolites is negligible, an assumption supported by unrewarded efforts to detect any GA_1 or GA_8 in extracts of 10 g fresh weight samples of young maize leaves. Specific radioactivities for extracted [^{3}H]GA_1 and [^{3}H]GA_1-X were assumed to be the same as for the [^{3}H]GA_1 supplied in the incubation medium. The assumption is based on the knowledge that prolonged acid hydrolysis of [^{3}H]GA_1-X yields only an acid-modified derivative of [^{3}H]GA_1 (3, 13), indicating that [^{3}H]GA_1 is in-

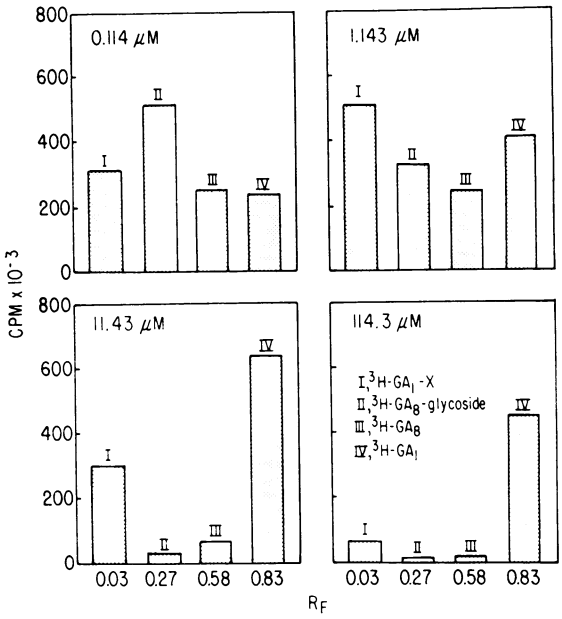

FIG. 3. Effects of specific activity on [^{3}H]GA_1 metabolism in leaf sections after 7 hr of incubation. Sections were incubated in four different concentrations of GA_1 (0.114 μM, 1.143 μM, 11.43 μM, and 114.3 μM) with the amount of radioactivity supplied as [^{3}H]GA_1 held constant at 4×10^6 cpm/ml. This procedure produced four [^{3}H]GA_1 specific radioactivities: 43, 4.3, 0.43 and 0.043 Ci/mmole, respectively.

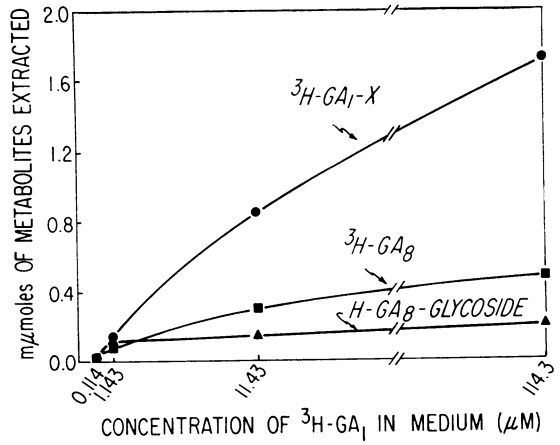

FIG. 4. Effects of GA_1 concentration in the medium on metabolism of [^{3}H]GA_1. The data showing production of radioactive metabolites in Figure 3 were used to calculate the yields of metabolites on a molar basis per 25 leaf sections.

