Preparation of Radioactive Gibberellin A_1 and its Metabolism in Dwarf Peas

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Summary. Gibberellin A_1-3,4-^{3}H was prepared by selective catalytic reduction of gibberellic acid with a mixture of tritium and hydrogen. ^{3}H-GA_1 was applied at physiological concentrations to dwarf peas and the metabolism of the hormone was investigated. ^{3}H-GA_1 was converted to an acidic, biologically active compound. Radioactive but biologically inactive compounds were also found in the neutral fraction and could not be converted to acidic gibberellins by hydrolysis. No attachment of gibberellin to any macromolecular fraction was evident.

While much work has been done on the physiological and biochemical effects of the gibberellins, very little attention was given to the fate of this hormone within the plant. One of the reasons for this may have been the lack of reliable methods to produce radioactive gibberellins. Zweig et al. (19) and McComb (11) prepared ^{14}C-gibberellin by growing the gibberellin-producing fungus *Fusarium moniliforme* in a medium containing ^{14}C-acetate. This method yields a hormone of low specific activity and the quantities obtained are too small for rigorous chemical purification. Baumgartner et al. (1) and Nitsch and Nitsch (14) used gibberellic acid which was randomly tritiated by the Wilzbach method. It is very difficult to predict the nature of the products of a Wilzbach tritiation. In many cases, addition of tritium to double bonds gives rise to highly radioactive compounds which would be very difficult to separate from the original substance. Much of the early work with labeled gibberellin dealt with the translocation properties of this compound (12, 19). McComb (12) re-isolated ^{14}C-gibberellic acid from pea seedlings 5 hours after treatment and found it to be unaltered. Nitsch and Nitsch (14), on the other hand, obtained a biologically active metabolite of gibberellic acid from beans and Rappaport et al. (16) found a conversion of ^{14}C-gibberellic acid to a neutral compound in bean seeds.

This communication describes the preparation of radioactive gibberellin A_1 (GA_1, II, fig 1) of a specific activity sufficiently high to study the metabolism as well as the cellular and subcellular fate of this hormone. It was deemed necessary to study first the metabolism of a gibberellin in a growing plant but, in other experiments, an attempt is being made to localize the hormone within the cell in the hope of obtaining clues on its site of action.

Materials and Methods

Preparation of Radioactive Gibberellin A_1. Controlled reduction of gibberellic acid (GA_3, I, fig 1) yielding gibberellin A_1 (GA_1, II, fig 1) and a dicarboxylic acid (III, fig 1) was reported by Jones and McCloskey (4). Selective hydrogenation of the cyclic double bond was achieved through partial poisoning of the palladium catalyst by adding pyridine to the reaction mixture. The procedures of Jones

[1] The hydrogenation was performed by New England Nuclear Corporation, but all further purifications were done by the author.

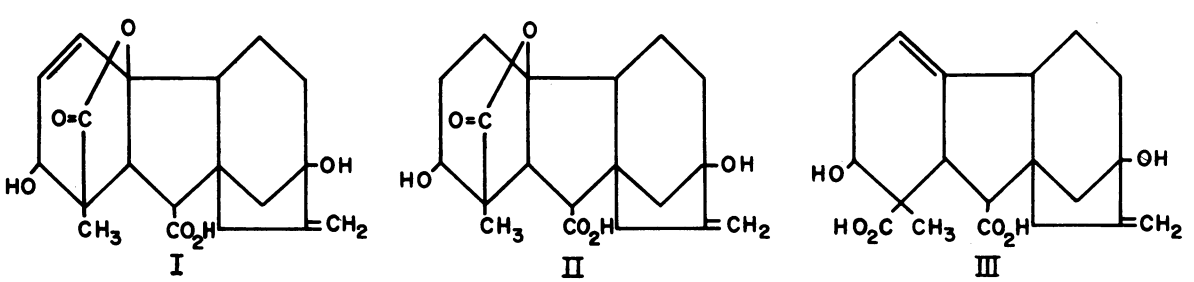

FIG. 1. Structures of gibberellic acid (GA_3, 1), gibberellin A_1 (GA_1, II) and the dicarboxylic acid (III).

