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Fate of Radioactive Gibberellin A_1 in Maturing and Germinating Seeds of Peas and Japanese Morning Glory

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Abstract. Radioactive gibberellin A_1 (^{3}H-GA_1) was injected into excised fruits of peas and Japanese morning glory. These were then grown in sterile culture to maturity and the label was followed in the seeds during further development and subsequent germination. During development of both pea and morning-glory seeds a large part of the radioactivity became associated with the aqueous fraction, while another part of the ^{3}H-GA_1 was converted into 2 new, acidic, biologically active compounds, designated X_1 and X_2. A relatively small part of the neutral compounds could be converted back to ^{3}H-GA_1, X_1, and X_2 by means of mild acid hydrolysis. During germination of pea and morning-glory seeds, part of the bound compounds was released in the form of ^{3}H-GA_1, X_1 and X_2 while, particularly during rapid seedling growth, a further conversion of ^{3}H-GA_1, mainly to X_1, took place. In pea seedlings, growth during the first 2 to 3 days after imbibition was not affected by Amo-1618, an inhibitor of gibberellin biosynthesis. This, in conjunction with the findings on the interconversions between free and bound ^{3}H-GA_1 suggests that, at least in peas, early seedling growth may at least partly be regulated by gibberellins released from a bound form which was formed during seed development.

An increasing number of reports describe the occurrence of so-called bound GA-like[2] substances (4, 5, 6, 8, 12, 14, 16, 18, 19, 20, 21, 24, 26), sometimes also called neutral (4, 5, 6), water-soluble or butanol-soluble (4, 16, 18, 21, 26) GA-like substances. These GAs have particularly been found in seeds. They are poorly soluble in organic solvents such as ethyl acetate and can be converted to free GAs by hydrolysis with a mineral acid (24) or by treatment with hydrolytic enzymes (14, 15). One water-soluble GA, from *Pharbitis* seeds, has recently been identified chemically as 2-*O*-β-glucosyl-gibberellin A_3 (26). However, very little is known about the origin and the physiological significance of these GAs. With respect to the physiological role, bound GAs in seeds can be considered either as a reserve form, from which they are released and used upon germination, or as "inert" GAs which have no function in the plant. Tritium-labeled GA_1 (^{3}H-GA_1) of high specific activity having become available (9) we have tried to elucidate some aspects of formation, fate, and function of bound GAs by injecting ^{3}H-GA_1 into growing fruits of dwarf peas and of Japanese morning glory and following the label during seed development and maturation, subsequent germination, and early seedling growth.

The detached fruits were grown in organ culture to avoid possible complications due to export of the applied GA from the fruits, or import of endogenous GAs into the latter from other parts of the plants.

Pea seeds and seedlings contain 2 extractable, acidic gibberellins. One of these seems to be identical with GA_5. The other appears to be an unknown GA but in both chemical (chromatographic) properties and biological activity it is very similar to GA_1 (see 10, 22). *Pharbitis* seeds contain "*Pharbitis* Gibberellin" which is dihydro-GA_5, and very probably GA_3 (gibberellic acid) (25), the latter being a GA which is chemically and biologically rather close to GA_1. Thus, results obtained with exogenous GA_1 can be assumed to reflect at least part of the endogenous situation, in peas as well as morning glory.

Materials and Methods

Radioactive GA_1. GA_1-3,4-^{3}H, prepared by selective catalytic reduction of the cyclic double bond of gibberellic acid (GA_3) with a mixture of tritium and hydrogen (9), was used in this investigation. Two batches of ^{3}H-GA_1 were available, with specific activities of 87 mc/mmole and 20 mc/mmole, respectively.

Plant Material. Plants of dwarf peas (*Pisum sativum* L., cv. Progress No. 9, Asgrow Seed Company) and of Japanese morning glory (*Pharbitis nil* Chois., cv. Violet) were grown in a gravel-vermiculite (1:1) mixture in a greenhouse under natural light and watered with half-strength Hoagland nutrient solution. The light period was extended

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[2] Throughout the text, GA stands for gibberellin and GAs for gibberellins.

with light from Grolux fluorescent lamps (Sylvania) to give a photoperiod of 16 hours. The *Pharbitis* seedlings, when 5 days old, received 1 week of 8-hour short days (dark-period temperature, 25°) in order to induce flowering.

Treatment of Excised Pea Pods and of Fruits of Morning Glory. The procedures, except for the injection of ^{3}H-GA_1, were similar to those used by Baldev *et al.* (1) with peas. Pods of the dwarf peas were detached from the plants 13 or 16 days after anthesis. Thirteen days after anthesis the pea seeds are still somewhat flattened and have not yet reached their maximum fresh weight. Sixteen days after anthesis they are at or near maximum fresh weight, ready to enter the phase of maturation.

