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An Amphoteric Conjugate of [^{3}H]Gibberellin A_1 from Barley Aleurone Layers[1,2]

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ABSTRACT

The major metabolite produced during incubation of [^{3}H]gibberellin A_1 ([^{3}H]GA_1) with barley aleurone layers is an amphoteric, water-soluble compound tentatively called [^{3}H]ampho GA_1. Formation of [^{3}H]ampho GA_1 in barley aleurones begins after a period of 2.5 hours. As judged by degradation studies as well as Sephadex column chromatography, GA_1 appears to be linked to a peptide; positions C-3 and C-7 were ruled out as conjugation sites.

Among the most studied effects of gibberellins on plant tissues is the induction of certain hydrolytic enzymes in barley aleurone layers (5, 7, 10, 11, 12). During an investigation (8) of the relation between metabolism of [^{3}H]GA_1 and the induction of α-amylase in barley aleurones, we confirmed previous reports (2, 3) that ABA suppresses this induction. In addition, we found that ABA enhances conversion of [^{3}H]GA_1 to more polar (and less bioactive) metabolites. Formation of one of these metabolites, [^{3}H]GA-X, here called [^{3}H]ampho GA_1 (*I*), was especially promoted by ABA. Its formation was proportional to the logarithm of the ABA concentration. Now *I* has been produced in relatively high radiochemical yield (3.2×10^5 dpm, 1.2 μg) in order that we may study its structure and physical properties.

MATERIALS AND METHODS

[^{3}H]GA_1 was prepared and purified by methods recently described in detail (8). Its specific radioactivity is 43 Ci/mmole, 2.6×10^8 dpm/μg (8). Barley seeds, *Hordeum vulgare* L., 'Himalaya', vintage 1972, were obtained from the Department of Agronomy, Washington State University. ChromAR, a glass fiber-silicic acid (30:70) mixture, was purchased from Mallinckrodt Chemical Works.

Generally, the incubation procedures folowed were those described by Chrispeels and Varner (3). The buffer was 2 mM in sodium acetate and 20 mM in $CaCl_2$, adjusted to pH 4.8. One hundred fifteen isolated aleurone layers were incubated at 25 C in 6 ml of buffer which was 48 μM in [^{3}H]GA_1 (10 μg, 2.6×10^9 dpm, per 6.0 ml) and 19 μM in ABA (30 μg per 6 ml). Most of the layers were incubated 24 hr, but nine lots of three layers each were withdrawn at earlier intervals to determine the onset of formation of *I* (Fig. 1). After 24 hr of incubation, radioactive materials in the remaining layers were extracted with methyl alcohol. The methyl alcohol extract was concentrated by distillation under high vacuum so that the temperature never exceeded 45 C. The concentrate was refined by TLC on ChromAR, using isopropanol-NH_4OH-water (8:1:1). In this system, *I* was the only radioactive material remaining at the origin.

The chromatographic systems and the R_F values of the compounds studied are shown in Table I. Details on these procedures have been reported previously (8) and are summarized in Table II along with the retention times of the compounds under investigation.

High voltage paper electrophoresis was done using sheets of Whatman No. 3 MM paper having basic weight = 185 g/m^2, 0.33 mm thickness, medium flow rate, and smooth surface. In all runs the voltage was 1500 v, and the time was 1.5 hr. Zones were detected by radiochromatogram scanning. Buffers were: pH 10.6, NH_4OH (0.065 M); pH 5.9, pyridine-acetic acid-H_2O (100:15:900); pH 2.9, pyridine-acetic acid-H_2O (3:100:1900); pH 2.2, 2.5% formic acid-8% acetic acid, with water.

RESULTS AND DISCUSSION

The first report of [^{3}H]ampho GA_1 was by Nadeau *et al.* (10) who referred to it as [^{3}H]GA-X. This most polar of GA_1 conjugates was found only as a metabolite of monocotyledonous plants, including barley (*Hordeum vulgare*), maize (*Zea mays*) (13; Davies and Rappaport, unpublished), and wheat (*Triticum aestivum*) (Davies, Stoddart, and Rappaport, unpublished). Although its function in plants is unknown, it was of interest that, as the major product of GA_1 metabolism in barley aleurones, it dramatically increased in concentration as a function of concentration of ABA. This led to the speculation that one feature of ABA action in inhibiting enzyme synthesis in the barley aleurone was the enhanced conversion of GA_1 to [^{3}H]GA-X (10).