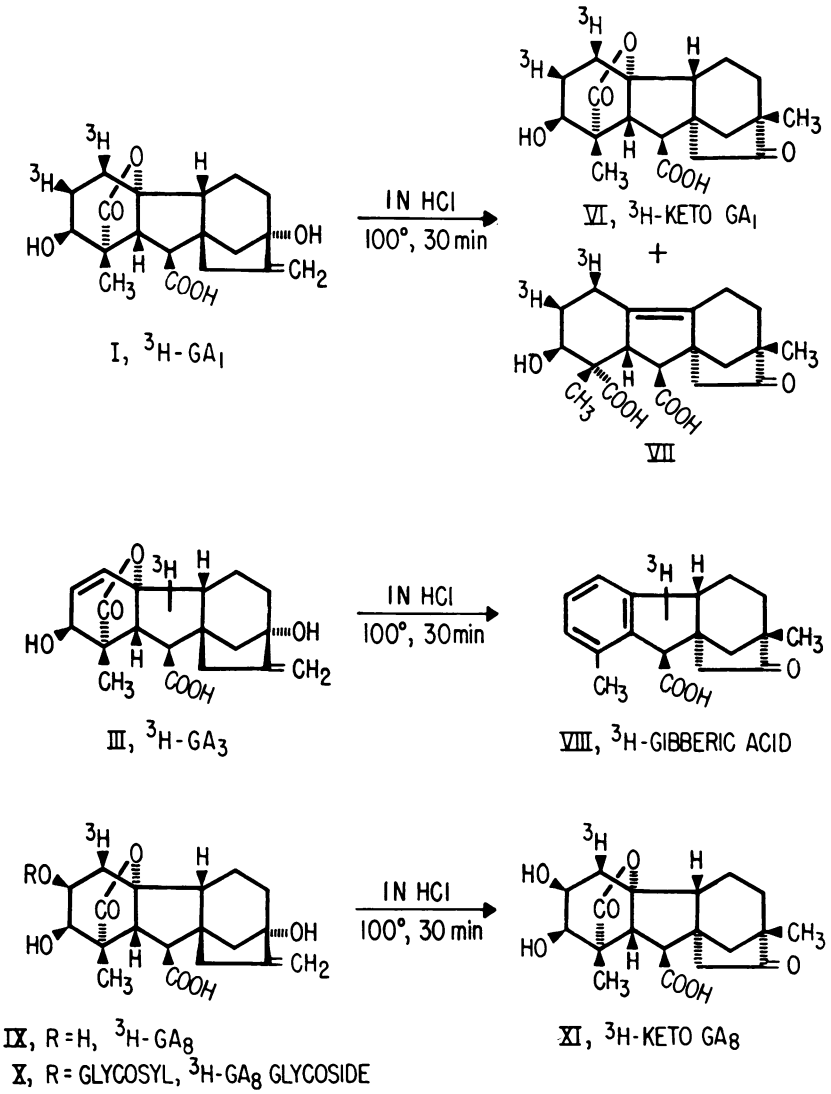

FIG. 5. Structures of [^{3}H]GA$_3$, [^{3}H]GA$_1$, and derivatives of [^{3}H]GA$_1$. I: [1,2-^{3}H]gibberellin A$_1$ ([^{3}H]GA$_1$); II: [1,2-^{3}H]pseudogibberellin A ([^{3}H]pseudoGA$_1$, the 3α–OH epimer of [^{3}H]GA$_1$); III: [^{3}H]gibberellin A$_3$ ([^{3}H]GA$_3$); IV: [1,2,16,17-^{3}H]tetrahydrogibberellin A$_3$ ([^{3}H]tetrahydroGA$_3$); V: [1,2-^{3}H]gibberellin A$_1$ methyl ester ([^{3}H]GA$_1$-Me); VI:*ent*-[1,2-^{3}H]-3α,10-dihydroxy-13-methyl-16-oxo-17,20-bisnor-13β gibberellane-7,19-dioic acid 19,20-lactone.

corporated into [^{3}H]GA$_1$-X without modification of the radioactive portion of the molecule. The specific radioactivity of both [^{3}H]GA$_8$ and [^{3}H]GA$_8$-glu was taken as 28, 2.8, 0.28, and 0.028 Ci/mmole when [^{3}H]GA$_1$ was supplied, respectively, at 0.114, 1.14, 11.43, and 114.3 μM in the medium. During the hydroxylation of [^{3}H]GA$_1$ to [^{3}H]GA$_8$, 35% of the tritium in [^{3}H]GA$_1$ is lost as tritiated water (3, 15); hence, the use of 28 Ci/mmole as the undiluted specific radioactivity of both [^{3}H]GA$_8$ and [^{3}H]GA$_8$-glu.

As the concentration of [^{3}H]GA$_1$ in the medium is increased, the production of [^{3}H]GA$_1$-X rises considerably (Fig. 4). The production of [^{3}H]GA$_8$-glu on a molar basis is virtually unaffected by the concentration of GA$_1$, although the synthesis of [^{3}H]GA$_8$ does show a slight increasing trend with increasing supply of substrate.