Table I. *Solvent Systems for Thin Layer Chromatography and R_F Values of GA_1*

All solvents were of chromatographic grade or redistilled. Chromatograms were developed over a distance of 10 or 15 cm.

Solvent	R_F of GA_1
1. Isopropanol-water (80:20, v/v)	0.67
2. Chloroform-ethyl acetate-acetic acid (60:40:5, v/v)	0.21
3. Benzene-*n*-butanol-acetic acid (70:25:5, v/v)	0.77
4. Di-isopropyl ether-acetic acid (95:5, v/v)	0.07
5. Di-isopropyl ether-acetone-acetic acid (90:30:1, v/v)	0.19

and McCloskey were followed except that the reduction was carried out with a mixture of tritium and hydrogen.[1] Gibberellin A_1-3,4-^{3}H obtained by this method at a yield of 30 % was separated from the dicarboxylic acid (III, fig 1) by thin-layer chromatography using silica gel H and solvent 1 (table I). This ^{3}H-GA_1 had a specific activity of 1.3 c/mmole and it showed the expected biological activity in the dwarf corn assay. Subsequently, unlabeled authentic GA_1 was added to it and the mixture was recrystallized 4 times from acetone-light petroleum ether (b.p. 40–50°) yielding a constant specific activity of 80.0, 87.1, 87.0 and 88.1 mc/mmole. The ^{3}H-GA_1 co-chromatographed as 1 single spot with authentic GA_1 on silica gel H thin layers using 5 different solvent systems (table I). ^{3}H-GA_1 (3.5 μc) was dissolved in 10 ml of ethanol and the solvent was distilled off under vacuum and trapped. No radioactivity was found in the distillate, proving that no exchangeable tritium was associated with the ^{3}H-GA_1. The radioactive hormone was stored at a concentration of 2 mM in ethyl acetate at −20°. The purity of the compound was checked regularly over a period of 1 year and no decomposition due to self-irradation could be observed. A second batch of GA_1 of lower specific activity (20 mc/mmole) was prepared in an identical fashion. The pure crystalline hormone was used without addition of carrier GA_1. All major experiments were repeated several times with this second lot of ^{3}H-GA_1 and identical results were obtained as with the first lot.

Growth and Treatment of Pea Plants. Dwarf peas (*Pisum sativum* L., var. Progress No. 9) were soaked overnight, planted in vermiculite and kept in the dark at 25°. After 3 days, the seedlings were selected for uniformity and transferred to plastic boxes containing half-strength Hoagland solution. They were kept under low-intensity red light (450 $\mu W \cdot cm^{-2}$) at 25° for 24 hours and were treated thereafter with ^{3}H-GA_1. After gibberellin treatment, the plants were returned to the red light chamber for a period of 24 to 72 hours. Dark-grown plants were handled under dim green light but otherwise treated identically.

^{3}H-GA_1 was dissolved in 0.05 % (v/v) Tween 20 (polyoxyethylenesorbitan monolaurate) and applied to the plumular hook in a 5 μl droplet at a concentration of 20 μM (0.035 μg/plant). Under our conditions, the growth response of the dwarf pea is half saturated at a GA_1 concentration of 30 μM (0.053 μg/plant), the optimum being reached at 100 μM GA_1 (0.175 μl/plant). Changing the concentration of the hormone applied to the plant did, however, not alter the pattern of its metabolism. Before harvesting, plants were surface-washed with methanol (50 %, v/v) or distilled water. Taking the average of 8 experiments, 23.5 % of the applied radioactivity could be recovered in this wash. Since only the internodes above the first node showed a marked growth response to gibberellin (fig 2), the shoots were harvested above the first scale leaf and immediately frozen in liquid nitrogen.

