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Variations in Endogenous Gibberellins in Developing Bean Seeds I. Occurrence of Neutral and Acidic Substances[1]

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Summary. Activities of separated and chromatographed substances in the nonacidic, acidic ethyl acetate and acidic butanol fractions from bean seeds, *Phaseolus vulgaris* L., cv. Bountiful and Kentucky Wonder, were measured in the Progress No. 9 dwarf pea bioassay grown under red light. Activity in the nonacidic fraction was shown to be attributable only to neutral substances and was free of acidic gibberellin-like substances. As the seed matures, neutral substances and one of the acidic butanol-soluble substances (B-I) increase in activity. The acidic ethyl acetate substances and butanol-soluble substance (B-II) initially increase and then almost disappear.

Changes in levels of gibberellin-like substances during ontogeny of seeds and buds are well documented. These substances are present in comparative abundance in immature seeds, but decrease in amount as the seeds mature (1, 7, 8, 11, 12, 17, 18). Acidic gibberellins are present in high concentration in potato stolons, but essentially disappear when the tubers form (19). They are present only in small quantity in buds of resting potato tubers, but they increase in level as the tubers sprout (13, 15, 19, 20). Recognition of these changes led us to the hypothesis (5) that gibberellins present in plant organs during periods of active growth are converted into forms that are relatively inactive during periods of arrested growth.

Radley (14), Corcoran and Phinney (1), and Skene and Carr (18) have shown that gibberellins extracted by the usual method, i.e., at low pH (2.5–3.0) in ethyl acetate, increase to a peak in the immature bean seed and then disappear as the seed dries. However, Skene (16) and Hashimoto and Rappaport (2) found both acidic and nonacidic gibberellin-like substances in green bean seeds. The latter investigators also showed that nonacidic gibberellin-like substances occur even in the mature dry seed; these compounds may resemble neutral substances reported to occur in peelings of potato tubers (3, 4). Additionally, Hashimoto and Rappaport found that gibberellin-like substances which are insoluble in ethyl acetate but soluble in butanol occur in bean seeds at various stages of development.

This paper presents a new analysis of changes in gibberellin-like substances during ontogeny of the bean seed.

Materials and Methods

Seed Materials. Seeds of *Phaseolus vulgaris* L., cv. Kentucky Wonder and cv. Bountiful were used. All seeds were harvested from plants grown at the University Farm at Davis, except the mature dry seeds of Bountiful. These were obtained from commercial sources.

Bean fruits were harvested and selected for uniformity; the seeds were removed from the pericarp as quickly as possible. Seeds of Bountiful were divided according to length and weight into 3 arbitrary developmental stages: immature, mature green, and mature dry. Developmental stages assigned to Kentucky Wonder were medium mature and mature green. The length and fresh weights of the seeds are shown in table I. Seed size is not a precise estimate of age but, nevertheless, is useful as an index to the stage of development and as a basis for comparing data with that of other workers.

Extraction and Fractionation. These procedures were similar to those used by Hayashi and Rappaport (3, 5). The seeds were covered with methanol, ground in a Waring blendor for 5 minutes, and the homogenate filtered through a Buchner funnel. The extraction was repeated by washing the residue with

Table I. *Length and Fresh Weight of the Seeds*

Data concerning Kentucky Wonder and Bountiful bean seeds of the stages of development studied: immature (I), medium mature (MM), mature green (MG), and mature dry (MD). The fresh weight of MD seed was measured after imbibition in water.

	Kentucky wonder		Bountiful		
	MM	MG	I	MG	MD
Length (mm)	11	17	9	16	19
Fr wt (mg/seed)	142	517	73	635	1100
Dry wt (mg/seed)	28	134	9	201	443

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methanol. Mature dry seed was extracted with 50 % methanol.