Specificity of Metabolism. To study the specificity of the [^{3}H]GA$_1$ metabolizing system in *d-5* maize, [^{3}H]GA$_1$ and five compounds structurally similar to it were fed to young leaf sections (Fig. 5). Of these compounds, [^{3}H]GA$_1$, [^{3}H]GA$_3$, and [^{3}H]tetrahydroGA$_3$ are tritiated forms of naturally occurring GAs, whereas the other three have not been found in plants. Incubating leaf sections for 12 hr in the six substrates, and analysis of the metabolites by TLC, yielded the radiochromatograms shown in Figure 6. The substances detected were eluted, and their radioactivity quantitated by scintillation counting. The radioactivity data were used to calculate the extent of metabolism of the six tritiated GAs (Table II).

It is apparent that [^{3}H]GA$_3$ and the four derivatives of [^{3}H]GA$_1$ were not metabolized in the same manner as was [^{3}H]GA$_1$. Of the compounds tested, only the natural GAs were rapidly metabolized. As indicated in Table II, [^{3}H]GA$_3$, was metabolized less extensively than GA$_1$, whereas [^{3}H]tetrahydroGA$_3$ was more extensively metabolized than [^{3}H]GA$_1$. [^{3}H]GA$_3$ was converted massively to a very polar compound detected at R_F 0.03 and slightly to two other metabolites detected on TLC at R_F 0.40 and 0.56 (Fig. 6). This metabolic pattern differed from that of [^{3}H]GA$_1$ from which [^{3}H]GA$_8$-glu was produced in amounts as large as that of [^{3}H]GA$_1$-X (Fig. 6). [^{3}H]TetrahydroGA$_3$ was converted to two metabolites, detected at R_F 0.27 and 0.62, possibly analogous to

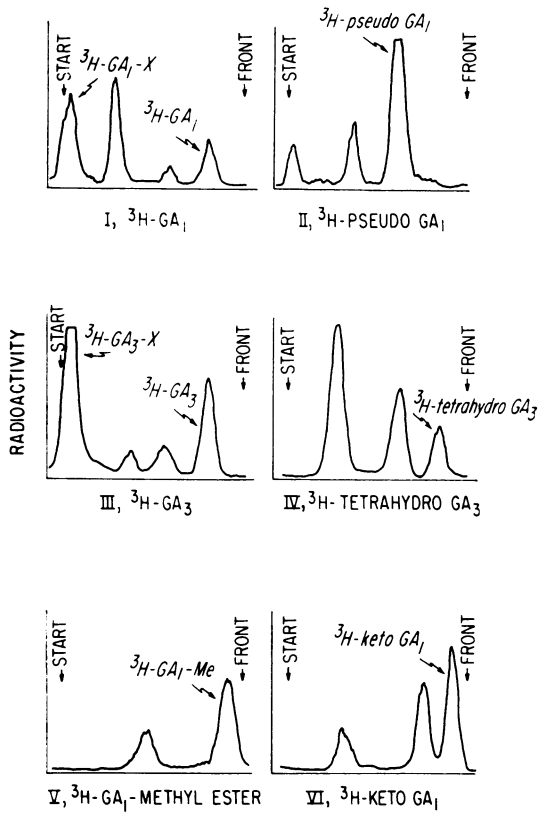

FIG. 6. Comparative radiochromatogram tracings of metabolites obtained by incubating leaf sections in [^{3}H]GA$_8$, [^{3}H]GA$_1$, and four derivatives of [^{3}H]GA$_1$ for 12 hr. The tritiated GAs were fed in separate media at the following concentrations: 0.024 μg/ml [^{3}H]GA$_1$ (43 Ci/mmole, 1×10^8 cpm/μg), 0.015 μg/ml [^{3}H]pseudoGA$_1$ (43 Ci/mmole), 0.067 μg/ml [^{3}H]GA$_3$ (13 Ci/mmole, 3×10^7 cpm/μg), 0.012 μg/ml [^{3}H]tetrahydroGA$_3$ (73 Ci/mmole, 1.7×10^8 cpm/μg), 0.023 μg/ml [^{3}H]GA$_1$-methyl ester (43 Ci/mmole), and 0.022 μg/ml [^{3}H]ketoGA$_1$ (43 Ci/mmole). Extracts of leaf sections were run on ChromAR sheet TLC developed (1) with isopropanol-ammonia-water (8:1:1, v/v/v) to 12 cm, and then (2) with benzene-acetic acid (4:1, v/v) for 70 min.