Extraction Procedures. Fifty to a hundred frozen plants were extracted with 100 ml of methanol using an "Omnimix" blender. The extract was filtered, the tissue residue kept for radioactive analysis and the filtrate evaporated. The filtrate residue was taken up in 50 ml of 0.5 M phosphate buffer (pH 8.4) and partitioned several times against equal volumes of light petroleum ether (b.p. 40–50°) until the organic phase was colorless. The combined petroleum ether phase was checked for radioactivity. The aqueous phase was further purified by repeated partitioning against ethyl acetate until no pigment was extracted by the solvent. The combined alkaline ethyl-acetate fraction was dried over Na_2SO_4 and the radioactivity was determined. The buffer was now adjusted with 2 N HCl to pH 2.5 and partitioned 7

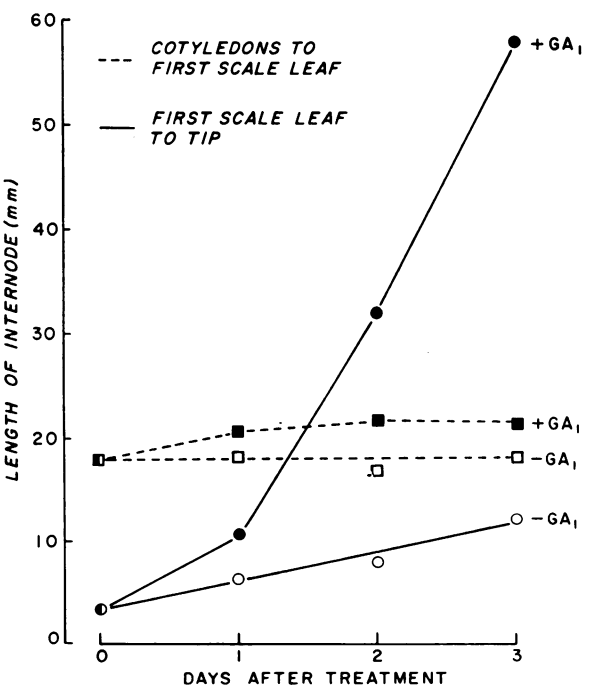

FIG. 2. Growth response of light-grown dwarf peas to ^{3}H-GA_1 (0.035 μg/plant). Each point on the curve is the average of 16 individual measurements.

times against ethyl acetate. After this, no radioactivity was extractable from the acidified buffer. The combined acidic ethyl-acetate phase was dried over Na_2SO_4 and the radioactivity was counted. The aqueous residue was brought to pH 7.0 and was also tested for radioactivity.

Chromatography. Thin-layer chromatography on silica gel H was performed according to MacMillan and Suter (9) and Sembdner et al. (17). Gibberellin standards were run as reference on the side of each plate. The portion of the chromatogram with the plant extract was divided into 10 equal zones, the silica gel was scraped into centrifuge vials and eluted 4 times with water-saturated ethyl acetate. These fractions were used for the subsequent assays. The reference chromatograms were sprayed with 5 % (v/v) H_2SO_4 in ethanol, heated at 100° for 10 minutes and the gibberellin spots were viewed under UV light (9).

Determination of Radioactivity. Radioactivity was determined by liquid scintillation spectrometry using Bray's solution (2) as scintillator. Quench corrections were made when necessary. Before counting, the vials were kept in the dark for 24 hours. The radioactivity of the chromatographic fractions was determined either by assaying aliquots of the eluates or by counting directly parts of the silica gel corresponding to the various R_F zones. The latter method proved to be very efficient as 85 to 90 % of the counts applied to the plate were detectable. The tissue residue was dried, weighed and 10 mg were immersed into the scintillation solvent.

Bioassays. For most part of this work, the dwarf-corn bioassay (d_5) was used as described earlier (6). The dwarf-pea test (6) was applied occasionally and gave similar results.

