Gibberellins and Light Inhibition of Stem Growth in Peas [1, 2]

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Light-induced inhibition of stem growth is a common phenomenon in plants. Particularly well investigated is the case of peas, *Pisum sativum,* mainly thanks to the work of Lockhart. Lockhart showed that growth of Alaska peas, a tall variety, was retarded when plants were transferred from darkness to low-intensity light but that, after a period of inhibition, the plants resumed growth at a rate equal to those grown continuously in the dark (5, 7). Applied gibberellic acid reversed the inhibition caused by light. The effect of irradiation on the growth of dwarf peas is even more drastic. Dwarf peas grow like, or nearly like, the tall types in darkness (1) but when they are exposed to light, stem elongation is strongly reduced (1, 5, 6) and the plants do not regain their original growth rate but stay dwarfed as long as kept in the light. Again the effect of illumination can be overcome by gibberellic-acid treatment. Light inhibition in dwarf peas is mediated by the phytochrome system, red light being most effective in causing suppression of growth and far red negating the effect of a previous irradiation with red light (5, 6).

Lockhart postulated that light interferes with the metabolism of endogenous gibberellins in plants. Our experiments were aimed at finding whether light- and dark-grown dwarf peas exhibit quantitative and qualitative differences in their gibberellin content and/or in their responses to applied gibberellins.

Materials and Methods

Bioassays. The main bioassay for pure gibberellins and for gibberellin-like materials in extracts was a dwarf-pea assay developed in this laboratory by E. Reinhard (4). Alaska peas and a dwarf corn mutant, d_5, served as complementary assay plants.

Dwarf peas, var. Progress No. 9 (Asgrow, New Haven, Connecticut), were soaked for 24 hours at room temperature in vermiculate and kept in darkness at 23°. On the third day after sowing seedlings were selected for uniformity and transferred to plastic boxes containing half-strength Hoagland solution. Plants were measured and selected again on the fourth day and then treated with test solutions. Gibberellins and plant extracts were dissolved in distilled and deionized water containing 0.05 % Tween 20 (polyoxyethylenesorbitan monolaurate) as a wetting agent and were applied to the epicotyl hook in 5λ droplets. Not less than 4 plants were used for any one assay. Test plants were kept under low-intensity red light at 27° until the seventh or ninth days after sowing (light source: six 96″ T8 red fluorescent tubes, General Electric; 12.5 cm apart; distance from plants approximately 105 cm). Gibberellin activities were expressed by measuring the height of the pea stems from the lowest to the highest node.

Alaska peas were grown and handled in the same manner as dwarf peas. d_5-corn seeds were soaked for 24 hours at 15°, planted in vermiculite, and grown under artificial light at an 8-hour photoperiod and 27°. The dwarf mutants were selected on the fifth day after sowing and transferred to plastic boxes containing half-strength Hoagland solution. Solutions and extracts were applied to the first leaf in 0.1 ml of water with 0.05 % Tween 20 added. Assays were evaluated 1 week after treatment by taking the sum of the lengths of the first and second leaf sheaths. Again not less than 4 plants were used for testing 1 fraction.

Application of Growth Retardants. In some tests, a growth retardant, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-pipiridinecarboxylate methyl chloride (Amo-1618), was used in place of red light to dwarf etiolated dwarf peas and Alaska peas. The growth retardant was added to the nutrient solution when the plants were transplanted to the latter on the third day after sowing. Concentrations were 100, 150, and 200 mg/liter, as specified in the different experiments.

Plant Material for Extraction. Dwarf peas to be used for extraction were grown in the same manner as those for bioassays, except they were not selected for uniformity a second time, and were kept in darkness or given only 24 hours of red light.