After surface sterilization with 0.1 % mercuric chloride (10 min) and subsequent washing with sterile distilled water, each pod was injected with 10 μl of labeled GA_1 and was placed into a 60-ml test tube with the cut pedicel inserted into a medium (20 ml) containing mineral nutrients according to Nitsch (17), 5 % sucrose and 1 % agar.

Fruits of morning glory were treated in the same way, except that they were excised and injected with labeled GA_1 18 days after anthesis. At this time, the seeds have almost but not entirely reached maximum size and fresh weight whereas dry weight continues to increase until maturity, *ca.* 35 days after anthesis. In 1 experiment, morning-glory fruits were injected while remaining on the intact plant until maturity.

Samples of seeds were harvested 2, 4, or 8 days after injection and immediately frozen in liquid nitrogen. The remaining cultures were harvested after the seeds had completely ripened. The seeds were dried in a desiccator, were imbibed for 24 hours in water, planted in vermiculite and grown in continuous darkness at 25° ± 2°. The morning-glory seeds were treated for 60 minutes with concentrated sulfuric acid and rinsed with water before imbibition. This is a standard procedure needed to overcome the hard-coatedness of the seeds. The seedlings were harvested at different time intervals after planting; shoots, cotyledons, and roots were frozen separately in liquid nitrogen. All frozen material was lyophilized, extracted, and analyzed for ^{3}H-GA_1, and its conversion products.

Extraction Procedures. A flow sheet of the extraction procedures is given in figure 1. The lyophilized tissue was extracted with 100 ml methanol in a Sorvall "Omni-Mixer." The extract was filtered, the tissue residue (Fr. 1) kept for radioactive analysis and the filtrate evaporated to dryness in a flash evaporator. The filtrate residue was taken up in 100 ml of 0.1 M phosphate buffer (pH 8.4), partitioned several times against equal volumes of light petroleum ether (b.p. 40–50°) and then against ethyl acetate until, in either case, the organic phase was colorless. The combined petroleum-ether phase and the combined alkaline ethyl-acetate phase were checked for radioactivity. The buffer phase was then adjusted with 2 N HCl to pH 2.5 and extracted 7 times with ethyl acetate. The combined acidic ethyl-acetate fraction (Fr. 2) was dried over Na_2SO_4 and evaporated to dryness. The residue was taken up in 5 or 10 ml of ethanol, and the radioactivity determined. The remaining aqueous phase was brought to pH 7.0, and its radioactivity was also determined (Fr. 3). In some experiments, the aqueous phase was hydrolyzed in 0.4 N HCl for 60 minutes at 60°. The pH was then adjusted to 2.5 and the aqueous solution again extracted 7 times with ethyl acetate. This "second" acidic ethyl-

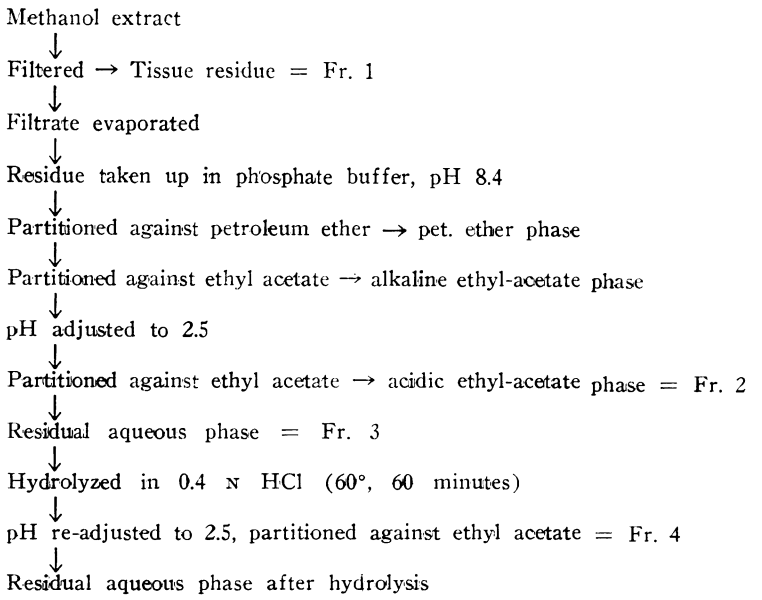

FIG. 1. Flow sheet showing procedure for extraction and separation of gibberellins.

acetate phase (Fr. 4) and the aqueous phase of the hydrolysate were also tested for radioactivity.