Results

In initial experiments, light and dark-grown dwarf peas treated with 20 μM ^{3}H-GA_1 were extracted and fractionated as described. No radioactivity was found in the petroleum ether and alkaline ethyl-acetate phases. The distribution of radioactivity in the tissue residue, acid ethyl-acetate fraction and the aqueous phase at 24, 48 and 72 hours after treatment is summarized in table II.

Acidic Ethyl Acetate Fraction. The bulk of the radioactivity was associated with this fraction. It decreased, however, with time and this was paralleled by an increase of the radioactivity in the other fractions (table II). When chromatographed with solvent 2, 1 peak of radioactivity was obtained which also showed biological activity and which co-chromatographed with authentic GA_1 (fig 3). A second zone of biological activity, most probably due to GA_5, was free of radioactivity (fig 3). In no case could a conversion of the ^{3}H-GA_1 to the GA_5-like fraction be observed. The radioactive hormone co-chromatographed with GA_1 as 1 single zone in 4 solvents, 1, 2, 4 and 5. It could, however, be separated into 2 fractions with solvent 3 (fig 4). Besides unaltered ^{3}H-GA_1 a second, biologically active compound appeared which increased in concentration with time (table II). The biological activity of this compound could be demonstrated best when peas were treated with a relatively high concentration of ^{3}H-GA_1 (60 μM, 0.105 μg/plant). The kinetics of the conversion of GA_1 to this unknown acidic compound varied from experiment to experiment. Table II summarizes the most extreme case when the half-life time of ^{3}H-GA_1 was about 3 days. In other experiments, the rate of conversion was somewhat slower.

Table II. *Distribution of Radioactivity in the Extracts of Light-grown Dwarf Peas 24, 48 and 72 Hours after Treatment with ^{3}H-GA_1*

For each extraction, 48 plants were treated with a total of 113,280 cpm and the hormone was applied at a concentration of 20 μM (0.035 μg/plant). The amounts of unchanged GA_1 and the unknown acidic metabolite were calculated from their relative distribution on a chromatogram developed with solvent 3.

Harvested after treatment	Fraction	Radioactivity recovered	Total recovered counts
hrs		cpm	%
24	Acidic ethyl acetate	70.712	95.1
	GA_1		(76.6)
	unknown substance		(18.5)
	Aqueous	2943	3.9
	Tissue residue	721	1.0
48	Acidic ethyl acetate	66,948	92.6
	GA_1		(61.6)
	unknown substance		(31.0)
	Aqueous	4320	6.0
	Tissue residue	1025	1.4
72	Acidic ethyl acetate	53,255	88.0
	GA_1		(48.4)
	unknown substance		(39.6)
	Aqueous	5734	9.5
	Tissue residue	1509	2.5

