Phytochemistry, 1975, Vol. 14, pp. 1195–1200. Pergamon Press. Printed in England.

METABOLISM OF GIBBERELLINS IN MATURING AND GERMINATING BEAN SEEDS

HISAKAZU YAMANE, NOBORU MUROFUSHI and NOBUTAKA TAKAHASHI

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

(*Received* 17 *September* 1974)

Key Word Index—*Phaseolus vulgaris*; Leguminosae; Kentucky Wonder; metabolism of gibberellins, A_1, A_4, A_5, A_8 and A_{20}.

Abstract—Tritium-labeled gibberellins (GA_1, GA_4, GA_5, GA_8 and GA_{20}) were fed to immature bean seeds 18 days after anthesis and their metabolic pathways were investigated. The results suggest that GA_4 and GA_{20} are both converted to GA_1, and the latter and GA_5 into GA_8. Conversions to corresponding glucosides and glucosyl esters also occurred. On germination, GA_1 was rapidly converted into GA_8 glucoside, and a slight decrease in radioactivity of GA_1 glucosyl ester was observed.

INTRODUCTION

Many studies on gibberellins in *Phaseolus vulgaris* revealed that the immature seeds contain GA_1, GA_4, GA_5, GA_6, GA_8, GA_{37}, GA_{38} and GA_8 glucoside, while the mature seeds contain GA_1, GA_8, GA_8 glucoside and glucosyl esters of GA_1, GA_4, GA_{37} and GA_{38} [1–4]. Since *P. vulgaris* contains a variety of free gibberellins in different oxidation stages and furthermore many glucosyl derivatives such as *O*-glucosides and glucosyl esters, it is useful for studying the metabolism of gibberellins in higher plants during seed development and germination.

Skene and Carr reported [5] that in the development of *Phaseolus* seeds there are two phases of rapid growth separated by a brief phase of very slow growth (the lag phase) and that the amount of gibberellins in the acidic EtOAc fraction correlates well with this diauxic pattern of seed development. The first phase corresponds with the rapid development of the embryo and the second, with that of seed maturity.

Our objective in this study was to clarify the metabolic pathways of the endogenous gibberellins in the maturing process of the seeds and their behaviour during germination. Therefore, five radioactive gibberellins (Fig. 1) were fed separately to the seeds of *Phaseolus vulgaris* cv. Kentucky Wonder 18 days after anthesis, when the developmental stage of the bean seeds corresponds either to the lag phase or the beginning of maturing period. Of these gibberellins, GA_1, GA_4, GA_5 and GA_8 are known to be endogenous in the seeds of *P. vulgaris*. Although GA_{20} has not been confirmed to be present in the seeds, it was included in the experiments because of its occurrence in the seeds of the closely related *P. coccineus* [2].

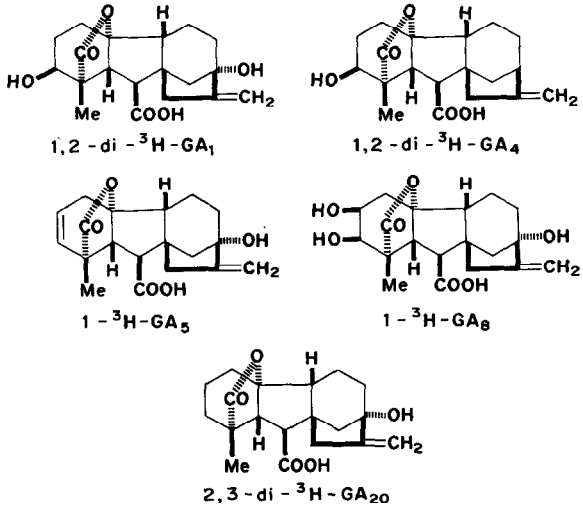

Fig. 1. Radioactive gibberellins fed to *Phaseolus vulgaris* seeds.

RESULTS AND DISCUSSION

In all the experiments (see Experimental), it was found that each of the ^{3}H-GAs fed to the seeds was mainly converted into ^{3}H-GA_8 glucoside. Since ^{3}H-GA_4 and ^{3}H-GA_{20} were converted into ^{3}H-GA_8 glucoside via ^{3}H-GA_1, the metabolism of these three precursors is discussed in parallel. On the other hand, ^{3}H-GA_5 was converted into ^{3}H-GA_8 glucoside via ^{3}H-GA_8, and so the metabolism of ^{3}H-GA_5 is discussed together with that of the latter compound.