Table II. *Extent of Metabolism of [^{3}H]GA$_3$, [^{3}H]GA$_1$ and Four Derivatives of [^{3}H]GA$_1$ in d-5 Maize*

Data obtained from measurement of radioactive substances detected on the radiochromatograms shown in Figure 6 were used to calculate extent of metabolism as the ratio of cpm in all recovered metabolites to cpm in recovered unmetabolized substrate. Specific radioactivities were 73 Ci/mmole for [^{3}H]tetrahydroGA$_3$; 43 Ci/mmole for [^{3}H]GA$_1$, [^{3}H]pseudoGA$_1$, [^{3}H]GA$_1$-methyl ester, and [^{3}H]ketoGA$_1$; and 13 Ci/mmole for [^{3}H]GA$_3$.

Substrate	CPM in all Metabolites/ CPM in Unmetabolized Substrates
	Ratio
[^{3}H]GA$_1$	6.69
[^{3}H]PseudoGA$_1$	0.73
[^{3}H]GA$_3$	3.76
[^{3}H]TetrahydroGA$_3$	9.52
[^{3}H]GA$_1$-methyl ester	0.52
[^{3}H]KetoGA$_1$	1.32

[^{3}H]GA$_8$-glu and [^{3}H]GA$_8$ (Fig. 6). Surprisingly, however, [^{3}H]tetrahydroGA$_3$ was not converted at all to a highly polar metabolite.

Metabolism of [^{3}H]ketoGA$_1$ was qualitatively similar to that of [^{3}H]tetrahydroGA$_3$. Metabolites were found at R_F 0.32 and 0.76 on TLC, however, without any production of a [^{3}H]GA$_1$-X-like metabolite. Quantitatively, metabolism of [^{3}H]ketoGA$_1$ was considerably less extensive than that of [^{3}H]GA$_1$ and [^{3}H]tetrahydroGA$_3$ (Fig. 6 and Table II). [^{3}H]PseudoGA$_1$ was slightly metabolized to two compounds, detected at R_F 0.36 and 0.03, the latter migrating on TLC in a like manner to [^{3}H]GA$_1$-X. The methyl ester of [^{3}H]GA$_1$ had the slowest metabolism of all (Table II), being converted to only one metabolite, detected at R_F 0.47 (Fig. 6).

DISCUSSION

Previously it was reported that *d-5* maize plants convert [^{3}H]GA$_1$ to [^{3}H]GA$_8$, [^{3}H]GA$_8$-glu, and an unknown metabolite referred to as [^{3}H]GA$_1$-X (4). Prolonged acid hydrolysis of [^{3}H]GA$_1$-X isolated both from barley aleurone layers (13) and from *d-5* maize (3), released acid-modified derivatives of [^{3}H]GA$_1$. Nadeau and Rappaport (13) provided strong evidence for direct conjugation of exogenously supplied [^{3}H]GA$_1$ with an unknown compound (likely a peptide) to produce [^{3}H]GA$_1$-X (now called amphoGA$_1$). In attempts to identify amphoGA$_1$, neither a [^{3}H]GA$_8$-X-like compound nor derivatives other than those produced from chemically degraded GA$_1$ were found. It is therefore most likely that [^{3}H]GA$_1$ is incorporated into [^{3}H]GA$_1$-X without prior modification. Thus it appeared there are two pathways of [^{3}H]GA$_1$ metabolism in maize, one leading to [^{3}H]GA$_8$-glu via [^{3}H]GA$_8$, and the other leading to [^{3}H]GA$_1$-X. Our present findings are in line with this conclusion as the production of [^{3}H]GA$_1$-X is decidedly different in response to changes in specific radioactivity and concentration of [^{3}H]GA$_1$ than is the production of [^{3}H]GA$_8$ and [^{3}H]GA$_8$-glu. Reducing the specific radioactivity of the [^{3}H]GA$_1$ fed to leaf tissues had much less effect on the conversion of radioactivity to [^{3}H]GA$_1$-X than it did on the conversion to [^{3}H]GA$_8$ and [^{3}H]GA$_8$-glu. This was true whether the specific radioactivity was reduced indirectly by preincubating tissues in cold GA$_1$ (Fig. 2), or directly by adding cold GA$_1$ to the [^{3}H]GA$_1$ before feeding it (Fig. 3). On a molar basis, production of [^{3}H]GA$_1$-X was thus greatly increased by raising the concentration of [^{3}H]GA$_1$ supplied, whereas the production of [^{3}H]GA$_8$ and [^{3}H]GA$_8$-glu was much less affected by [^{3}H]GA$_1$ concentration (Fig. 4). These observations are consistent with the notion that [^{3}H]GA$_1$-X formation is independent of [^{3}H]GA$_8$ synthesis, and point to two separate pathways for [^{3}H]GA$_1$ metabolism.