Here we report that [^{3}H]ampho GA_1 was detected within 2.5 hr, a time roughly coincidental with a number of biochemical

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[2] Ernest Sondheimer's research was characterized by his consistent efforts to provide a chemical basis for an understanding of plant hormone action. He understood that simply measuring a change in concentration of a hormone is not sufficient to explain its function in a process. Not only was he concerned with the occurrence of hormones but also with their identity. The dedication of this volume to mark his lamentably early death should serve to focus the attention of those engaged in growth regulator research on the need to attain a fuller comprehension of the role of hormone biosynthesis and turnover in relation to physiological processes. If this occurs his contribution will remain as much more than a footnote in the annals of hormone research.

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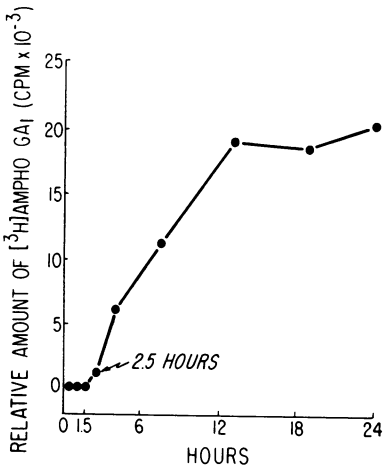

FIG. 1. Time course of formation of [^{3}H]ampho GA_1. At each of the indicated times, three aleurone layers were withdrawn from the incubation medium, washed briefly with water, stored at 0 C, and later homogenized and analyzed by TLC for [^{3}H]ampho GA_1 content.

Table I. *Mobilities of Compounds on TLC*

Compound	TLC System		
	A[1]	B[2]	C[3]
	R_F		
GA_1	0.49	0.78	ND[4]
I	0	0	0.53
IIa, b	0.59	ND	ND
IIIa, b	0.73	ND	ND
IV	0.21	ND	ND
V	0.33	ND	ND
VIa, b	0.43	ND	ND
VIIa, b	0.54	ND	ND

[1] System A: Stationary phase, ChromAR; solvent, Et_2O-benzene-HOAc (135:65:10). During 15 min of development the front migrated 15.2 cm.

[2] System B: Stationary phase, ChromAR; solvent, isopropanol-concentrate d NH_4OH-H_2O (8:1:1). During 1.5 or of development the front moved 15.3 cm.

[3] System C: Stationary phase, ChromAR; solvent, butyl alcohol-acetic acid-H_2O (4:1:4, organic phase). During 1.5 hr of development the front moved 13.5 cm.

[4] Not determined.

events that take place in cells of the aleurone layer (6). It is not known whether ampho-GA_1 occurs naturally in aleurone layers. The 2.5 hr lag period might indicate that applied GA_1 was necessary to induce an enzyme system responsible for formation of the conjugate. In a single test, [^{3}H]ampho GA_1 was shown to have only slight activity in inducing α-amylase synthesis in barley aleurones, but further tests are required to establish its bioactivity. In view of the timing of its formation, and its unique chemical characteristics, knowledge of the structure of [^{3}H]ampho might yield important insights into its mode of action. Therefore, attempts were made to elucidate the structure of this compound. After an initial rapid increase, formation leveled off at 12 hr (Fig. 1). Hence a 12-hr incubation time was sufficient to obtain *I*.