The growth curves of etiolated and illuminated dwarf peas are shown in figure 1. Growth in darkness reaches a steady state the fourth day after sowing. Growth in low-intensity red light proceeds at another, lower but also steady rate within 24 hours after the beginning of irradiation, indicating that all light-induced biochemical changes which affect the growth of the plants must have occurred by that time. On the basis of these observations, etiolated plants were grown in darkness for 7 days. Plants which were to be given a light treatment were taken from the dark cabinets on the sixth day, illuminated with red

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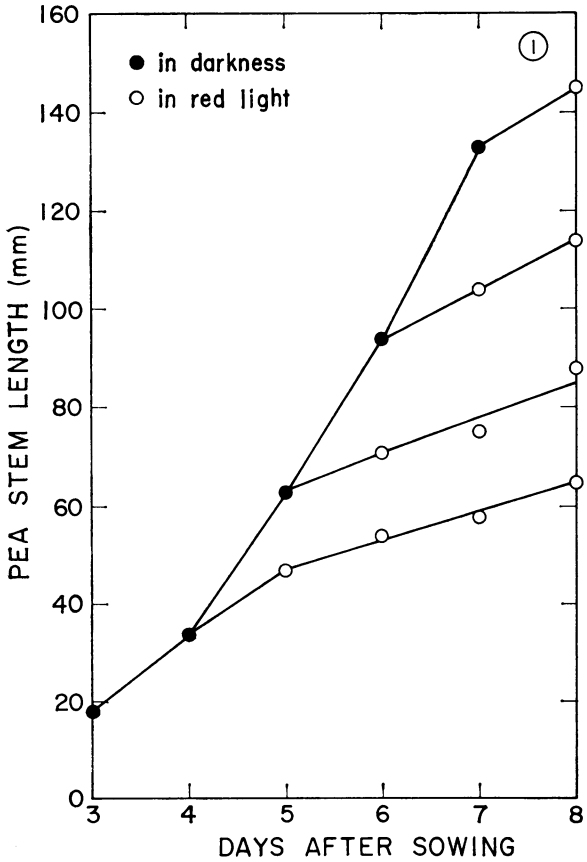

FIG. 1. Growth curve of dwarf peas which were grown in darkness or transferred to red light 4, 5, 6, and 7 days after sowing. Each point represents the average value obtained from 48 observations.

light for 24 hours, and harvested at the same time as the dark-grown plants. Either material was instantly killed in liquid nitrogen. The light-treated plants were harvested in red light; otherwise, harvesting and all necessary handling was carried out in dim green light.

Extraction of Gibberellins. Plant material to be extracted was freeze-dried and ground in a mortar. Dry powder (50 g) was shaken overnight with 1000 to 1200 ml of methanol at 1°. The extract was filtered and the residue extracted with the same volume of methanol for another 6 hours. The combined methanol extracts were dried in a rotary vacuum evaporator. The residue was taken up in 1-M sodium phosphate buffer (pH 8.4) and petroleum ether (B.R. 30–60°). A total of not more than 250 ml of buffer was partitioned several times against equal volumes of petroleum ether until the organic phase was colorless. The buffer phase was further purified by partitioning twice against ethyl acetate and then adjusted with half-concentrated hydrochloric acid to a pH of 2.5. The acidic fraction was extracted from the aqueous phase by partitioning 3 times with ethyl acetate. The 3 ethyl acetate extracts were combined, dried over sodium sulfate, and evaporated under reduced pressure.

Partition Chromatography. Partition chromatography on a buffered celite column was used as the initial step for the chromatography of gibberellin-like materials in plant extracts. This method was first applied by Stodola et al. (10) and Grove et al. (2) to separate gibberellic acid (GA_3) and gibberellin A_1 (dihydrogibberellic acid, GA_1). In either case, phosphate buffer was used as stationary and ether as moving phase. Celite (diatomaceous earth) served as carrier for the buffer.

Our method is a modification of these procedures, with ethyl acetate instead of ether serving as the moving phase.

Celite 535 was stirred into one-third concentrated hydrochloric acid. Twenty-four hours later the acid was decanted and the celite washed with distilled deionized water until the pH of the water was neutral. The celite was then rinsed with methanol and dried in the oven at 100°. These procedures are necessary to obtain celite free of inorganic and possible organic contaminations. A glass tube (17 mm I.D.) was packed under suction with 18 g of celite to a height of 20 cm. Seventy-five milliliters of 0.5-M phosphate buffer (KH_2PO_4/NaOH), pH 6.4, were shaken with 150 ml of ethyl acetate. First the buffer saturated with ethyl acetate, then the ethyl acetate saturated with buffer were passed through the column.