The extract was evaporated to the water phase under reduced pressure. To make partitioning as quantitative as possible, the water phase was supplemented with water when needed, providing a ratio of about 150 ml to 50 g of seeds, fresh weight. The water phase was adjusted to pH 7.5 with $NaHCO_3$ and extracted 3 times with equal volumes of ethyl acetate which were combined. This was the nonacidic fraction. The remaining water phase was adjusted to pH 3.0 with dilute H_3PO_4 and shaken 3 times with ethyl acetate. This gave the acidic ethyl acetate fraction. Since gibberellin activity was found in the remaining water phase, it was further extracted with *n*-butanol, giving the acidic-butanol fraction. The 3 fractions thus obtained were dehydrated by passing through anhydrous Na_2SO_4 columns and were concentrated under reduced pressure. Additional details of fractionation are supplied in the discussion of individual experiments under Results.

Paper Chromatography. The fractions were streaked on Whatman No. 3 MM paper and chromatographed. An isopropyl alcohol-ammonium hydroxide (28 %)-water (10:1:1, v/v) solution was the developing solvent for the acidic ethyl acetate and butanol fractions. A solution in which *n*-butyl alcohol was substituted for the isopropyl alcohol was used with the nonacidic fraction. The solvent front was allowed to run a distance of 23 cm in descending chromatography at 25°. The chromatogram then was dried and cut into 10 equal sections parallel to the origin at each 0.1 R_F value. Sometimes the origin was used as an additional section. The material on each section (2.3 × 57.3 cm) was eluted with absolute methanol in descending paper chromatography for 2 or 3 days, and the eluates that dripped off the end of the strips were collected. The final volume of each eluate was about 15 ml. The eluates were dried under reduced pressure and prepared for bioassay by dissolving in a solution of equal volumes of ethanol and water containing 0.5 % Tween-20 (polyoxyethylene sorbitan monolaurate). When activity in 1 R_F zone is discussed, only the upper edge of the histogram is reported. If activity occurred over more than 1 R_F zone, the lower and upper edges of the entire zone are mentioned.

Bioassay. The pea bioassay used in this study was originated by E. Reinhard and A. Lang (unpublished) and was utilized by Kende and Lang (7). It was modified by Hayashi, Blumenthal-Goldschmidt, and Rappaport (4). Seeds of dwarf peas (*Pisum sativum* L., cv. Progress No. 9) were soaked for 6 to 8 hours in running tap water, sown in trays of wet vermiculite, and allowed to germinate in total darkness for 4 days at 23 to 25°. Then seedlings measuring 2.75 to 3.25 cm from the cotyledonary node to the hook were transferred to trays with water at 23 to 25° under red light. The red light was obtained by filtering the light from four 40-w fluorescent tubes through 3 layers of red cellophane. Based on spectrophotometric examination of the cellophane, the bulk of transmitted light was in the 640- to 700-mμ region. Twenty-four hours after transfer of seedlings, 5μl of test solution were applied with a microsyringe to the apex of each plant. The length of the shoot from the cotyledonary node to the highest visible node was measured 5 days after treatment.

Data for elongation were expressed according to the following equation:

$$\text{Percent of control} = \frac{\text{Length control plant} - 3.0\text{ cm}}{\text{Length treated plant} - 3.0\text{ cm}} \times 100.$$

The 3.0 cm values represent the average initial length of the seedlings. A standard curve of activity for GA_1 is shown in figure 1.

Dosage is expressed as extract equivalent to x number of seeds or extract equivalent to x *g* of seeds. In figures 2 and 3 the 5 % confidence limits, determined according to Snedecor (21), are indicated by unshaded areas. The shaded areas show statistically significant activity.