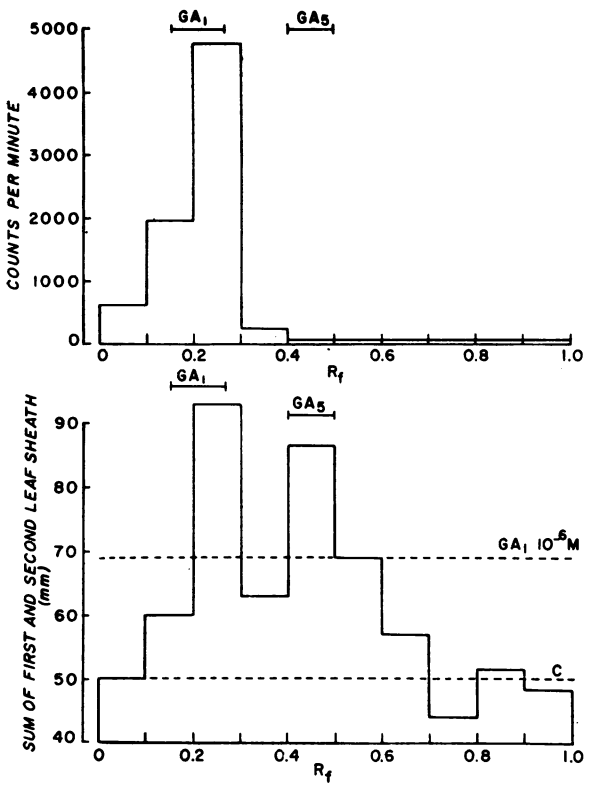

FIG. 3. Chromatography of the acidic ethyl acetate fraction from 70 peas extracted 48 hours after treatment with ^{3}H-GA$_1$ (0.035 μg/plant) on silica gel H with solvent 2. Top) distribution of the radioactivity. Bottom) distribution of the biological activity as measured by the dwarf corn (d_5) bioassay. The horizontal bars indicate the R_F values of GA$_1$ and GA$_5$, the dotted lines indicate the height of control plants (C) and plants treated with 1 μM GA$_1$.

Only 7 % of the GA$_1$, was converted to the unknown acidic substance after 24 hours and 30 % after 3 days. The reason for this variation is not known.

Aqueous Phase. After exhaustive extraction of the acidified buffer, some radioactivity still remained in the aqueous phase (table II). The presence of neutral bound gibberellins has been reported by a number of authors (3, 10, 13, 18). Evidence was presented that acidic gibberellins could be recovered from such complexes by treatment with glycolytic (13) and proteolytic (10) enzymes as well as by acid hydrolysis (18). The aqueous fraction was either incubated with hydrolytic enzymes, or hydrolyzed with acid as specified in table III. Thereafter, the aqueous phase was re-acidified and extracted 7 times with ethyl acetate. An equal part of the aqueous phase was incubated without enzymes or left unhydrolyzed. These controls were also acidified and partitioned against ethyl acetate. Table III shows that none of the hydrolytic treatments resulted in a significant release of radioactivity into the acidic fraction. The radioactivity obtained from hydrolyzed and control aqueous phases was probably a small residue not extracted during the first fractionation.

The aqueous phase was lyophylized and the residue chromatographed on a Sephadex G-10 column. All the radioactivity was retarded on this column which excludes compounds of a molecular weight above 700. Blue Dextran 2000 served as a marker to determine the chromatographic properties of substances with a molecular weight higher than 700. This experiment showed that the radioactivity was associated with at least 2 compounds of low molecular weight (fig 5).

The residual radioactivity could be extracted from the acidified aqueous phase by partitioning with n-butanol. The butanol fraction was chromatographed on Whatman No. 3 paper using isopropanol-NH_4OH-water (10:1:1, v/v) and gave 3 peaks of radioactivity which, however, were biologically inactive.

Tissue Residue. Residual radioactivity in the extracted tissue was very small but increased in the course of a 3-day experiment (table II). After 24 hours, the radioactivity associated with the tissue amounted to 0.3 % to 1 % of the total recovered