Chromatography. Thin-layer chromatography on silica gel H was carried out according to MacMillan and Suter (13) and Sembdner *et al.* (23) using mainly a solvent (No. 1) consisting of chloroform-ethyl acetate-acetic acid (60:40:5, v/v; R_F GA_1 = 0.21), and another (No. 2) consisting of benzene-*n*-butanol-acetic acid (70:25:5, v/v; R_F GA_1 = 0.77). GA standards were run as references on the side of each plate. The part of the chromatogram with the plant extract was divided into 10 equal zones, the silica gel was scraped off into centrifuge tubes and eluted 3 times with water-saturated ethyl acetate, followed by 2 elutions with 80 % methanol. The combined ethyl acetate-methanol eluates of each zone were evaporated and used for bioassays. The reference portions of the chromatograms were sprayed with 5 % (v/v) sulfuric acid in ethanol, heated at 100° for 10 minutes and the GA spots were detected under ultraviolet light (13).

Determination of Radioactivity. The radioactivity was determined by means of liquid scintillation spectrometry, using Bray's solution (2) as scintillator. Procedures for counting the different fractions and zones of the chromatograms were described earlier (9).

Bioassay. The dwarf-corn bioassay (d_5) was used, as described in Kende and Lang (10).

Results

Maturing and Germinating Peas: Fate of ^{3}H-GA_1. In the following experiment the pea pods were detached 13 days after anthesis and injected with 10 µl ^{3}H-GA_1 (70 µM, 0.245 µg/pod). Developing seeds were extracted 2 and 4 days and dry seeds 12 days after the GA application. Part of the dry seeds were germinated and the seedlings harvested 1, 3, 5, and 7 days later.[3] The extracts were partitioned according to figure 1 yielding petroleum ether, alkaline ethyl acetate, acidic ethyl acetate (Fr. 2) and aqueous (Fr. 3) fractions.

The distribution of radioactivity in Fr. 2, Fr. 3, and the tissue residue (Fr. 1) is shown in figure 2. No significant radioactivity was found in any other fraction. It can be seen that the relative amount of radioactivity in the acidic ethyl-acetate fraction (Fr. 2) decreased during seed maturation and that, at the same time, the radioactivity in the aqueous phase (Fr. 3) increased. There was also a slight increase of the radioactivity in the tissue residue (Fr. 1).

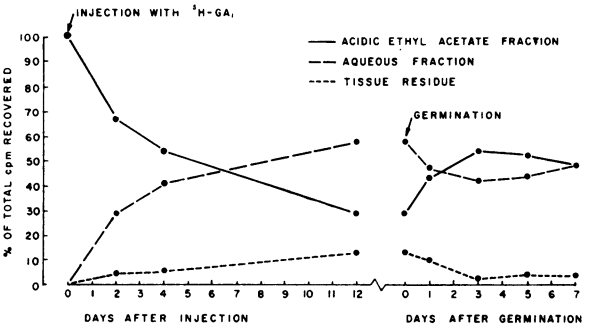

FIG. 2. The distribution of the radioactivity in extracts of developing and germinating pea seeds. The pea pods were detached from the plant 13 days after anthesis. Each pod was injected with 10 µl ^{3}H-GA_1 at a concentration of 70 µM (0.245 µg/pod) and subsequently grown on a sterile medium. The distribution of the total recovered radioactivity over the acidic ethyl-acetate fraction, the aqueous fraction and the tissue residue was determined at different time intervals after injection and germination.

When the seeds were germinated the trend was at first reverse. During the first 3 days of germination, the radioactivity of Fr. 2 increased while that of Fr. 1 and Fr. 3 decreased. After the third day of germination there was a slight decrease in the radioactivity of the acidic ethyl-acetate fraction (Fr. 2) and at the same time an increase in the radioactivity of the aqueous fraction (Fr. 3).

These results indicate that, during the development of pea seeds to maturity, the injected GA_1 was partially converted to a compound or to compounds which remained in the aqueous phase and probably represented, at least partly, so-called bound GA(s). During the first 3 days of germination the opposite process took place, the bound GA being converted, at least partially, back to the free form.

The Acidic Ethyl-Acetate Fraction. GA_1, when applied to dwarf-pea seedlings, is partially converted to an acidic, biologically active but chemically unidentified compound which can be separated from the unaltered GA_1 by thin-layer chromatography using solvent No. 2 (9). The following experiments were undertaken to determine whether a similar conversion occurs in maturing pea seeds.