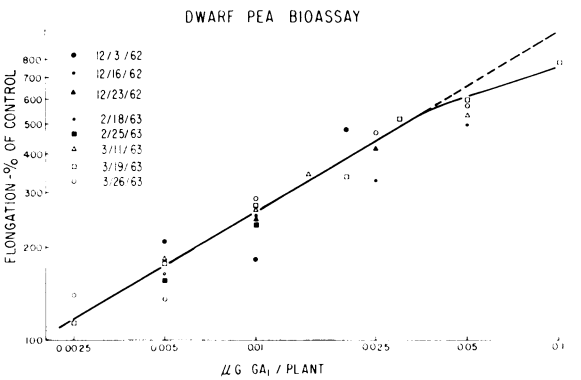

FIG. 1. Response curve of Progress No. 9 dwarf peas grown under red light following treatment with several concentrations of GA_1. Points represent values of observations made in 8 tests over a period of 4 months. All concentrations were not tested in each test.

Results

Acidic Ethyl Acetate Fraction. The native gibberellins in mature dry seed were examined first. For bioassay, an eluate from an R_F zone of the chromatogram obtained from an extract equivalent to 5 g of seed was applied to each pea plant (fig 2, upper histograms). The original extract was diluted 3 times, and the data are shown in the lower histograms. In the original acidic ethyl acetate fraction, activity was found at R_F 0.1, 0.3, and 0.5. In the lower histogram slight activity is apparent at R_f 0.2. The total activity in the acidic ethyl acetate fraction

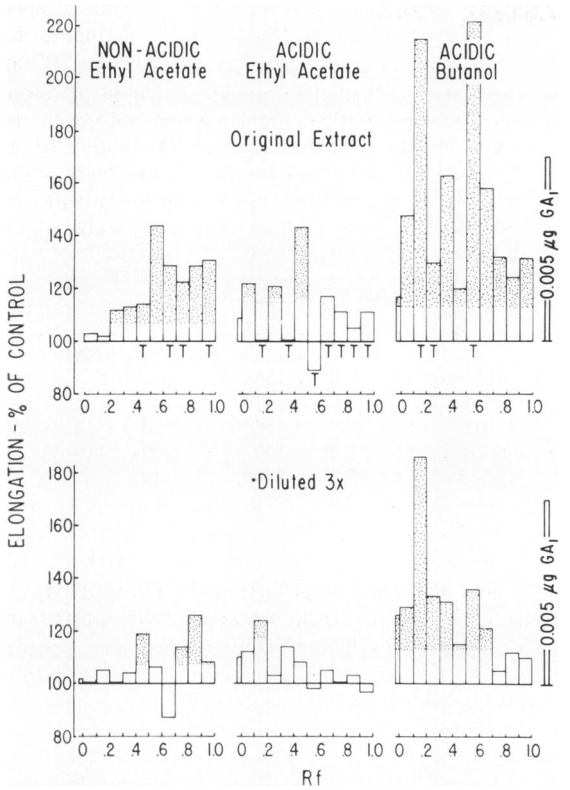

FIG. 2. Response of Progress No. 9 pea plants, grown under red light, to gibberellin-like substances extracted from mature dry seeds of *Phaseolus vulgaris* L., cv. Bountiful. Dosage: Original extract represents 5 g of seed equivalent fresh weight; 10 plants per treatment. Shaded portions show activity above the 5 % confidence limits of the control. The narrow strip to the left of the acidic ethyl acetate and butanol fractions represents activity of the origin of the chromatogram. T refers to toxicity symptoms.