The amounts of the ^{3}H-GAs fed to the seeds (0·33–100 μg per seed) were chosen from a chemical point of view and not on physiological grounds. However, the seeds metabolized the ^{3}H-GAs in similar way regardless of the quantity applied over this range.

A portion of the seeds treated with radioactive gibberellins was harvested on the second day after treatment (immature seeds) and the rest was grown to maturity. Each sample was extracted with methanol and fractionated into an acidic ethyl acetate (AE), a neutral ethyl acetate (NE), an acidic *n*-butanol (AB), a neutral *n*-butanol (NB) and an aqueous residue (Aq) fraction. The radioactivity of each fraction was determined and its distribution is summarized in Table 1.

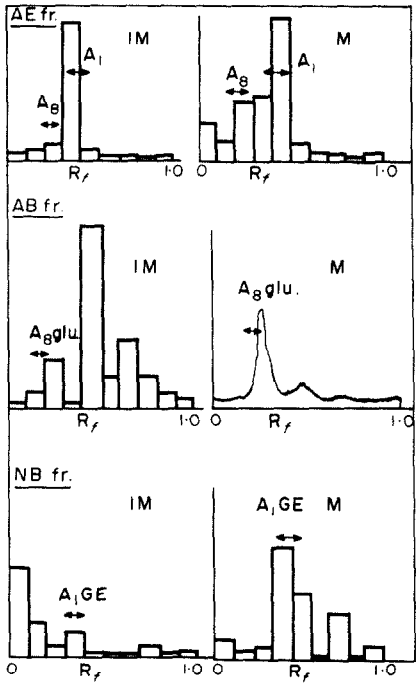

Fig. 2. Radiochromatogram tracing of each fraction from the immature (IM) and mature (M) seeds treated with ^{3}H-GA_1. Scintillation counts of eluted R_f strips were used when the radioactivity was low. TLC on Kiesel gel G was developed with the solvent systems described in Experimental. Since adequate quantity of each sample to study the location of radioactivity was spotted on the TLC plates, the volume of aliquot used in each experiment is variable. Abbreviation: GAs glu., GAs glucoside; GAs GE, GAs glucosyl ester.

Comparison of radioactivity between fractions from the immature and mature seeds obtained by treatment with ^{3}H-GA_1 showed a decrease in the relative radioactivity of the AE fraction and an increase of those of the AB and NB fractions during the maturing process of the seeds. A portion of each fraction was chromatographed on TLC and radioactivity located either by radiochromatogram scanning or liquid scintillation counting of each strip (Fig. 2). The chromatogram of the AE fraction from the immature seeds showed that radioactivity was mainly due to ^{3}H-GA_1 and ^{3}H-GA_8. This fact was confirmed by GLC-radiocounting (GC–RC) [6]: the trimethylsilyl (TMS) ether of the methyl ester of the AE fraction from the immature seeds gave two radioactive peaks on GLC corresponding to authentic TMS ethers of methyl esters of GA_1 and GA_8.

Radioactive components in the AE fraction from the mature seeds also consisted mainly of ^{3}H-GA_1 and ^{3}H-GA_8, but the relative radioactivity of GA_8 to GA_1 increased (Fig. 2). The AB fraction from the mature seeds showed two peaks on TLC and the R_f value of the larger more polar peak corresponded to that of authentic GA_8 glucoside. However, enzymatic hydrolysis of this peak afforded both ^{3}H-GA_1 and ^{3}H-GA_8 as aglycones, identified by GC–RC as TMS ethers of its methyl esters. Thus this peak proved to consist of glucoside-like conjugates of ^{3}H-GA_1 [7] and ^{3}H-GA_8. The smaller less polar peak contained unknown products. The occurrence of a ^{3}H-GA_1 glucoside-like conjugate was further supported by the fact that the zone containing this compound on the TLC plate gave ^{3}H-gibberellin C on acid hydrolysis [8]. Analysis by liquid scintillation counting after preparative TLC with double development (solvent system, $CHCl_3$–MeOH–HOAc–H_2O, 75:20:3:2) of the AB fraction indicated that glucoside-like conjugates of ^{3}H-GA_1 and ^{3}H-GA_8 were in the ratio 1:2. The AB fraction from the immature seeds also contained glucoside-like conjugates

of ^{3}H-GA$_1$ and ^{3}H-GA$_8$ (1:1) together with unknown products (Fig. 2).