Gibberellins move on the partition column according to their solubility properties in water. Gibberellin A_5 (GA_5), which contains 1 hydroxyl group, appears in the second and third fractions while GA_1 with 2 hydroxyl groups does not appear until the eighth fraction (fig 2).

In work with plant extracts the dried acidic fraction was dissolved in small volumes of ethyl acetate saturated with the pH 6.4 buffer and was pipetted on top of the column; this was then developed with dry

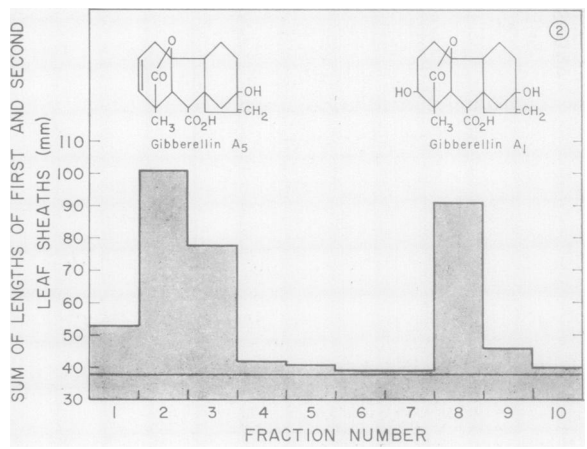

Fig. 2. Partition chromatogram of gibberellin A_1 (1 μg) and gibberellin A_5 (1 μg) on a buffered celite column. Fractions were tested on d_5 corn which is 3 times more sensitive to applied GA_5 than to GA_1.

ethyl acetate, collecting fractions of 50 ml each. The fractions were evaporated in front of a fan. No biological activity ever appeared later than the tenth fraction. After the termination of development the column was washed with 150 ml acetone but no gibberellin-like activity was found in the acetone wash either.

Thin-layer Chromatography. The celite column was usually used for prepurification of extracts. Further purification was accomplished by rechromatographing fractions with gibberellin-like activity on silica-gel thin layers according to the method of MacMillan and Suter (9). Diisopropylether-acetic acid (95: 5) and benzene-acetic acid-water (8: 3: 5) were used as solvents. Reference chromatograms were sprayed with 5 % sulphuric acid in ethanol and heated to 100° for 10 minutes. Gibberellins are oxidized by this treatment to compounds which are visible as fluorescent spots under UV light. The silica gel was removed from the glass plates in zones corresponding to R_F units of 0.1 and eluted with 4 ml of ethyl acetate saturated with water. (Dry ethyl acetate is very ineffective as eluant.) The silica gel was centrifuged off and washed twice with wet ethyl acetate, and the fractions were then used in bioassays as usual.

Results

Gibberellin-like Substances in Dark-grown Dwarf Peas. When an extract of etiolated dwarf-pea seedlings was chromatographed on a buffered celite column and tested on dwarf peas in red light, one clear peak with gibberellin-like activity was obtained at fraction No. 6 (fig 3). There was a slight indication of another active region which ran faster on the column (fraction No. 2). Bioassays on d_5 revealed 2 zones of gibberellin-like activity, at fractions No. 2 and 3, and 5 to 7. The earlier peak, which stimulated growth of dwarf corn but not of dwarf peas, will be referred to as fraction I. The second peak will be called subsequently fraction II.

It was remarkable that fraction I, although extracted from dwarf peas, was inactive when tested on the same plant in red light. It could be a gibberellin or gibberellin precursor which could not be utilized as or converted to an active hormone by dwarf peas in the light. If this assumption was correct, fraction I should exhibit gibberellin-like activity in bioassays using dwarf peas grown in total darkness or normal peas in red light.