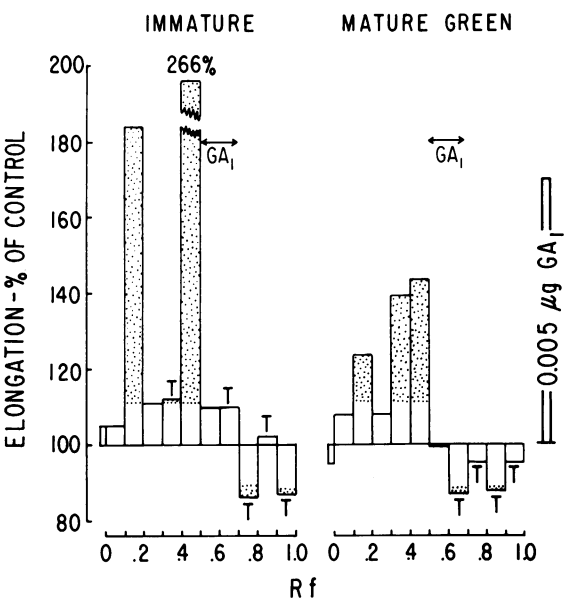

FIG. 3. Activity of gibberellin-like substances in the acidic butanol fraction of immature (9 mm) and mature green (16 mm) seeds of cv. Bountiful as measured on Progress No. 9 pea plants grown under red light. Each of 10 plants received extract equivalent to 0.5 g of seed. Shaded portions show activity above the confidence limits of the control. GA_1 markers show the position to which authentic GA_1 traveled in co-chromatography. T refers to toxicity.

was very low, corresponding to the data reported by several workers (1, 8, 11, 12, 16, 17, 18).

Acidic Butanol Fraction. The exceedingly high activity in the butanol fraction (fig 2) is most striking, especially since the fraction was extracted from dry seed. The activity at zone R_F 0.0 to 0.4 was especially strong, even after dilution. Since toxicity (T) appeared in some zones of the upper histogram, the lower histogram is considered to present a more reliable picture of the distribution of activity although dilution revealed no important qualitative differences.

Zones of activity were present at R_Fs 0.2 and 0.5 in immature seed and at R_Fs 0.2 and 0.3 to 0.5 in mature green seed (fig 3). These zones probably contain more than 1 active substance. However, for convenience the lower and upper R_F zones in both stages of development are designated as substance B-I and substance B-II, respectively.

Quantitative changes in B-I and B-II eluates from immature, mature green and mature dry seed of cv. Bountiful were tested on the pea bioassay (fig 4). This figure shows that activity of B-I per g fresh weight decreased with maturation, but activity per seed increased as the seed developed. Activity of B-II decreased on a fresh weight basis, and activity per seed was low in immature seed, high in mature green seed, and decreased as the seed matured.

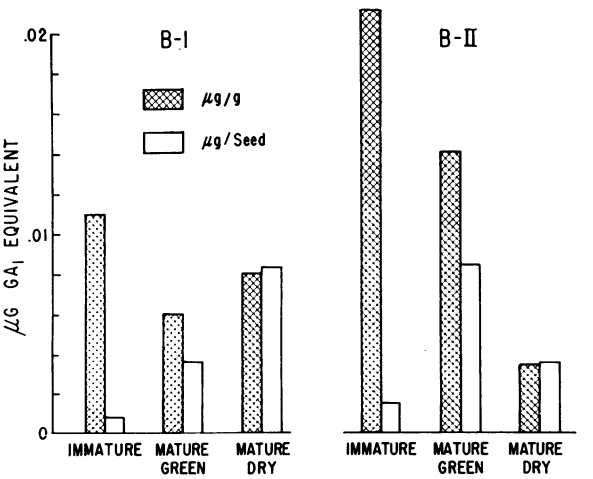

FIG. 4. Activities of butanol-soluble gibberellin-like substances B-I and B-II at 3 stages of seed development of cv. Bountiful. Data are expressed on per seed and on fresh weight bases. Bioassay: Progress No. 9 dwarf peas grown under red light, 10 plants per treatment.