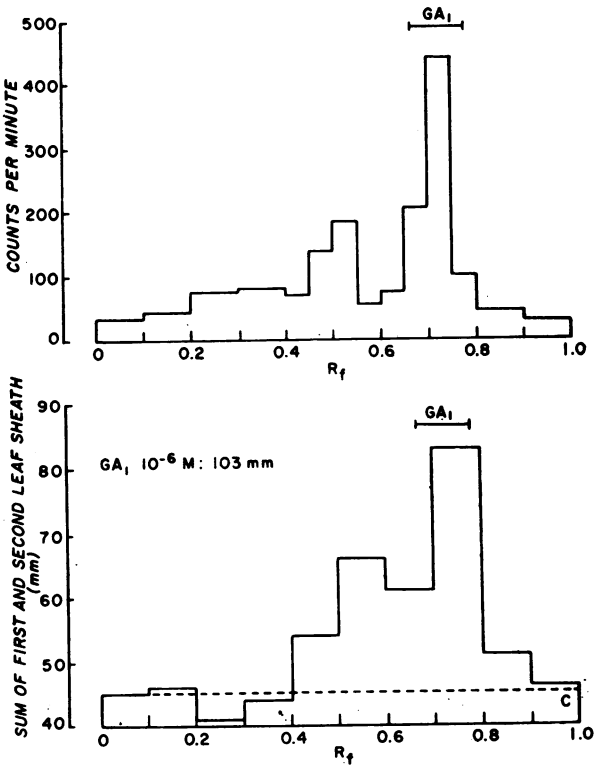

FIG. 4. Rechromatography of the radioactive GA$_1$-region obtained from a previous chromatogram (developed with solvent 2) on silica gel using solvent 3. The acidic ethyl acetate fraction was obtained from 48 light-grown pea plants 72 hours after treatment with ^{3}H-GA$_1$ (0.105 μg/plant). Top) distribution of radioactivity. Bottom) distribution of biological activity as measured by the dwarf corn (d_5) assay. The horizontal bars indicate the R_F value for GA$_1$, the dotted line the height of control plants (C).

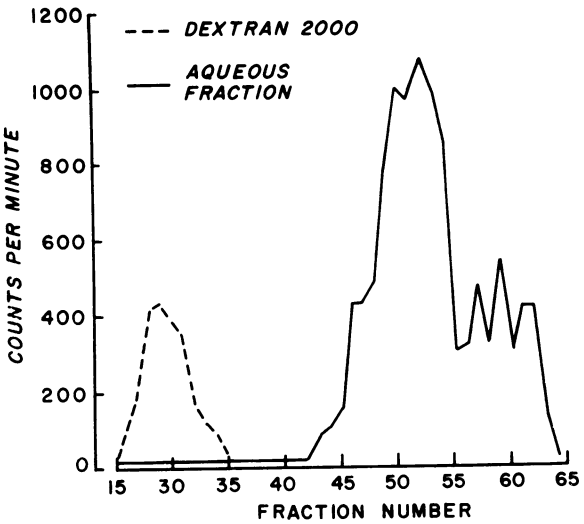

FIG. 5. Chromatography of the aqueous fraction from 92 light-grown pea plants extracted 48 hours after treatment with ^{3}H-GA$_1$ (0.035 μg/plant) on a Sephadex G-10 column (108 cm long, 18 mm diameter). The column was eluted with distilled water and 3 ml fractions were collected. The OD of dextran 2000 was measured at 630 mμ.

radioactivity (tables II and IV). Extensive washing of the residue with methanol removed all detectable counts.

Gibberellin Metabolism in Dark and Light-Grown Peas. Reversion of light inhibited stem growth by gibberellic acid in peas was demonstrated by Lockhart (7). It was concluded that light interacts somehow with the native gibberellin. Measuring the effect of unlabeled GA$_1$ on light and dark-grown dwarf peas, Kende and Lang (6) concluded that the hormone was utilized to the same extent under both conditions. In a re-examination of this question, ^{3}H-GA$_1$ was found to be metabolized by light and dark-grown plants to the same extent (table IV). Thus, light-mediated growth inhibition is not due to a faster breakdown of the hormone during illumination.

Discussion

Pea seedlings contain at least 2 gibberellins, having biological and chromatographical properties of GA$_1$ and GA$_5$ (6). Since GA$_1$ and GA$_5$ were originally isolated from seeds of leguminous plants (8) it seems very likely that GA$_1$, or a gibberellin very similar to it, is in fact a natural hormone of peas. Studying the fate of applied ^{3}H-GA$_1$ is thus relevant to the metabolism of at least 1 of the endogenous gibberellins of peas.

In the experiments reported here, no strong binding of the hormone to any large molecules could be found. After 24 hours, essentially all radioactivity was extractable with methanol. In other experiments, no radioactivity was found to be associated with either the nucleic acid or the protein fractions extracted from peas 24 hours after treatment with ^{3}H-GA$_1$ of concentrations up to 200 μM (Kende, unpublished results). The results indicate that the attachment of the hormone to its site of action must be of a very loose and possibly of non-covalent nature.