In order to remove the GA_5-like endogenous factor which is known to be present in peas (10, 22), the acidic ethyl-acetate fraction (Fr. 2) was chromatographed in solvent No. 1. This resulted in 1 peak of radioactivity near the origin, co-chromatographing with authentic GA_1. No significant radioactivity was found in the GA_5 region. The radioactive fraction from these chromatograms was then rechromatographed with solvent No. 2. This procedure yielded 2 major radioactive peaks. One of these co-chromatographed with authentic GA_1 while the second peak had chromatographic properties (R_F 0.55–0.65) of the unidentified acidic compound described earlier (9). In addition, a minor

[3] For convenience sake, germination times are counted, throughout the text, from the start of imbibition of the seeds. Thus 1 day after germination means 24 hours after start of imbibition; the first 3 days of germination means the 72-hour period starting with imbibition.

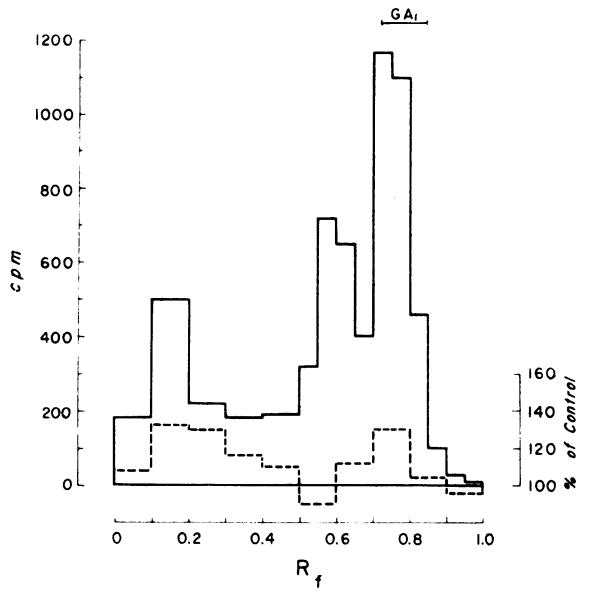

FIG. 3. The distribution of radioactivity (———) and biological activity (----) in the acidic ethyl-acetate fraction of an extract of 30 dry seeds from pea pods previously injected with ^{3}H-GA$_1$ (1.225 μg GA$_1$/pod). The acidic fraction was chromatographed in benzene-*n*-butanol-acetic acid (70:25:5, v/v).

third peak was observed near the origin, particularly in experiments in which a high amount of ^{3}H-GA$_1$ had been injected into the pods (1.225 μg/pod). The histogram in figure 3 shows the distribution of radioactivity and biological activity in the acidic ethyl-acetate fraction (Fr. 2) of an extract of 30 pea seeds after chromatography with solvent No. 2.

Biological activity was associated with the radioactive GA$_1$ peak and with the radioactive peak near the origin. None was found in the region of the radioactive peak at R_F 0.55 to 0.65. The unidentified GA$_1$ conversion factor with this R_F value which was previously found in pea seedlings (9) did possess biological activity. Either the factor now found in the developing pea seeds is, despite the similar R_F value, not the same as the seedling factor, or more likely, biological activity was not detected because the amount of material extracted was relatively small and the acidic fraction was subjected to chromatography twice. The levels of biological activity found in the pea-seed extracts were generally low, very probably because of these same reasons. The unidentified substance with R_F 0.55 to 0.65 to which GA$_1$ is converted will be referred to as X$_1$, the one with R_F 0.1 to 0.3 as X$_2$.

Table I summarizes the results of a more detailed analysis of the distribution of radioactivity in the acidic ethyl-acetate fraction (Fr. 2) of developing pea seeds, and table II a similar analysis for germinating ones. In the experiment of table I, the pea pods were detached 16 days after anthesis and each was injected with 10 μl ^{3}H-GA$_1$. (350 μM, 1.225 μg/pod). Twenty immature seeds were harvested 2, 4, and 8 days after injection and 30 seeds after maturation. The different fractions were analyzed for radioactivity. The results indicate that increasing amounts of GA$_1$ were converted with time to the unidentified acidic compounds X$_1$ and X$_2$ and, in mature seeds, less than one-third of the radioactivity of the acidic fraction represented unaltered GA$_1$.

In the experiment of table II, the pods had been injected with 0.245 μg ^{3}H-GA$_1$ each. Shoots, cotyledons, and roots were analyzed separately, but the data for the roots have been omitted since the results were similar to those for the shoots.