The key to an interesting characteristic of [^{3}H]ampho GA_1 was analysis by high voltage paper electrophoresis (Fig. 2). We found that *I* is isoelectric at pH 2.9, and that it moves toward the cathode at pH 2.2, indicating that *I* might contain at least one protonizable nitrogen-containing function. This possibility was substantiated by the fact that *I* reacted quantitatively with dinitrofluorobenzene (14), yielding a derivative which remained at the neutral point during electrophoresis at pH 2.2. On electrophoresis at pH 5.9, *I* had greater mobility toward the anode than did [^{3}H]GA_1, indicating that it contains more acidic groups than GA_1. [^{3}H]Ampho GA_1 has a higher mol wt than GA_1, as shown by Sephadex chromatography, using NADPH (mol. wt. 746) and [^{3}H]GA_1 (mol wt 350) as markers (Fig. 3).

To test the possibility that *I* might be a conjugate of [^{3}H]GA_1,

Table II. *GLC Retention Times on 3% SE-30 of the Methylester-Trimethylsilyl Ether Derivatives*

Three per cent SE-30 adsorbed on 100–120 mesh Gas Chrom Q was packed in stainless steel columns measuring 83 cm × 3 mm. Conditions were: oven temperature, 212 C; injector temperature, 225 C; detector temperature, 230 C; carrier gas flow, N_2, 44 ml/min.

Compound-Trimethylsilyl Ether	Retention Time
	min
GA_1	9.9
IIa, b	9.0
IIIa, b	6.7
IV	19.0
V	14.3
VIa, b	13.2
VIIa, b	7.4

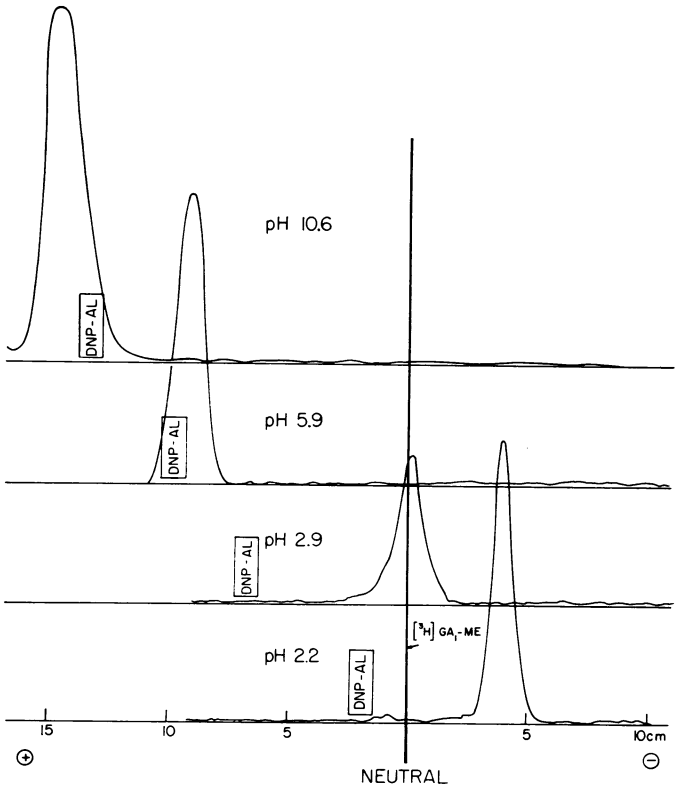

FIG. 2. High voltage paper electrophoresis of [^{3}H]ampho GA_1. In each run, ~10^6 dpm of *I* were applied to a 4-cm portion of the center line, together with dinitrophenolalanine as reference marker. On a separate portion of the sheet, [^{3}H]GA_1Me (GA_1-methyl ester) was applied in order to ascertain the neutral point. Radioactive zones were detected by radiochromatogram scanning.

I was combined with nonradioactive GA$_1$. The mixture was subjected to increasingly stringent acid conditions until treatment with 1 N HCl at 100 C for 12 hr (Fig. 4, reaction A) gave a 66% yield of ethyl acetate-soluble radioactive material (*I* is water-soluble and ethyl acetate-insoluble). GLC-RC (9) revealed that the ethyl acetate extract contained nonradioactive IIa and IIIa, the products expected (1) from acid treatment of GA$_1$, plus the radioactive IIb and IIIb. In addition, a radioactive peak appeared at longer retention time, but this peak did not have a corresponding nonradioactive component. Retention times are given in Table II.