The significance of the acidic metabolite of GA$_1$ which has some biological activity is not clear. Three possibilities have to be considered. A) The formation of this compound may be unrelated to the action of GA$_1$; B) it may be formed during the binding of the hormone to its site of action, and C) the hormone may have to be converted to this compound before it can be active. On circumstantial evidence, the first possibility is favored. Half-grains of barley were incubated with 1 μM ^{3}H-GA$_1$ for 24 hours according to the procedures of Jones and Varner (5). The half-grains as well as the incubation medium were extracted thereafter and no metabolites of ^{3}H-GA$_1$ were evident (Kende, unpublished results). This confirms earlier results by Paleg and Coombe (15)

Table III. *Enzymatic and Acid Hydrolysis of the Aqueous Fraction of Light-grown Dwarf Peas*
The plants were extracted 48 hours after treatment with ^{3}H-GA$_1$ (0.035 μg/plant).

Treatment	Original counts in aqueous phase	Counts extractable into acid ethyl acetate fraction after treatment
	cpm	cpm
Ficin*	9150	1760
Control	9150	1692
β-Glucosidase**	2130	193
Control	2130	195
0.4 N HCl***	4195	933
Control	4320	737

* Extract of 35 peas, 54 ml of aqueous phase at pH 6.2 (0.5 M phosphate buffer) incubated with 500 mg ficin (Sigma Chemical Co.) and 1 mg cysteine at 27° for 12 hr. Control incubated without enzyme.

** Extract of 24 peas, 35 ml of aqueous phase at pH 5.0 (0.5 M phosphate buffer) incubated with 5 mg β-glucosidase (Worthington Biochemical Corp.) at 27° for 24 hr. Control incubated without enzyme.

*** Extract of 48 peas, aqueous phase hydrolized in 0.4 N HCl at 80° for 30 min. Control acidified and immediately partitioned with ethyl acetate.

Table IV. *Distribution of Radioactivity in the Extract of Light and Dark-grown Dwarf Peas*

48 Plants were extracted 24 hours after treatment with ^{3}H-GA$_1$ (0.035 μg/plant). A total of 113,000 cpm were applied. The plants were grown in nutrient solution containing 100 mg/l Amo-1618*. The amounts of unchanged GA$_1$ and the unknown acidic metabolite were calculated from their relative distribution on a chromatogram developed with solvent 3.

Growth conditions	Fraction	Radioactivity recovered	Total recovered counts
		cpm	%
Red light	Acidic ethyl acetate	67,704	95.2
	GA$_1$		(79.7)
	unknown substance		(15.5)
	Aqueous	3160	4.4
	Tissue	235	0.3
Darkness	Acidic ethyl acetate	68,200	94.3
	GA$_1$		(79.8)
	unknown substance		(14.5)
	Aqueous	3900	5.4
	Tissue	224	0.3

* Amo-1618 was added in order to increase the sensitivity of dark-grown peas to applied GA$_1$. For control purposes, Amo-1618 was included in the growth medium of the light-grown peas as well.

who could not find a decline in the biological activity of gibberellic acid after incubation of barley half seeds. In this system, ^{3}H-GA$_1$ does not need to be altered for its action and, apparently, is not changed while it acts. Radioactive GA$_1$ incubated at 27° in buffers of pH 4.5, 7.0 and 9.5 does not decay spontaneously for at least 4 days. The simplest explanation would then be that the unknown acidic compound is a metabolite of ^{3}H-GA$_1$ and is unrelated to the action of this gibberellin. From the experiments with peas and barley, it seems very likely that GA$_1$ itself is the immediate biologically active hormone.

The radioactive compounds in the aqueous phase were biologically inactive and had a molecular weight below 700. They could not be converted into an acid gibberellin by hydrolysis and can, most likely, be regarded as further degradation products of the hormone.