The ratio of radioactive acidic to non-acidic compounds was higher in the shoots than in the cotyledons, indicating a preferential movement of GA$_1$ and its acidic conversion products from cotyledons to shoots. In the first 3 days after germination the ratio between unaltered ^{3}H-GA$_1$, X$_1$, and X$_2$ remained essentially unchanged, but from day 3 to day 5 the relative amount of radioactivity associated with GA$_1$ declined sharply in both cotyledons

Table I. *Distribution of Radioactivity in Extracts of Developing Peas*

Pea pods were detached 16 days after anthesis and each pod was injected with 10 μl ^{3}H-GA$_1$ at a concentration of 350 μM (1.225 μg/pod) and subsequently grown on a sterile medium. Harvests were made at 2, 4, and 8 days after injection (20 seeds each) and at maturity (30 seeds). The acidic ethyl-acetate fraction, the aqueous fraction and the tissue residue were analysed for radioactivity. X$_1$ and X$_2$ are unidentified substances to which ^{3}H-GA$_1$ converts.

Time after injection	Total radioactivity recovered[1]	Amount of total recovered radioactivity					
		Acidic (GA$_1$, X$_1$, X$_2$)				Aqueous	Tissue
Days	*cpm*	%				%	%
2	166,721	70.1	(55.7,	8.7,	5.6)	22.7	7.2
4	141,855	60.4	(29.7,	23.4,	7.3)	28.7	11.0
8	125,816	48.4	(18.6,	21.2,	8.6)	40.5	11.0
Mature seeds	396,842	49.7	(15.5,	13.9,	15.5)	45.7	4.6

[1] In order to economize on labeled material, the GA$_1$ was not added to the medium but was injected into the pods. The precise locus of gibberellin injection could not be controlled accurately, *i.e.*, the radioactive substance was sometimes injected into a young seed, sometimes into the pod proper or into the free space within the pod. For this reason, the distribution of radioactivity between pod and seeds varied. This led to the observed variation in the total amount of radioactivity recovered from the relatively small numbers of seeds.

Table II. *Distribution of Radioactivity in Extracts of Seeds, Cotyledons, and Shoots of Germinating Peas*

Pea pods were detached 13 days after anthesis and each pod was injected with 10 µl ^{3}H-GA_1 at a concentration of 70 µM (0.245 µg/pod) and subsequently grown on a sterile medium. Analyses for radioactivity were made of the acidic ethyl-acetate fraction, the aqueous fraction and the tissue residue of extracts from 25 mature seeds, from 25 seeds soaked in water for 24 hours, from 30 seedlings harvested after 3 days of germination and 50 seedlings harvested after 5 days of germination. X_1 and X_2 are unidentified substances to which ^{3}H-GA_1, converts.

Time after start of imbibition	Total radioactivity recovered	Amount of total recovered radioactivity				
		Acidic (GA_1, X_1, X_2)			Aqueous	Tissue
Days	*cpm*	%			%	%
0 (seeds)	86,226	28.8	(5.1,	19.4, 4.3)	58.8	13.2
1 (seeds)	25,528	43.3	(8.0,	27.8, 7.5)	46.9	9.9
3 (cotyledons)	88,470	52.6	(12.7,	30.9, 9.0)	43.6	3.8
5 (cotyledons)	277,010	49.4	(3.0,	38.5, 7.9)	45.7	4.8
3 (shoots)	4,790	71.1	(20.8,	41.7, 8.5)	28.1	0.8
5 (shoots)	10,050	78.6	(6.5,	62.9, 9.4)	17.3	4.0

and shoots while it increased in the X_1 fraction. The X_2 fraction showed little change. This result suggests a rapid conversion of ^{3}H-GA_1 to X_1 during this period of seedling growth. Since the growth of the seedling in the first 3 days of germination is slow and becomes rapid thereafter (see fig 4A), there seems to exist a correlation between growth and conversion of GA_1 to X_1.

The Aqueous Fraction. It had been found (fig 2) that an increasing part of the radioactivity from applied ^{3}H-GA_1 became associated with the aqueous fraction during seed development, suggesting partial transformation of GA_1 to a bound gibberellin. In some experiments one-half of the aqueous fraction (Fr. 3) was hydrolyzed while the other half was kept as control at room temperature. The hydrolysis did not prove very effective as not more than 10 % of the counts present in the aqueous fraction (Fr. 3) could be extracted from the hydrolysate with ethyl acetate; in the non-hydrolyzed control part the value was, however, only 1 %. Chromatography of the control in solvent 2 gave radioactivity at the origin which was also found if the aqueous phase (Fr. 3) was chromatographed directly, *i.e.* without hydrolysis.