The NB fraction from the mature seeds contained ^{3}H-GA$_1$ glucosyl ester (A$_1$ GE) as a major component together with a minor unknown product. The ^{3}H-GA$_1$ GE, purified by TLC, was acetylated in usual way, followed by preparative TLC to give ^{3}H-GA$_1$ GE pentaacetate and crystallized to constant activity from ethyl acetate–hexane. The constant specific radioactivity of purified product provided strong confirmation of the occurrence of ^{3}H-GA$_1$ GE in the NB fraction.

In the NB fraction from the immature seeds, the occurrence of a small quantity of ^{3}H-GA$_1$ GE was suggested by TLC tracing. Radioactivity at R_f 0–0·1 of the histogram was found to be due to radioactive glucosyl ethers from their behaviour on TLC, which remained in the NB fraction because of incomplete fractionation.

In a similar manner, it was found that ^{3}H-GA$_4$ was converted rapidly to ^{3}H-GA$_1$ in the seeds, which was identified by GC–RC as a TMS ether of its methyl ester. ^{3}H-GA$_4$ almost disappeared about 48 hr after the treatment. Consequently ^{3}H-GA$_4$ may be considered to follow the same fate as ^{3}H-GA$_1$, although some unknown products were present in each fraction. The NE fraction would be expected to contain ^{3}H-GA$_4$ glucosyl ester from the view of finding of Hiraga *et al.* [3], but unfortunately its occurrence in significant amounts could not be confirmed.

The radiochromatograms of each fraction from the immature and mature seeds which had been treated with ^{3}H-GA$_{20}$ at the immature stage showed that it was also converted to ^{3}H-GA$_1$ which was identified in the AE fraction from the immature seeds by dilution method. Some ^{3}H-GA$_8$ was also present which were identified by TLC tracing. The radioactivity of the AB fraction of the immature seeds consisted of glucoside-like conjugates of ^{3}H-GA$_1$ and ^{3}H-GA$_8$ (R_f 0·25) together with an unknown compound (R_f 0·6). The AB fraction of the mature seeds on the other hand yielded glucoside-like conjugates of ^{3}H-GA$_1$, ^{3}H-GA$_8$ and a small quantity of ^{3}H-GA$_{20}$, which were identified after enzymatic hydrolysis by TLC tracing, together with an unknown product. The radiochromatogram of the NB fraction from the mature seeds also suggested the presence of ^{3}H-GA$_1$ GE. The identification of an unknown product in the AB fractions, which was also detected at R_f 0·1–0·2 in the NB fractions because of incomplete fractionation, is now in progress.

The radiochromatograms of the AE and AB fractions from the immature seeds treated with ^{3}H-GA$_8$ showed unusual patterns with major unknowns, while those from the mature seeds suggested that the AE and AB fractions contained mainly ^{3}H-GA$_8$ and its glucoside respectively. The unusual patterns found in the case of the immature seeds may be attributed to abnormal metabolism caused by excess feeding of ^{3}H-GA$_8$ and the resulting unknown metabolites may have been excreted from the seeds during the development process. This is compatible with the observation that the recovery of radioactivity from the mature seeds was low.

Examination of radiochromatograms and comparison of R_f values with authentic samples suggested that ^{3}H-GA$_5$ was mainly converted to ^{3}H-GA$_8$, which was identified in the AE fraction by

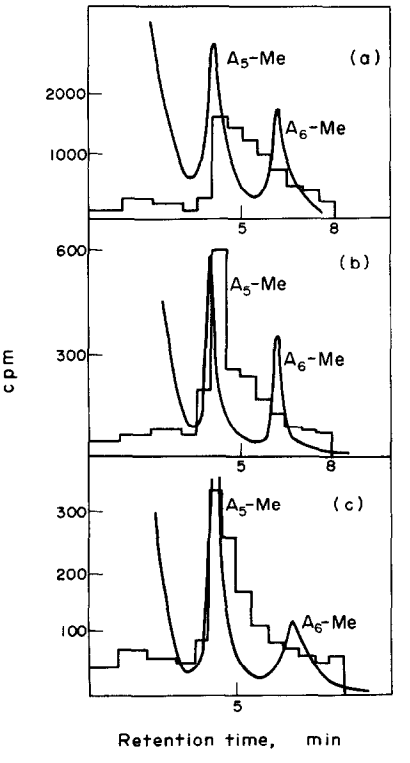

Fig. 3. GC–RC of methyl esters of GA$_5$ zones obtained from TLC plates; (a) the AE fraction from mature seeds treated with ^{3}H-GA$_5$ at the immature stage, (b) aglycones from the AB fraction, (c) aglycones from the NB fraction. Each zone was diluted with authentic methyl esters of GA$_5$ and GA$_6$.