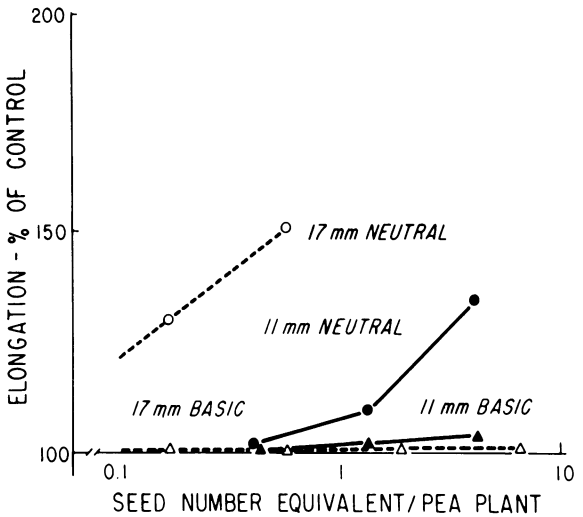

FIG. 5. Response of Progress No. 9 dwarf peas to unchromatographed extracts of the neutral and basic fractions from medium mature and mature green seed of Kentucky Wonder. Bioassay: Progress No. 9 dwarf peas grown under red light, 10 plants per treatment.

Nonacidic Fraction. Distinctly active zones are seen in the nonacidic ethyl acetate fraction from dry seed (fig 2). In the upper histogram they occur at R_F 0.5 to 1.0, and in the lower histogram at R_Fs 0.5 (N-II) and 0.7 to 0.9 (N-III). The small discrepancies in R_F values in the original and diluted extracts probably are attributable to toxicity.

When levels of the nonacidic gibberellin-like substances in nonchromatographed extracts of medium mature and mature green seeds were compared (fig 5), it was apparent that the large seeds contained about 10 times more activity than did the medium mature seeds.

Does the Nonacidic Fraction Contain Acidic Substances? Despite intensive efforts to separate neutral and acidic substances, one might suspect that gibberellin activity in the nonacidic fraction of bean seeds is due to contamination by acidic gibberellins. To investigate this possibility, eluates from 3 different nonacidic fractions were refractionated into nonacidic and acidic fractions. The activity of these 2 fractions in the pea bioassay was examined (table II). Eluates from zones R_F 0.6 (N-II) and 0.8 (N-III) of mature dry seeds of cv. Bountiful and R_F 0.2 to 0.4 from Kentucky Wonder were used. A discussion of the fraction from Kentucky Wonder will appear elsewhere. It appeared as a broad band on the chromatogram at R_F 0.0 to 0.5 and was designated N-I. The refractionation was by the same method described in the original procedure.

The gibberellin activity of each of the 3 refractionated eluates appeared only in the nonacidic portion (table II). Activity of 113 % of the control in the acidified fraction was not statistically significant. The data show clearly that the nonacidic fraction does not contain acidic gibberellin-like substances.

Table II. *Test for Occurrence of Acidic Substances in the Nonacidic Fraction*

The water phase of the methanol extract of bean seeds was reextracted with ethyl acetate at pH 8.0, yielding the nonacidic fraction. Active chromatographic eluates were reextracted by the same procedure and a dilution series was run. The original eluate and its dilutions were tested on Progress No. 9 dwarf peas grown under red light, 10 plants per treatment.

Nonacidic eluates and cultivar	pH	Dilution		
		1	1/2	1/3
		(% of control)		
R_F 0.2–0.4	8.0	136	...	100
Kentucky wonder	3.0	113	...	106
R_F 0.6	8.0	158	128	...
Bountiful	3.0	100	99	...
R_F 0.8	8.0	128	144	...
Bountiful	3.0	102	111	...

Are Nonacidic Gibberellin-like Substances Neutral or Basic? To determine whether the nonacidic gibberellin-like substances in bean seeds are neutral or basic in nature, the nonacidic fraction was separated into neutral and basic fractions. Nonacidic fractions from 11- and 17-mm seed of cv. Kentucky Wonder were dissolved separately in ethyl acetate and shaken with pH 2.8 phosphoric acid. Under these conditions it would be expected that the basic substances would move into the water phase, leaving the gibberellin-like neutral substances in the ethyl acetate phase. The partitioned water phase was adjusted to pH 8.0 with sodium bicarbonate and extracted with ethyl acetate to separate the basic substances. The neutral and basic fractions thus obtained were evaporated to dryness, and total gibberellin activity was determined (fig 6). All the activity appeared in the neutral fraction of the medium mature and mature green seeds. It may be concluded that the non-acidic gibberellin-like substances in bean seeds are neutral in nature. Therefore, in the subsequent discussion, these substances in the nonacidic ethyl acetate fraction will be termed neutral gibberellin-like substances to unify terminology with that of previous papers (3, 4, 5).