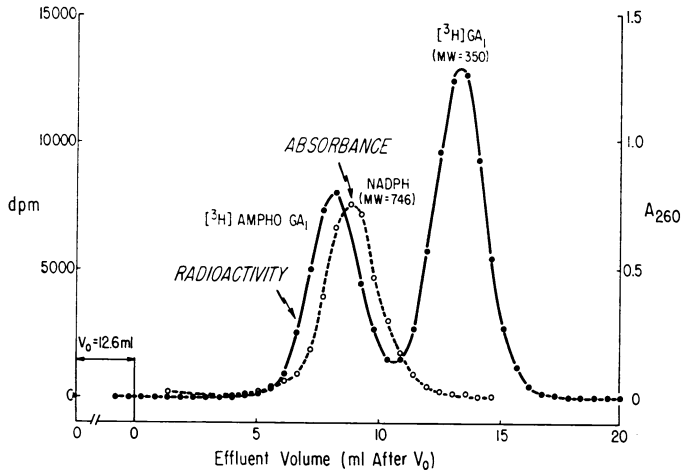

FIG. 3. Sephadex G-25 chromatography: comparison of *I*, [^{3}H]GA$_1$ and NADPH. The profiles of *I* and [^{3}H]GA$_1$ were detected by scintillation counting of a portion of each 0.5-ml fraction, and that of NADPH by measuring absorbance at A_{260}. Elution buffer was 0.1 M NaCl. Column dimensions 0.9 × 60 cm.

Having found that *I* is a conjugate of [^{3}H]GA$_1$, we sought to determine the site of conjugation. There are five possible conjugation sites in GA$_1$: C-7 carboxyl, C-3 hydroxy, C-13 hydroxy, C-10 (potential) hydroxy, and lactone (potential) carboxy. Amide and ester linkages were ruled out because strong alkaline treatment of a mixture of *I* and GA$_1$ did not produce an ethyl acetate-soluble radioactive substance, but did give an EtOAc-soluble mixture of nonradioactive GA$_1$ and 3αOH-GA$_1$ (pseudo GA$_1$). This indicated a possible ether linkage at C-3, C-13, or C-10. An unusual feature in the chemistry of the GA$_1$ molecule made it possible to eliminate from consideration an ether linkage at C-3. When GA$_1$ is exposed to mild alkali, epimerization of the C-3 hydroxyl occurs (4). This can happen only when the C-3 hydroxyl is not conjugated. Treatment of a mixture of *I* and GA$_1$ with 0.1 N NaOH (Fig. 4, reaction B) did not cleave the conjugate, a result shown by electrophoresis at pH 5.9. However, the mild alkali treatment did cause a change in *I* as subsequent acid hydrolysis (Fig. 4, reaction C) of the equilibrium mixture and extraction into ethyl acetate produced not only IIb and IIIb, but also the pseudo forms VIb and VIIb, as indicated by GLC-RC. Naturally, since GA$_1$ was present as a component from the start, IIa, IIIa, VIa, and VIIa were also detected in the final product mixture. We con-