Table 1. Recovery and distribution (%) of radioactivity

	^{3}H-GA$_1$		^{3}H-GA$_4$		^{3}H-GA$_{20}$
	Immature	Mature	Immature	Mature	Immature
Recovery	6·5	9·0	9·3	11·6	39·3
Total dpm ($\times 10^5$)	4·0	5·6	2·5	3·1	110·0
AE fraction	75·1	10·9	50·5	31·8	64·7
GA$_8$	9·2	1·8	4·7	2·7	10·7
GA$_1$	58·8	6·2	26·6	6·6	35·3
GA$_4$					
GA$_5$					
GA$_{20}$					11·9
Others	7·1	2·9	19·2	22·5	6·8
NE fraction	0·8	0·8	6·3	7·8	1·0
AB fraction	13·3	66·7	12·6	45·7	30·7
GA$_8$ glucoside	1·1	33·2	1·6	20·9	2·9
GA$_1$ glucoside-like conjugate	1·1	18·8	1·4	11·0	2·8
GA$_5$ glucoside-like conjugate					
GA$_{20}$ glucoside-like conjugate					
Others	11·1	14·0	9·6	13·8	25·0
NB fraction	8·4	12·3	7·7	8·3	0·7
GA$_1$ glucosyl ester	1·2	7·8	1·0	3·5	
GA$_5$ glucosyl ester-like conjugate					
Others	7·2	4·5	6·7	4·8	0·7
Aq. residue	2·4	9·3	22·9	6·3	2·9

dilution method. Since enzymatic hydrolysis of the AB fraction from the mature seeds gave ^{3}H-GA$_5$ and ^{3}H-GA$_8$, which were identified by TLC tracing, and alkali hydrolysis of the NB fraction from the mature seeds gave ^{3}H-GA$_5$, the AB fraction obviously contained glucoside-like conjugates of ^{3}H-GA$_5$ and ^{3}H-GA$_8$ (^{3}H-GA$_8$ glucoside was identified by GC–RC as the TMS derivative of its methyl ester), and the NB fraction, ^{3}H-GA$_5$ glucosyl ester-like compound.

In view of the structural relationship between GA$_5$ and GA$_8$, GA$_6$ may be a biosynthetic intermediate in the interconversion. Indeed, Sembdner *et al.* [9] reported that ^{3}H-GA$_6$ was converted to ^{3}H-GA$_8$ and its glucoside in the immature seeds of *P. coccineus*, and we therefore investigated whether ^{3}H-GA$_6$ or its glucosyl derivatives were involved in the conversion of GA$_5$ to GA$_8$ in *P. vulgaris*. Since GA$_5$ and GA$_6$ cannot be separated clearly on TLC even as their methyl esters, GC–RC analysis was applied. The AE fraction and the aglycone fractions obtained by hydrolyses of the AB and NB fractions from the mature seeds were first subjected to TLC and the ^{3}H-GA$_5$ zones were scraped from the plates, eluted with ethyl acetate, and methylated with etherial diazomethane. However shown in Fig. 3, ^{3}H-GA$_6$ methyl ester did not appear to be present in these zones.

It is noteworthy that the mature seeds treated with ^{3}H-GA$_5$ contained equivalent amounts of ^{3}H-GA$_8$ and ^{3}H-GA$_8$ glucoside, but the relative radioactivity of these components was extremely low in the mature seeds treated with ^{3}H-GA$_1$, ^{3}H-GA$_4$ or ^{3}H-GA$_{20}$. Recently Nadeau and Rappaport reported that ^{3}H-GA$_1$ was converted to ^{3}H-GA$_8$ and ^{3}H-GA$_8$ glucoside in the germinating seeds of *P. vulgaris*, but that 28% of the radioactivity from the seeds was in the glucoside as against 0·9% in the aglycone [6]. These facts suggest that GA$_1$ is converted directly into GA$_8$ glucoside without release of the aglycone from the enzyme system, while the conversion of GA$_5$ into GA$_8$ glucoside involves GA$_8$ as a free intermediate.