The following features make gibberellins suitable candidates for an attempt to find the site of action of the hormone. In peas, a gibberellic acid-enhanced growth rate can be measured after 2 to 4 hours (12) and after 24 hours the effect is plainly visible. During this time the hormone is little metabolized. Gibberellins, unlike IAA or cytokinins, are not related to basic metabolites such as tryptophan and purines and this should facilitate the interpretation of results. Current work focusses on the intracellular localization of ^{3}H-GA$_1$.

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Literature Cited

1. Baumgartner, W. E., L. S. Lazer, A. M. Dalziel, E. V. Cardinal, and E. L. Varner. 1959. Determination of gibberellins by derivative labeling with diazomethane-^{14}C and by isotopic dilution analysis with tritium-labeled gibberellins. J. Agr. Food Chem. 7: 422–25.
2. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279–85.
3. Hayashi, F., S. Blumenthal-Goldschmidt, and L. Rappaport. 1962. Acid and neutral gibberellin-like substances in potato tubers. Plant Physiol. 37: 774–80.
4. Jones, D. F. and P. McCloskey. 1963. Selective reduction of gibberellic acid. J. Appl. Chem. 13: 324–28.
5. Jones, R. L. and J. E. Varner. 1967. The bioassay of gibberellins. Planta 72: 155–61.
6. Kende, H. and A. Lang. 1964. Gibberellins and light inhibition of stem growth in peas. Plant Physiol. 39: 435–40.
7. Lockhart, J. A. 1956. Reversal of the light inhibition of pea stem growth by the gibberellins. Proc. Natl. Acad. Sci. 42: 841–48.
8. MacMillan, J., J. C. Seaton, and P. J. Suter. 1960. Isolation of gibberellin A$_1$ and gibberellin A$_5$ from *Phaseolus multiflorus*. Tetrahedron 11: 60–66.
9. MacMillan, J. and P. J. Suter. 1963. Thin layer chromatography of the gibberellins. Nature 197: 790.
10. McComb, A. J. 1961. 'Bound' gibberellin in mature runner bean seeds. Nature 192: 575–76.
11. McComb, A. J. 1964. The preparation of ^{14}C-labeled gibberellic acid. J. Gen. Microbiol. 34: 401–11.
12. McComb, A. J. 1964. The stability and movement of gibberellic acid in pea seedlings. Ann. Botany London 28: 669–87.

13. MURAKAMI, Y. 1961. Formation of gibberellin A_3 glucoside in plant tissues. Botan. Mag. Tokyo 74: 424–25.
14. NITSCH, C. AND J. P. NITSCH. 1963. Etude du mode d'action de l'acide gibbérelique au moyen de gibbérelline A_3 marquée. I. Action sur les substances de croissance du Haricot nain. Bull. Soc. Botan. France 110: 7–17.
15. PALEG, L. G. AND B. G. COOMBE. 1967. Physiological effects of gibberellic acid. IX. Recovery of gibberellic acid following incubation with endosperm. Plant Physiol. 42: 445–49.
16. RAPPAPORT, L., A. HSU, R. THOMPSON, AND S. F. YANG. 1967. Fate of ^{14}C-gibberellin A_3 in plant tissues. Ann. N. Y. Acad. Sci. In press.
17. SEMBDNER, G., R. GROSS, AND K. SCHREIBER. 1962. Die Dünnschichtchromatographie von Gibberellinen. Experientia 18: 584–85.
18. SEMBDNER, G., G. SCHNEIDER, J. WEILAND, AND K. SCHREIBER. 1964. Über ein gebundenes Gibberellin aus *Phaseolus coccineus* L. Experientia 20: 89–92.
19. ZWEIG, G., S. YAMAGUCHI, AND G. W. MASON. 1961. Translocation of ^{14}C-gibberellin in red kidney bean, normal corn, and dwarf corn. Advan. Chem. Ser. 28: 122–34.