When the acidic ethyl-acetate fraction of the hydrolysate (Fr. 4) was chromatographed in solvent No. 2, 3 radioactive peaks were obtained, corresponding to those of the original acidic fraction (Fr. 2). In an experiment with the aqueous fraction of immature seeds all 3 radioactive peaks coincided with regions of biological activity but in experiments with the same phase but of mature seeds or seedlings no biological activity was detected.

Tissue Residue. In most cases a significant although minor amount of radioactivity was found in the tissue residue (Fr. 1). This had also been observed in work with seedlings, and it had been found that almost all of this radioactivity could be removed by further washing with methanol (9). We also found that further washing of the residue with methanol, or longer grinding of the tissue before extraction resulted in removal of additional

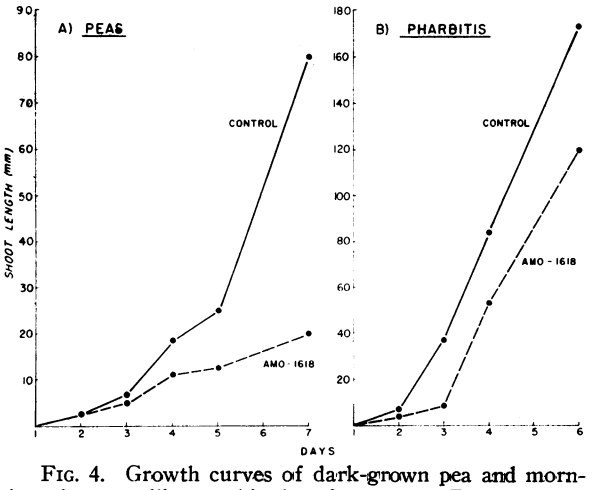

FIG. 4. Growth curves of dark-grown pea and morning-glory seedlings. A) dwarf peas, cv. Progress No. 9; B) Japanese morning glory, cv. Violet. The seeds were either soaked for 24 hours in water (control) or in a solution of Amo-1618 (250 mg/l) and then planted in vermiculite. The morning-glory seeds, prior to soaking, were treated for 1 hour with concentrated sulfuric acid. Each point on the curve represents the average of 20 individual measurements.

Table III. *Biological Activity in Extracts of Germinating Peas*

The total biological activity of the acidic ethyl-acetate fraction of peas at time 0 and 1 day and of cotyledons of seedlings after 3, 5, and 7 days of germination was determined by means of the dwarf corn (d_5) assay. The peas used for this experiment were previously injected with ^{3}H-GA_1 as described in the legend of table II.

Time from start of imbibition	Part studied	GA_3 equivalents per 100 peas or cotyledons
Days		*µg*
0	Whole seed	0.170
1	Whole seed	0.154
3	Cotyledons	0.223
5	Cotyledons	0.164
7	Cotyledons	0.075

amounts of radioactivity, up to less than 1 % of the total. Thus, most of the activity in Fr. 1 is bound in a relatively easily removable manner. It was not investigated whether the remaining activity could also be extracted.

Biological Activity. The total biological activity of the acidic ethyl-acetate fraction (Fr. 2) of mature seeds, of seeds imbibed in water for 24 hours and of cotyledons at 3, 5, and 7 days after imbibition was determined with the dwarf-corn (d_5) assay. The results are shown in table III. There appeared to be a maximum of biological activity after 3 days of germination, significantly higher than in the mature seeds. After the third day, the biological activity decreased and was, after 7 days of germination, well below the initial level in the seeds. These data fit very well with those obtained with the acidic ethyl-acetate fraction (Fr. 2), namely, an increase of radioactive GA_1 in the first 3 days of germination followed by an apparent rapid conversion of GA_1 to the biologically less active acidic compound X_2 (table II).

Fate of ^{3}H-GA_1 in Maturing and Germinating Seeds of Japanese Morning Glory. When fruits of morning glory were detached 18 days after anthesis, injected with 10 μl ^{3}H-GA_1 each (70 μM, 0.245 μg/pod), and harvested 2, 4, and 8 days later, a distribution of radioactivity was found as shown in table IV. As in peas, the relative amount of radioactivity in the acidic ethyl-acetate fraction (Fr. 2) decreased with time while that in the aqueous fraction (Fr. 3) underwent a simultaneous increase. After chromatography of Fr. 2 in solvent No. 2, 3 radioactive peaks were obtained, with the same R_F's as those found in peas, *i.e.* unaltered ^{3}H-GA_1, X_1 and X_2.