Discussion

In agreement with the results of other investigations (1, 8, 11, 12, 14, 17, 18), we found that the level of acidic ethyl-acetate-soluble gibberellin-like substances was very low in the dry bean seed. It was of interest, therefore, to find that bean seeds, at all stages of development tested, contained gibberellin-like substances which were insoluble in ethyl acetate but soluble in *n*-butanol. The substances were overlooked until recently, primarily because conventional fractionation methods generally used ethyl acetate

as solvent, and the water phase usually was discarded. These butanol-soluble substances migrated to 2 zones (B-I and B-II) on paper chromatography (fig 2, 3). Activity of B-I increased as bean seeds matured and it was particularly high in mature dry seeds (fig 4). It is possible that substances in B-I are less active forms of gibberellins.

An interesting parallel to the data concerning variation in level of B-II (fig 4) is seen in the work of Ogawa (11, 12). He detected a substance (Factor II) in extracts of seed of *Pharbitis nil* which showed certain of the characteristics of GA_3, and probably of B-II of bean seeds. Both Factor II and B-II are low in level in immature seeds and then increase after ethyl-acetate-soluble gibberellin-like substances decrease. The increase in Factor II corresponds to the period of rapid embryo development, and Ogawa suggested that it was functional in embryo growth. The data concerning changes in level of B-II supports Ogawa's conclusion.

Neutral gibberellin-like substances increased late in seed development and were present in fairly large amounts in mature dry seeds (fig 2, upper histogram). These findings indicate that the neutral substances in bean seeds may be reserve forms of gibberellins, conceivably the products of conversion from acidic substances (Hashimoto and Rappaport, in preparation).

Murakami first reported the occurrence of water-soluble gibberellin-like substances; he recorded their presence in the seeds of *Pharbitis* and *Wisteria*. These substances remained in the water phase after exhaustive extraction with ethyl acetate and were soluble in butanol. Sembdner, Schneider, and Weiland and Schreiber (16) isolated a butanol-soluble, neutral gibberellin from bean seeds (*Phaseolus coccineus* L.). It consisted of sugars (one of which was glucose), ninhydrin-positive substances, and a gibberellin. The substance was present abundantly in mature dry seeds; this contrasted with other gibberellins, which were soluble in ethyl acetate and disappeared as the seeds matured. Murakami (9) also showed that a glucoside of gibberellin formed in cucumber (*Cucumis*) leaves floated on a solution of GA_3. The glucoside was insoluble in ethyl acetate but soluble in butanol. The gibberellin-like substances in our butanol fraction were similar in solubility and chromatographic behavior to the substances reported by the above workers. Weaver and Pool, using the technique described in this publication, reported the occurrence of active, butanol-soluble substances in grape berries (22). McComb (8) found that treating homogenates of mature pea seeds with ficin, a proteolytic enzyme, resulted in larger amounts of acidic ethyl-acetate-soluble, gibberellin-like substances than were present in extracts not treated with the enzyme. Although the nature of the bound material was not determined by McComb, it is undoubtedly different from both our neutral and butanol-soluble substances.

It is possible that there are several reserve forms of gibberellins in resting organs. The acidic ethyl-acetate-soluble gibberellins and substance B-II, which increase early in seed development and then decrease, may be required for seed growth. B-I and the neutral substances, which increase steadily in activity as the seeds mature, may act as reserve forms of gibberellins. Interconversions of these substances may account for the different activities and types of gibberellin-like substances during development. The possible occurrence of such conversion systems is being investigated. One such system is the subject of a paper now in press.

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