Fraction I was accordingly purified further on a thin-layer chromatogram and tested in 3 assays, on dark-grown Progress No. 9 peas, on red-grown Progress No. 9 and on red-grown Alaska peas. The etiolated dwarf peas and the Alaska peas were artificially dwarfed with Amo-1618. These plants grow otherwise at a fast rate and exhibit relatively low sensitivity to applied gibberellin. Amo-1618 and another growth retardant, (2-chloroethyl)-trimethylammonium chloride (CCC or Cycocel) suppress strongly gibberellin production by *Fusarium moniliforme* (3) but they do not affect the gibberellin-induced activation of amylase in barley endosperm (L. Paleg, H. Kende, H. Ninnemann, and A. Lang. 1964. Plant Physiol. 39. In press.); thus, they seem to inhibit the biosynthesis of gibberellin but not to compete with the latter at its sites of action.

As can be seen from figure 4, the R_F 0.2 to 0.3

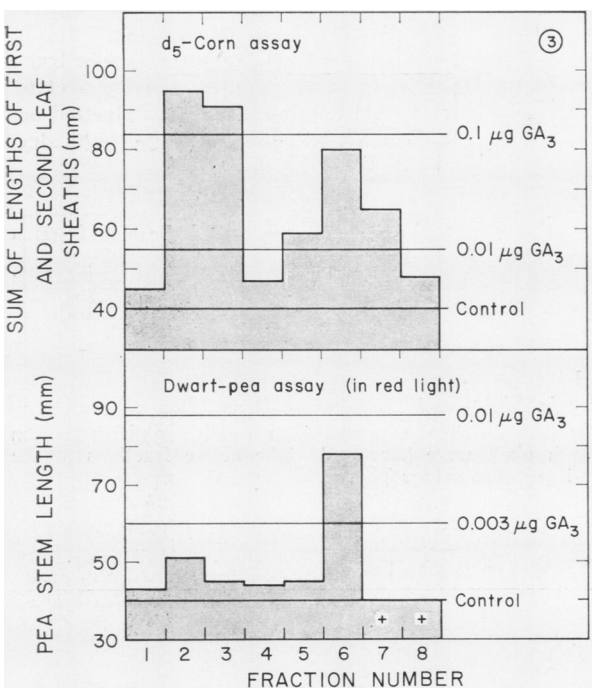

FIG. 3. Partition chromatogram of the acid fraction extracted from 1500 dark-grown dwarf peas (whole plants; dry wt, 50 g). + indicates killing of test plants.

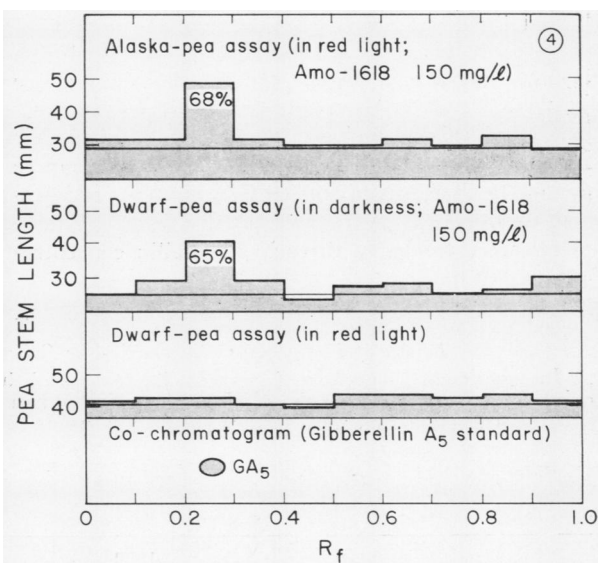

FIG. 4. Chromatogram of fraction I on silica-gel thin layer, solvent diisopropyl-ether-acetic acid (95: 5). Percentages indicate relative responses over control. A reference chromatogram of GA_5 standard is shown on the bottom of the histogram. Alaska peas and etiolated dwarf peas were dwarfed with Amo-1618.

region of the thin-layer chromatograms of fraction I overcame the effect of Amo-1618 in etiolated dwarf peas and in red-grown tall peas but did not reverse the light-induced inhibition in dwarf peas.