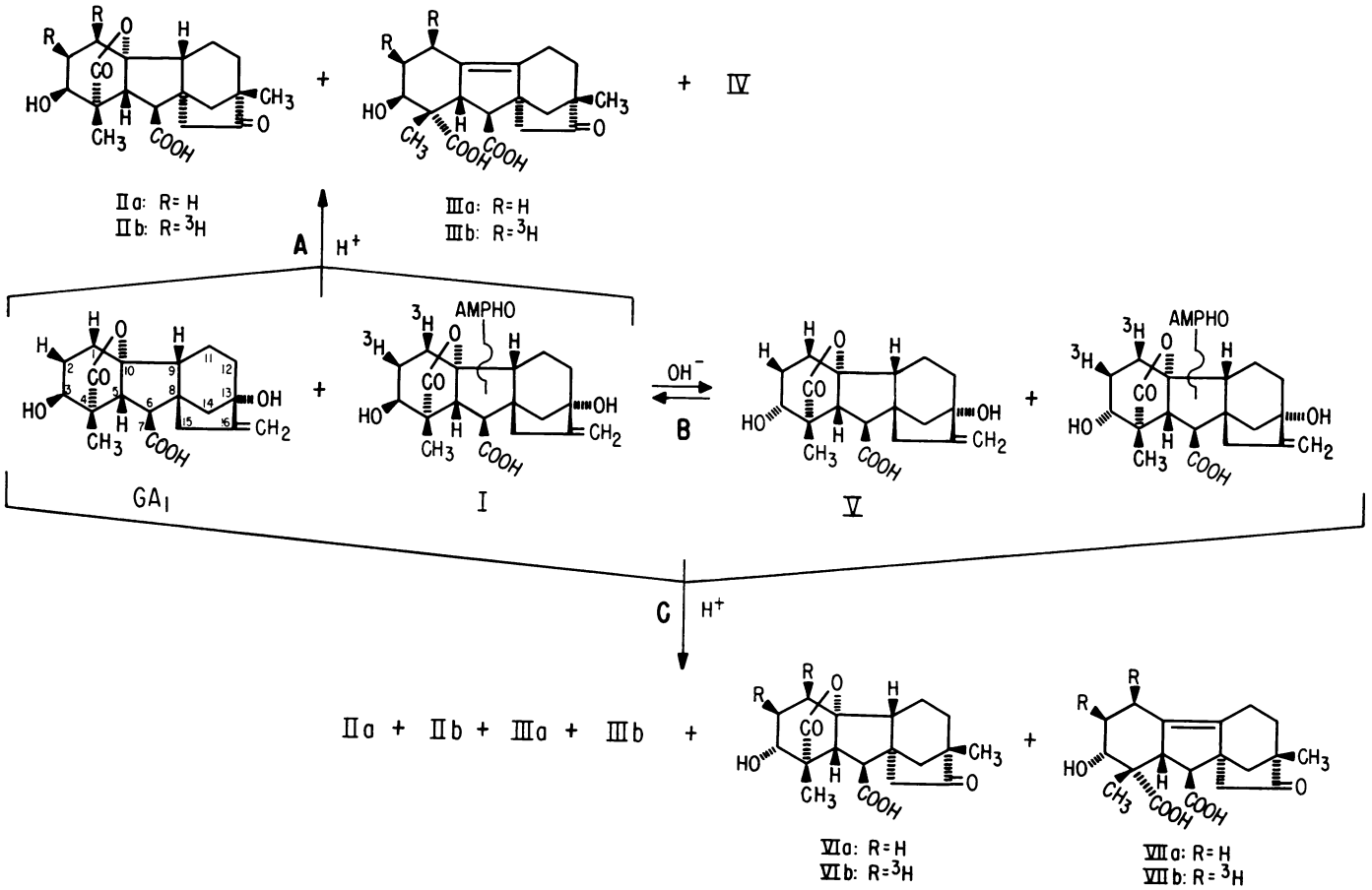

FIG. 4. Reactions of [^{3}H]ampho GA$_1$ (*I*) and GA$_1$ with acid and base. Reaction A: acid hydrolysis of *I*/GA$_1$ mixture; reaction B: mild alkali treatment of *I*/GA$_1$ mixture to produce equilibrium mixture of 3-OH-epimers; reaction C: acid hydrolysis of the epimeric equilibrium mixture from B.

cluded that in *I* the C-3 hydroxyl is in a "free" form, and thus is not the site of conjugation.

The question of conjugation at C-10 or C-13 was not pursued further in this study since none of the gibberellin conjugates so far reported have linkages at C-10 or C-13. All such conjugates are either glycosyl esters at C-7 or glycosyl ethers at C-2, C-3, or C-11 (15, 16). From the above findings it seems evident that [^{3}H]ampho GA_1 is an especially interesting conjugate of [^{3}H]GA_1, as it undoubtedly contains nitrogen. In view of this information, and of the mol wt of ampho GA_1 (700–800 by Sephadex), it is tempting to speculate that the conjugate moiety is a short peptide chain. Full characterization of ampho GA_1 will require production on a larger scale.

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