Mature seeds derived from excised fruits of morning glory proved to germinate very erratically. In order to study the fate of GA_1 during seed germination in this plant, ^{3}H-GA_1 was injected into fruits *in situ*, each fruit receiving 10 μl at a concentration of 167 μM (0.585 μg/fruit), and the fruits allowed to mature on the plant. Analysis of radioactivity was made on extracts from 20 mature seeds, from 30 seeds that were allowed to imbibe in water for 24 hours, and from 30 3-day-old seedlings. The results are summarized in table V.

Imbibition of the seed resulted in an increase of the relative amount of radioactivity in the acidic ethyl-acetate fraction (Fr. 2) while that in the aqueous fraction (Fr. 3) and particularly the tissue residue (Fr. 1) declined. In contrast, from the first to the third day of germination there was a sharp decrease in the relative amount of radioactivity in the acidic ethyl-acetate fraction and substantial increase in the aqueous fraction, suggesting a renewed binding of the free acidic compounds and/or a rapid metabolism of these compounds by the plant.

Within the acidic fraction (Fr. 2) the unaltered ^{3}H-GA_1 appeared to increase during imbibition relatively more than compounds X_1 and X_2, but afterwards this trend was reversed, suggesting a conversion of GA_1 particularly to X_1. Since morning-glory seedlings are making rapid growth between days 1 and 3 after imbibition, it appears again, as

Table IV. *Distribution of Radioactivity in Extracts of Maturing Seeds of Japanese Morning Glory*

Fruits were detached from the plant at 18 days after anthesis and each fruit was injected with 10 μl ^{3}H-GA_1 at a concentration of 70 μM (0.245 μg/fruit) and subsequently grown on a sterile medium. The acidic ethyl-acetate fraction, the aqueous fraction and the tissue residue of the extracts of 25 immature seeds at 2 days and of 30 seeds at 4 and 8 days after injection were analysed for radioactivity. X_1 and X_2 are unidentified substances to which ^{3}H-GA_1 is converted.

Time after injection	Total radioactivity recovered	Amount of total radioactivity recovered					
		Acidic (GA_1,	X_1,	X_2)	Aqueous	Tissue
Days	*cpm*	%			%	%
2	23,805	56.9	(39.7,	12.5,	4.7)	38.4	4.7
4	49,807	33.6	(18.8,	8.9,	5.8)	61.8	4.5
8	24,431	27.2	(11.5,	10.8,	4.9)	64.4	8.4

Table V. *Distribution of Radioactivity in Extracts of Germinating Seeds of Japanese Morning Glory*

Fruits on intact plants were injected with 10 μl ^{3}H-GA_1 at a concentration of 167 μM (0.585 μg/fruit) and seeds were harvested when mature. The acidic ethyl-acetate fraction, the aqueous fraction and the tissue residue of extracts of 20 mature seeds, of 30 seeds imbibed in water for 24 hours, and of 30 seedlings after 3 days of germination were analysed for radioactivity. X_1 and X_2 are unidentified compounds to which ^{3}H-GA_1 is converted.

Time after start of imbibition	Total radioactivity recovered	Amount of total radioactivity recovered					
		Acidic (GA_1,	X_1,	X_2)	Aqueous	Tissue
Days	*cpm*	%			%	%
0	6,503	36.1	(2.7,	16.7,	16.7)	56.2	7.7
1	16,967	47.7	(9.6,	16.2,	21.9)	51.6	0.7
3	12,989	29.5	(2.2,	12.2,	15.1)	69.5	1.0

with peas, that rapid growth and rapid conversion of ^{3}H-GA$_1$ to X$_1$ are somehow correlated.

Bioassay (dwarf corn d_5) of some of the acidic ethyl-acetate fractions (Fr. 2) obtained from morning-glory seeds and subjected to thin-layer chromatography showed, as with peas, at least 2 biologically active regions, corresponding to the radioactive peaks representing unaltered GA$_1$ and X$_2$. On 1 occasion only, a third region of relatively low biological activity was found, corresponding to X$_1$.

When the aqueous fractions (Fr. 3) were subjected to mild acid hydrolysis and the resultant second acidic ethyl-acetate fractions (Fr. 4) were chromatographed in solvent No. 2, the same 3 radioactive peaks (unaltered GA$_1$, X$_1$, and X$_2$) were found as with Fr. 2. Bio-assay of these regions gave the same pattern as that of Fr. 2, the X$_1$ region again having little if any biological activity in the assay used.