Gibberellin-like Substances in Light-grown Dwarf Peas. Dwarf-pea seedlings which were exposed to 24 hours of red light contained 2 fractions with gibberellin-like activity when the chromatographed extract was tested on d_5 corn or etiolated dwarf peas. The R_F values corresponded to those of fractions I and II from dark-grown dwarf peas. Only fraction II stimulated growth of dwarf peas in red light (fig 5). Extractions of dark- and light-grown plants were repeated 5 times each. No quantitative or qualitative differences in gibberellin contents could be found. Gibberellin levels in extracts of etiolated and illuminated dwarf peas obtained in 2 experiments, one with column chromatography and the other with column and thin-layer chromatography, are summarized in table I. Since dwarf peas are extremely sensitive to toxic substances which are still present in the partly purified extracts and which lower the response of the test plants to gibberellins, only assays on d_5 corn were used for quantitative evaluations. The variations evident in the experiments (relatively somewhat more gibberellins in dark-grown than in light-treated peas in experiment 5/11, somewhat less in 19/25) are within the range of experimental error. Table I also shows that thin-layer chromatography after partition chromatography results in further purification of biologically active compounds, yielding 2 to 3 times higher levels of activity in subsequent bioassays.

Activity of Gibberellin A_1 and Gibberellin A_5 in Different Pea Assays. Fraction I behaved biologically like gibberellin A_5. It was highly active on d_5 corn but showed very little activity on dwarf peas grown in red light. It also cochromatographed with GA_5 on a celite partition column and on silica-gel thin layers using the 2 solvent systems described above (fig 4). Fraction II on the other hand showed chromatographical and biological properties characteristic for gibberellin A_1. Thus it was obvious to compare the activity patterns of applied GA_1 and GA_5 in light- and dark-grown dwarf and Alaska peas. Twelve plants were treated with each gibberellin concentration and each experiment was repeated 5 times. Table 2 summarizes the results obtained in 2 representative experiments which were conducted simultaneously and under identical conditions. In red light GA_1 was more than 10 times as active as GA_5 in inducing stem elongation in dwarf peas. In darkness, however, GA_5 was as effective as GA_1. This held both for artificially dwarfed etiolated plants and for etiolated plants not treated with growth retardant. In light-grown

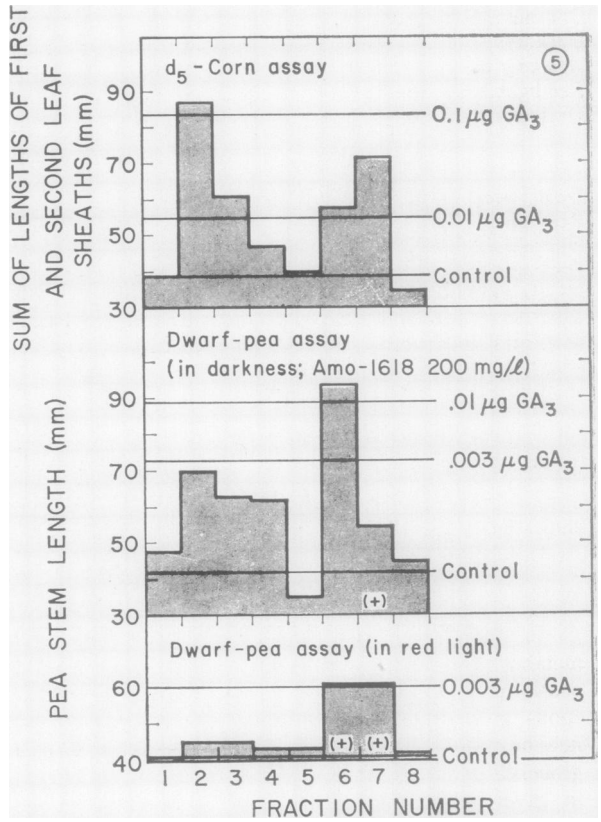

FIG. 5. Partition chromatogram of the acid fraction extracted from 1500 light-grown dwarf peas (whole plants; dry wt, 51 g). Etiolated dwarf peas were treated with Amo-1618. (+) indicates injury in test plants.