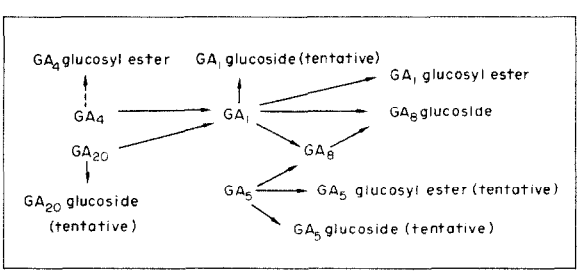

Fig. 4. Metabolic pathways of radioactive gibberellins fed to the bean seeds. The significant incorporation of GA$_4$ into GA$_4$ glucosyl ester was not confirmed in this experiment.

extracted from the seeds and seedlings fed with ^{3}H-GAs

^{3}H-GA$_{20}$	^{3}H-GA$_5$		^{3}H-GA$_8$		Seedlings (^{3}H-GA$_1$)	
Mature	Immature	Mature	Immature	Mature	1 Day	6 Days
24·6	8·8	9·8	83·6	3·5	5·3	4·8
70	41	46	74	3·1	3·3	3·0
33·1	46·4	49·3	70·3	31·6	7·6	1·2
4·7	28·2	18·6	8·2	13·7	1·5	0·2
12·1					2·7	0·6
	9·3	6·1				
2·2						
14·1	8·9	24·6	62·1	17·9	3·4	0·6
0·8	0·9	2·7	2·4	1·2	0·5	0·3
45·0	43·0	42·6	17·9	55·9	63·9	72·5
13·0	26·1	13·3	2·7	33·3	38·0	43·0
6·0					13·9	15·6
		12·2				
6·0						
20·0	16·9	17·1	15·2	22·6	12·0	13·9
1·7	1·4	3·7	4·0	3·3	12·1	7·4
0·6					9·7	6·5
		1·5				
1·1	1·4	2·2	4·0	3·3	2·4	0·9
19·4	8·3	9·8	4·9	8·5	15·9	18·6

The presence of a small quantity of ^{3}H-GA$_8$ in seeds treated with ^{3}H-GA$_1$ may be attributed either to a separate biosynthetic route to release GA$_8$ from the enzyme surface because of excess feeding of precursor or to subsequent hydrolysis of the glucoside.

The results of all these experiments suggest that radioactive gibberellins fed to the immature bean seeds are metabolized along the biosynthetic pathways illustrated in Fig. 4. The presence of glucosides of GA$_1$, GA$_5$ and GA$_{20}$, and of GA$_5$ glucosyl ester, which have not been reported to be naturally occurring, is also suggested. Their presence may be attributed to the nonspecificity of glucosyl ester and/or glucosyl ether synthetase.

The role of glucosides of gibberellins in higher plants has been discussed since their initial characterization and they are generally considered to be translocation inactive storage products of gibberellins. The conversion of GA$_1$ into its glucosyl derivatives in developing seeds may suggest that they are inactive storage products. Since gibberellin glucosyl esters are known to undergo hydrolysis in plant tissues [10], it is interesting to note the behaviour of glucosyl esters together with other gibberellin-like substances in the germinating stage.

Mature seeds which had been treated with ^{3}H-GA$_1$ at the immature stage were germinated in

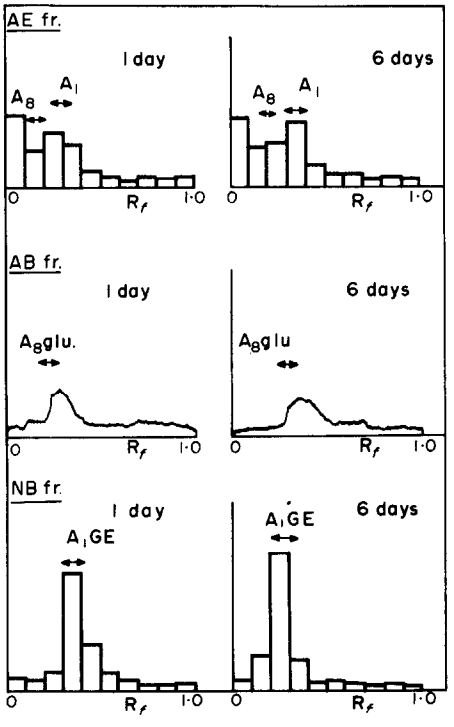

Fig. 5. Radiochromatogram tracing of each fraction from 1 and 6 days seedlings after sowing mature seeds which had been treated with ^{3}H-GA$_1$ at immature stage. Chromatography as in Fig. 2.