An important question is whether the observed metabolism is indicative of the normal endogenous metabolism in *d-5* maize. A fraction of the [^{3}H]GA$_1$ could conceivably enter cellular compartments inaccessible to endogenous GAs, there either inducing enzymes capable of GA$_1$ metabolism, or undergoing nonspecific reactions unrelated to endogenous GA metabolism. The finding that the system producing [^{3}H]GA$_1$-X has a much greater capacity to metabolize [^{3}H]GA$_1$ than does the system producing [^{3}H]GA$_8$ and [^{3}H]GA$_8$-glu (Fig. 4) is of significance here. When the low endogenous GA concentration usually found in leaf tissues is taken into account, it is obvious the [^{3}H]GA$_1$-X-synthesizing system is capable of metabolizing GA$_1$ in amounts far in excess of the availability of any endogenous GAs in leaves. One interpretation of this result is that [^{3}H]GA$_1$-X is elaborated by a system unrelated to endogenous GA metabolism.

[^{3}H]GA$_8$ was present within 30 min of exposure of leaf tissue to [^{3}H]GA$_1$, and [^{3}H]GA$_8$-glu appeared shortly afterwards. It is most likely that the hydroxylating and glucosylating enzymes are constitutive. Other plant species are known to contain enzymes capable of hydroxylating [^{3}H]GA$_1$ (21), and a cell-free system capable of carrying out this hydroxylation

has been isolated from bean seeds (15, 16). The appearance of [^{3}H]GA_1-X, on the other hand, takes at least 1 hr, sufficient time for the induction of the enzyme(s) synthesizing [^{3}H]GA_1-X (Fig. 1). Of interest in relation to possible enzyme induction is the finding that the protein synthesis inhibitor CHI markedly inhibited the appearance of all metabolites (Table I), indicating a protein synthesis requirement for [^{3}H]GA_1 metabolism. This result also indicates that the enzyme(s) producing [^{3}H]GA_1-X from [^{3}H]GA_1 is either inducible, or is catabolized rapidly. As the time-course data (Fig. 1) established that the enzyme which hydrolates [^{3}H]GA_1 is unlikely to be induced, the protein synthesis requirement for its activity may mean that the enzyme is turned over rapidly. It is also possible, however, that CHI affects the system indirectly by interfering with the production of ATP and NADH, both of which could be required for [^{3}H]GA_1 metabolism. Cycloheximide has been reported to inhibit respiration and energy transfer in some plant systems (2, 8), possibly by the effects of inhibition of protein synthesis on the enzymes involved; however, this inhibition is less likely in the green tissues (9) used here. Our results, although not conclusive, are consistent with the hypothesis that [^{3}H]GA_1]-X is produced by some system unrelated to the usual metabolism of endogenous GAs, and which may be induced by exogenous [^{3}H]GA_1. This hypothesis can be proved only by demonstrating that GA-X type compounds do not occur endogenously in maize. The conversion of [^{3}H]GA_1 to [^{3}H]GA_8 and [^{3}H]GA_8-glu more likely represents the normal type of metabolism of endogenous GAs in maize.