Table I

Levels of Endogenous Gibberellin-like Substances in Light- and Dark-grown Dwarf Peas

In experiment 5/11 whole plants were harvested and the extract was chromatographed on a celite partition column only. With respect to experiment 19/25 the upper 50 mm of each plant were harvested. The active fractions of the partition chromatogram were further purified by means of thin-layer chromatography.

Expt.	Growth conditions	Number of plants	Dry wt., g	μg GA_3 equivalents/1500 plants		
				Fraction I	Fraction II	Total
5/11	Darkness	1500	50.0	2.0	0.5	2.5
	Red light	1500	51.0	1.0	0.4	1.4
19/25	Darkness	1500	38.0	3.5	0.9	4.4
	Red light	1500	40.5	5.4	1.5	6.9

Table II

Activity of Gibberellin A_1 and Gibberellin A_5 on Dwarf Peas and Alaska Peas Grown in Red Light and Darkness

Dwarf peas were dwarfed with 150 mg/liter Amo-1618, Alaska peas with 100 mg/liter Amo-1618.

Plants, Growth conditions	Height of pea stem, cm			
	GA_1 (μg/plant)			
	0	0.01	0.03	0.1
Dwarf peas				
Red light	3.9 ± 0.05*	5.7 ± 0.11	9.7 ± 0.28	12.0 ± 0.28
Darkness, Amo-1618	4.8 ± 0.19	7.8 ± 0.38	10.6 ± 0.44	10.5 ± 0.47
Darkness	8.5 ± 0.36	12.2 ± 0.28	13.4 ± 0.31	14.2 ± 0.27
Alaska peas				
Red light Amo-1618	3.9 ± 0.06	6.3 ± 0.18	11.1 ± 0.35	13.0 ± 0.26
Darkness, Amo-1618	4.8 ± 0.26	9.8 ± 0.42	11.0 ± 0.33	12.3 ± 0.29
	GA_5 (μg/plant)			
Dwarf peas				
Red light	3.5 ± 0.04	4.2 ± 0.07	4.8 ± 0.12	5.4 ± 0.16
Darkness, Amo-1618	4.1 ± 0.18	8.3 ± 0.19	9.3 ± 0.32	10.5 ± 0.22
Darkness	9.8 ± 0.33	11.8 ± 0.21	13.3 ± 0.19	13.8 ± 0.29
Alaska peas				
Red light, Amo-1618	3.8 ± 0.05	5.8 ± 0.10	7.4 ± 0.10	8.5 ± 0.18
Darkness, Amo-1618	4.7 ± 0.31	9.1 ± 0.30	11.1 ± 0.25	11.5 ± 0.27

* Standard deviation of the mean.

Alaska peas twice the amount of GA_5 was needed to match the response caused by treatment with GA_1 but in dark-grown Alaska peas both gibberellins showed comparable activity as growth stimulators.

Discussion

In discussing light interference with gibberellin metabolism in plants Lockhart (5, 6) considered 3 possibilities: Light might cause a deficiency of endogenous gibberellins by inhibiting the biosynthesis of new hormone; it might trigger the breakdown of these gibberellins; it might render the tissue less responsive to given amounts of endogenous gibberellins. Of these possibilities, the first 2 become improbable in view of our findings. Since equal amounts of gibberellins were extractable from dark- and light-grown dwarf peas, red light seems to affect neither the biosynthesis nor the destruction of gibberellins in these plants. In contrast, our results are compatible with the third interpretation.

Two fractions exhibiting gibberellin activity were found in dwarf peas. Fraction II stimulated growth when applied to both dark- or light-grown dwarf peas, but fraction I could be effectively utilized as a growth hormone only by etiolated dwarf peas.

The differential behavior of fractions I and II with respect to light is of considerable interest since it offers a basis for explaining the growth pattern of peas in light and darkness. In order to do this, however, certain possibilities concerning the influence of light on the activity of fraction I must be considered first.