darkness at 25° and harvested 1 day and 6 days after sowing. The distribution of radioactivity in each fraction is summarized in Table 1. Radioactivity of the AE fraction from the seedlings decreased rapidly after sowing, while that of the AB fraction slightly increased. The results from radioscanning of TLC separations suggest that ^{3}H-GA$_1$ is converted into ^{3}H-GA$_8$ glucoside in the seedlings (Fig. 5). The AB fractions from the seedlings contained some ^{3}H-GA$_1$ glucoside, but the possibility of conversion of ^{3}H-GA$_1$ directly into the glucoside in the seedlings is excluded by the results of Nadeau *et al.* [6]. The decrease of relative radioactivity of the NB fraction from 1 day after sowing to 6 days may be due to enzymatic hydrolysis of ^{3}H-GA$_1$ GE, although it is not clear whether this decrease has some significance. Further study is in progress to clarify the role of gibberellin glucosyl derivatives in the process of germination.

EXPERIMENTAL

Material. Phaseolus vulgaris cv. Kentucky Wonder was used. Ammonium salts of tritium labelled gibberellins were dissolved in H_2O. A 5 μl aliquot of the soln was injected 18 days after anthesis directly into seeds within the plant pod. Preparation of ^{3}H-GA$_1$ [10], ^{3}H-GA$_5$, ^{3}H-GA$_8$ and ^{3}H-GA$_{20}$ are reported elsewhere [11]. ^{3}H-GA$_4$ was kindly supplied by Professor R. P. Pharis of The University of Calgary. Specific radioactivity and quantities of radioactive gibberellins fed to the seeds were: ^{3}H-GA$_1$, 2·4 mCi/mM, 0·7 μCi/seed; ^{3}H-GA$_4$, 8 mCi/mM, 0·3 μCi/seed; ^{3}H-GA$_5$, 5·3 Ci/mM, 5·3 μCi/seed; ^{3}H-GA$_8$, 0·5 Ci/mM, 1 μCi/seed; ^{3}H-GA$_{20}$, 3·2 Ci/mM, 3·2 μCi/seed. A portion of the seeds was harvested on the 2nd day after injection and the rest allowed to grow up to maturity. Mature seeds treated with ^{3}H-GA$_1$ at immature stage were also allowed to imbibe flowing H_2O overnight and germinated in darkness at 25° and harvested on the 1st and 6th day after sowing.

Extraction and fractionation. Four seeds or seedlings from each treatment were extracted with MeOH. After filtration, the extract was fractionated by the procedure as described in [10] to give 5 fractions (AE, NE, AB, NB and Aq).

TLC. Adsorbent, Kiesel gel G (Merck): solvent systems; AE, EtOAc–$CHCl_3$–HOAc, 20:8:1; NE and NB, $CHCl_3$–MeOH, 3:1; AB, $CHCl_3$–MeOH–HOAc–H_2O, 45:15:3:2.

Determination of radioactivity. The location of radioactivity on TLC plates was determined by a radiochromatogram scanner. Quantitative measurements were by liquid scintillation spectrometry using Bray's solution [12] as scintillator.

GC–RC methods. A gas chromatograph with a hydrogen ionization detector was used. A silanized glass column, 1 m × 3 mm with 2% QF-1 on Chromosorb W (mesh 80–100) and N_2 (33 ml/min) was used. Mass peaks were detected by FID and effluent from the detector was trapped directly into scintillator, which was composed of 300 ml of nonione, 700 ml of toluene and 4 g of PPO [13], and analysed by a liquid scintillation.

Acid hydrolysis of ^{3}H-GA$_1$ glucoside-like conjugate. ^{3}H-GA$_1$ glucoside-like conjugate, purified by preparative TLC, was hydrolyzed with 2 N HCl at 100° for 1 hr. The usual work up, followed by methylation with ethereal CH_2N_2 and subsequent preparative TLC, yielded ^{3}H-gibberellin C-Me, the identity of which was further confirmed by dilution.