The identity of [^{3}H]GA_1-X is unknown at this time. This metabolite, first found as a major product of [^{3}H]GA_1 metabolism in barley aleurone layers (13, 14), has so far been found only in monocotyledonous plants such as barley (13), maize (4), and wheat (Davies, Stoddart, and Rappaport, unpublished). Dicotyledons do not appear to produce [^{3}H]GA_1-X, as it was not found as a metabolite of [^{3}H]GA_1 in bean (*Phaseolus vulgaris*) (11), olive (*Olea europaea*) (21), or pea (*Pisum sativum*) (25). Nadeau and Rappaport (13) subjected the [^{3}H]GA_1-X isolated from barley aleurone layers to acid hydrolysis. The products were separated by paper electrophoresis, and [^{3}H]GA_1-X was found to be an amphoteric, conjugated derivative of [^{3}H]GA_1. On Sephadex G-25 chromatography, it was found to have a mol wt of about 700 to 800. The same techniques applied to [^{3}H]GA_1-X isolated from *d-5* maize (3) revealed that it was very similar to the barley [^{3}H]GA_1-X.

Modification of the D ring of [^{3}H]GA_1 appears to prevent its conversion to [^{3}H]GA_1-X, although metabolites with chromatographic properties similar to those of [^{3}H]GA_8 and [^{3}H]GA_8-glu are formed. Thus [^{3}H]ketoGA$_1$ and [^{3}H]tetrahydroGA$_3$, differing in D ring structure from [^{3}H]GA_1, were not converted to the [^{3}H]GA-X-type compound, although they were metabolized to two other products possibly analogous to [^{3}H]GA_8 and [^{3}H]GA_8-glu. In the case of [^{3}H]ketoGA$_1$, however, metabolism was at a very low level, similar to that found in barley (Stolp, Nadeau, and Rappaport, unpublished) and in peas (25). Failure of compounds with different D ring conformation to convert to their [^{3}H]GA_1-X analogs may indicate that synthesis of [^{3}H]GA_1-X involves conjugation of [^{3}H]GA_1 with another compound at a site in the D ring.

Any minor modification of the [^{3}H]GA_1 molecule affects its metabolism qualitatively, quantitatively, or both. [^{3}H]GA_3 with the same D ring structure as [^{3}H]GA_1 was converted to a highly polar compound, migrating on TLC and paper electrophoresis in a manner similar to [^{3}H]GA_1-X (13, 21) (Fig. 6). The [^{3}H]GA_3 molecule has a C-1,8 double bond in the A ring (Fig. 5), and this is likely to prevent hydroxylation at the C-2 position. The greatest limits to metabolism, however, were obtained by modifying the orientation of the hydroxyl group at position C-3 in the A ring (from axial to equatorial) to form [^{3}H]pseudoGA$_1$, or by methylating the carboxyl group at position C-7 (Fig. 6 and Table IV). It is apparent that the C-2 hydroxyl and the C-7 carboxyl groups on [^{3}H]GA_1 are very important for substrate recognition by the enzymes involved in [^{3}H]GA_1 metabolism.

The two minor [^{3}H]GA_3 metabolites (Fig. 6) were not characterized, although one could be [^{3}H]GA_3-glu, as GA_3 is converted to GA_3-glu in other plants (23, 24). In time-course studies reported elsewhere (3), dwarf and normal plants of *d-5* maize metabolized [^{3}H]GA_3 to the same extent, indicating that [^{3}H]GA_3 metabolism is not related to dwarfism in *d-5* maize. Metabolism of [^{3}H]GA_1 is likewise unrelated to dwarfism in *d-5* maize (4).

Of the six GAs used in this study (Fig. 5), [^{3}H]GA_1, [^{3}H]GA_3, and [^{3}H]tetrahydroGA$_3$ are tritiated forms of naturally occurring GAs; and these appear to be preferred substrates as they were more extensively metabolized than the other derivatives (Fig. 6 and Table II). This metabolism is carried out by enzymes specific for the GA molecule, and which are not likely to mediate a variety of conjugation reactions with a whole range of unrelated organic compounds. Substrate specificity is important, as it is possible that enzymes specifically metabolizing GAs are also precisely regulating plant GA status. Characterization of the enzyme systems involved should help clarify this point.

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