The simplest assumption would be that only fraction II is the active growth hormone while fraction I is its precursor, but inactive per se, and that conversion from I to II is blocked or greatly reduced in light. However, the level of fraction II did not decrease in light-grown plants; thus, this interpretation is improbable. It rather seems that the plants contain 2 materials, both of them with hormonal activity, but acting independently from each other, and that light lowers the sensitivity of the cells to endogenous fraction I. Our data, as far as they go at present, do not provide a clue for the mechanism of this light action. One possibility is that light is interfering with one of the reactions leading from fraction I to the growth response proper. It may be assumed that either the utilization of fraction I is affected, or the availability of a cofactor with which this hormone must combine is reduced. However, fraction I did not accumulate in light-treated plants; thus, the light-sensitive reaction would have to be separated from fraction I by one or more reaction step. It is indeed possible that some reaction product of fraction I (and of GA_5) was accumulating in light-grown dwarf pea plants but our methods were not designed to detect such a material.

Another possibility that may be visualized is light-induced formation of an inhibitor which is specific for fraction I. In this respect, it may be of interest that evidence for substances which reduce the response of dwarf peas to applied gibberellin has recently been found in several plants, including tall and dwarf peas, and that the level of these substances in the latter was higher in light than in dark (4). However, the effect of these inhibitory substances has been tested only against gibberellic acid (GA_3); it is not known whether they affect the response of plants to different gibberellins in a differential manner.

However, although the available experimental information is not sufficient to propose a precise explanation for the effect of light on the activity of fraction I, it is sufficient for advancing the following interpretation of the growth behavior of peas in light and darkness. Dwarf peas do grow in light, although at a considerably reduced rate. This growth may be attributed to fraction II. In this connection, it is noteworthy that fraction II is always present in the plants at a lower level (in terms of physiological activity) than fraction I. Since fraction I is almost inactive in light at physiological concentrations, light-grown plants are physiologically deficient in endogenous growth hormone and grow in the characteristic dwarf habit.

In tall peas, inhibition caused by low-intensity red light is less drastic than in dwarf ones and is not persistent over longer periods of time (1, 5, 7). Our experiments showed that GA_5, when applied to illuminated Alaska peas, was about half as active in causing stem elongation as was GA_1. In light-grown dwarf peas GA_5 was 10 times less active in promoting growth than GA_1. Both gibberellins were equally effective as growth hormones when etiolated Alaska or dwarf peas were treated. From this it may be estimated that the light-sensitive reaction in dwarf peas is about 5 times more sensitive to irradiation than in tall peas.

The chromatographic behavior of fraction I was similar to that of gibberellin A_5, that of fraction II close to that of gibberellin A_1. The effect of GA_1 in peas was the same as that of fraction II, both being equally active in darkness and in light. GA_5 promoted growth of dwarf peas in the dark much more than in light, thus being quite similar to fraction I. In other biological effects (their action on d_5 corn), fractions I and II also behaved like GA_5 and GA_1, respectively. Since both GA_1 and GA_5 have been shown to occur in leguminous plants (8), it seems highly probable that fraction I from peas is indeed identical with GA_5, and the same holds for fraction II with respect to GA_1.

Summary

Two fractions with gibberellin-like activity were obtained from dwarf peas by means of partition chromatography and thin-layer chromatography.

No differences could be detected in the levels of these substances when extracted from light- and dark-grown plants.

One of the 2 gibberellin-like fractions (fraction II) behaved chromatographically and biologically like gibberellin A_1 (GA_1), the other (fraction I) like gibberellin A_5 (GA_5).

Fraction II and GA_1 were highly active on dwarf peas grown in light and in darkness. Fraction I and GA_5 were highly active in the dark but 10 times less active when the test plants were exposed to red light.

In light-grown Alaska peas GA_5 was half as active as GA_1 in promoting stem elongation. Again both gibberellins were equally active when applied to etiolated Alaska peas.

It is concluded that illumination lowers the sensitivity of the tissue to fraction I and to GA_5 while not affecting the sensitivity to fraction II and GA_1. It is estimated that the reaction affected by light is 5 times more sensitive to irradiation in dwarf peas than in Alaska peas.

It is probable that fraction I is identical with GA_5 and fraction II identical with GA_1.

Acknowledgments

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