Acetylation of ^{3}H-GA$_1$ glucosyl ester. The NB fraction from mature seeds treated with ^{3}H-GA$_1$ at the immature stage was diluted with GA$_1$ GE tetraacetate [10] (30 mg) and resulting mixture acetylated with C_5H_5N–Ac_2O (2:1) overnight at room temp. Crude acetate was purified by preparative TLC to yield GA$_1$ GE pentaacetate (17·6 mg, mp 178–181°) as fine needles from EtOAc–*n*-hexane (orig. mixture, 17·6 mg, 423 cpm/mg; first crystal, 15·3 mg, 413 cpm/mg; second, 11·9 mg, 415 cpm/mg; third, 7·8 mg, 427 cpm/mg).

Enzymatic and alkaline hydrolysis. AB fractions were hydrolyzed with cellulase (Sigma) by the same method as described in the previous paper [14]. The NB fraction from the mature seeds treated with ^{3}H-GA$_5$ at immature stage was hydrolyzed with 0·05 N aq. NaOH in a sealed tube at 100° for 1 hr. The aglycones were recovered from acidic EtOAc fractions respectively.

Identification of ^{3}H-GA$_8$ and ^{3}H-GA$_1$ in the AE fractions from the immature seeds treated with ^{3}H-GA$_5$ and ^{3}H-GA$_{20}$. Each AE fraction was purified by preparative TLC. ^{3}H-GA$_8$ zone from the immature seeds treated with ^{3}H-GA$_5$ thus obtained was diluted with 3·5 mg of cold GA$_8$ and repeatedly crystallized to constant sp. radioactivity: orig. mixture, 3·5 mg, 2681 cpm/mg; first crystal, 3·2 mg, 2756 cpm/mg; second, 2·7 mg, 2667 cpm/mg; third, 1·7 mg, 2659 cpm/mg. ^{3}H-GA$_1$ zone from the immature seeds treated with ^{3}H-GA$_{20}$ was methylated with ethereal CH_2N_2. The presence of ^{3}H-GA$_1$-Me in the product was confirmed by diln: original mixture, 19·2 mg, 8789 cpm/mg; first crystal, 16·0 mg, 8884 cpm/mg; second, 12·4 mg, 8970 cpm/mg; third, 9·3 mg, 8761 cpm/mg.

Acknowledgement—The authors are grateful to Professor R. P. Pharis and Dr. R. C. Durley of The University of Calgary for supply of ^{3}H-GA$_4$.

REFERENCES

1. West, C. A. and Phinny, B. O. (1959) *J. Am. Chem. Soc.* **81,** 2424.
2. Durley, R. C., MacMillan, J. and Pryce, R. J. (1971) *Phytochemistry* **10,** 1891.
3. Hiraga, K., Yokota, T., Murofushi, N. and Takahashi, N. (1972) *Agr. Biol. Chem.* **36,** 345.
4. Hiraga, K., Yokota, T., Murofushi, N. and Takahashi, N. (1974) *Plant Growth Substances 1973* (The Organizing Committee of the 8th International Conference on Plant Growth Substances, eds.) in press.
5. Skene, K. G. M. and Carr, D. J. (1961) *Austr. J. Biol. Sci.* **14,** 13.
6. Nadeau, R. and Rappaport, L. (1972) *Phytochemistry* **11,** 1611.
7. Nadeau, R., Rappaport, L. and Stolp, C. F. (1972) *Planta (Berl.)* **107,** 315.
8. Kawarada, A., Kitamura, H., Seta, Y., Takahashi, N., Takai, M., Tamura, S. and Sumiki, Y. (1955) *Bull. Agr. Chem. Soc. Japan* **19,** 27.
9. Sembdner, G., Welland, J., Aurich, O. and Schreiber, K. (1968) in *Plant Growth Regulators, SCI Monograph* Vol. 31, 70.
10. Hiraga, K., Yamane, H. and Takahashi, N. (1974) *Phytochemistry* **13,** 2371.
11. Murofushi, N., Durley, R. C. and Pharis, R. P. (1974) *Agr. Biol. Chem.* **38,** 475.
12. Bray, G. A. (1960) *Analyt. Biochem.* **1,** 279.
13. Ishibashi, T. and Kametaka, M. (1974) *Jap. J. Zootech. Sci.* **45,** 165.
14. Yamane, H., Yamaguchi, I., Murofushi, N. and Takahashi, N. (1974) *Agr. Biol. Chem.* **38,** 694.