It had been found (Barendse, unpublished data) that the biological activity in the acidic fraction from morning-glory seeds increased significantly during imbibition of the seeds while the biological activity of the aqueous fraction (after hydrolysis) underwent a decline. During the first 3 days of germination, the trend was reversed, the biological activity of the acidic fraction decreasing and that of the hydrolyzed aqueous fraction increasing. These results are again in close agreement with the data for the increase and subsequent decrease of acidic compounds during germination of morning glory seeds as obtained by the use of ^{3}H-GA$_1$ (table V).

Effect of an Inhibitor of Gibberellin Biosynthesis on Early Seedling Growth in Peas and Morning Glory. In order to study the possible role of GAs, either free or bound, present in the seeds for the subsequent growth of the seedling it was important to assess the role of *de novo* GA synthesis during germination. For this purpose, pea and morning-glory seeds were soaked for 24 hours in either distilled water (control) or a solution of the growth retardant Amo-1618 (250 mg/l). Amo-1618 [2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate] has been shown to inhibit GA biosynthesis in both higher plants and in *Fusarium moniliforme* (3, 11) and to reduce the gibberellin content of developing pea seeds (1). The seeds were planted in vermiculite and subsequently grown in continuous darkness. The length of the shoots was measured and plotted as shown in figure 4. Each point represents the average of 20 individual measurements. From figure 4A it can be concluded that germination and growth of peas is independent of *de novo* synthesis of GA during a period of 2 to 3 days after start of imbibition. From then on, however, Amo-1618 becomes increasingly inhibitory, suggesting dependence of continued growth on GA synthesis in the seedling.

The results with morning glory are shown in figure 4B. Amo-1618 caused an immediate growth inhibition, although from the third day after imbibition onwards the growth rate, at least of plants treated with this Amo concentration, recovered and was only slightly less than that of the control. It appears that germination and early growth in morning glory are more dependent on *de novo* GA synthesis than in young pea seedlings. However, at least in peas, the gibberellins already present in the seeds appear to be sufficient for sustaining normal development during the first 2 to 3 days of germination.

Discussion

Our results show that during seed development GA$_1$ is partially converted to bound substances. The conversion of labeled gibberellic acid to non-acidic compounds has been reported earlier (22) and it was suggested that neutral or "bound" GAs are reserve forms of the hormone (4). A similar conversion of ^{3}H-GA$_1$ to non-acidic components occurs in growing pea seedlings, but at a much slower rate than in seeds (9). Growth of pea seedlings is independent of *de novo* GA synthesis during the first 2 to 3 days of germination. During the same period, free GA is released from the aqueous fraction and is preferentially transported to the growing shoot. These facts suggest that growth of the young pea seedling is, at least partly, supported by GAs which have been laid down in a bound form during seed development and maturation. Free GA is, however, also present in the mature seed and it may also participate in the regulation of germination and the early stages of seedling growth in peas.

The nature of the radioactive substances in the aqueous fraction has not been investigated in detail. In young pea seedlings, the aqueous fraction seems to contain at least 3 labeled substances (9). It is likely that the aqueous fraction of seeds is also heterogeneous, consisting partly of breakdown products of GA and partly of a "bound" form (or forms) of active GA(s). The following facts support this interpretation. Only 10 % of the radioactivity of the neutral fraction was released into the acidic ethyl-acetate fraction (Fr. 4) upon acid hydrolysis. The rest remained in the aqueous fraction even after repeated hydrolysis. During germination, only a fraction of the labeled compounds in the aqueous phase (10–15 %) was converted to acidic substances. This is in good agreement with the acid-hydrolysis results. From this it appears that a relatively small part of the radioactive non-acidic compounds may be bound GA while the bulk of the radioactivity is associated with conversion or breakdown products which may not have any further physiological significance. The entire situation is very similar in the Japanese morning glory, except that the latter seems to depend more on newly-synthesized gibberellin during the early growth of the seedling.

Another part of GA$_1$ was converted to 2 uniden-

tified, acidic compounds, X_1 and X_2. For X_2, biological activity was proven, while for X_1 it was demonstrable in only a few experiments. The significance of these conversion products is unknown. The formation of X_1 seems to be correlated with rapid growth, and it may be a product of GA utilization, but this is at present clearly a conjecture. Like GA_1 itself, the 2 conversion products can occur in "bound" form since acid hydrolysis of the aqueous fraction (Fr. 3) released not only unaltered GA_1 but also X_1 and X_2. Pea and morning glory seeds exhibited in all these conversions a remarkably similar pattern, suggesting that the observed picture, or a similar one, may hold in seed development, seed germination and seedling growth in many plants.

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