Evidence for Substances in Higher Plants Interfering with Response of Dwarf Peas to Gibberellin [1, 2]

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Following the discovery of the growth-promoting effects of gibberellins several attempts have been made to explain certain growth differences, such as those between tall and dwarf varieties of a species or between light- and dark-grown plants of the same genotype, in terms of their gibberellin content. For example, Lockhart (8–14) showed in a series of impressive experiments that the depressing action of light on the growth of many plants can be completely overcome by application of the appropriate gibberellin, and using a kinetic analysis concluded that light reduces the effective gibberellin level in the plant. In such situations one can envisage 3 possibilities: a) the slower-growing plant has less growth-promoting substance(s); b) it contains more growth-inhibiting substances which in some way counteract the production or function of the former; c) its sensitivity to the growth promoters has been lowered.

While evaluating methods for extraction and partial purification of gibberellin-like substances from higher plants we found evidence for substances which, when applied to dwarf peas together with gibberellin A_3 (gibberellic acid), reduce the growth-promoting effect of the latter. These substances may thus qualify for the inhibitory substances postulated under explanation b. Their chemical and physiological characterization and their actual role require much further study, but a brief report is indicated.

Materials and Methods

The plant materials that were used in our work, and the extraction and partitioning procedures will be described in the experimental part because they are an essential part of the actual experiments. Here, we shall describe the dwarf-pea bioassay used for determining both gibberellin and inhibitor activity, and the chromatographic procedures, since these were used in all experiments.

Dwarf pea Bioassay. The bioassay was developed in this laboratory by E. Reinhard (unpublished work) and is based on the growth-depressing action of red light and its reversal by applied gibberellin (8). It is conducted as follows: Seeds of dwarf peas (*Pisum sativum* L., var. Progress No 9, obtained from Asgrow Seed Company, New Haven, Conn.) were soaked (Sunday), planted in vermiculite on the next day (Monday) and grown for 3 days in darkness at 27°. They were then (Thursday) selected visually into 3 different sizes and transferred to water culture, kept overnight in darkness at 11 to 19° (depending on the size of the plants) and treated on the next day (Friday) with the test and standard solutions, each plant receiving 1 drop of 5 μliters onto the terminal bud. The plants were then grown in continuous weak red light at 27° and measured 5 days later (Wednesday). The red-light source was 4 F96-T8 Red fluorescent tubes, spaced 15 cm apart, 115 cm from test plant level.

The quantitative estimation of the activity of gibberellin-like substances was done by determining the amount present in each fraction of an extract (see below) on the standard dose-response curve for gibberellin A_3 (gibberellic acid), and totalling these amounts. Since the activity of different gibberellins on dwarf peas is quite different (1, 19, and Reinhard, unpublished data), this procedure may provide only relative levels of gibberellin activity. However, since we were interested only in the quantitative comparison of the levels of the same gibberellin-like or inhibitory substances, this fact does not affect our results.

Chromatography of Plant Extracts. Extracts were chromatographed in the following way: the method is a modification of one developed by Reinhard (unpublished work) which in turn is a modification of procedures used by MacMillan et al. (15) for the extraction of different gibberellins from runner-bean (*Phaseolus multiflorus*) seeds. The chemicals used here and for the extraction and partitioning procedures were reagent grade (A.C.S.).

Celite (L-665-A) and Norit-A were weighted in ratios of 1:1 or 2:1 (1–6 g Norit), washed in a breaker for 2½ hours with 0.44 N HCl, poured into the column, pressed down and washed 3 times with water. The following column sizes were used: a) 22 mm diameter, height of Celite-Norit mixture 10 cm, b) 10 mm diameter, 5.5 or 8 cm column height, c) 30 mm diameter, 2 cm height. The first 2 will be referred to as long columns, the third as short column. Aqueous extracts which had been adjusted to

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pH 3 with dilute HCl were added, the columns washed twice with water and eluted with increasing concentrations of acetone, each concentration (5 or 10 ml) being collected as a single fraction. The concentration steps were 2.5 or 5%, yielding a total of 20 or 40 fractions. At the end of the elution, the column was usually washed with an excess of pure acetone. The acetone in the fractions was removed at room temperature with the aid of a fan; the aqueous residues were lyophilized and taken up each with 0.1 ml of water containing 0.05% Tween 20 or, in the case of inhibitor studies, with solutions of gibberellin A_3 in Tween 20. One twentieth (5 μliter) of each fraction was applied per test plant.

It should be emphasized that the position of the activity peaks on the chromatograms in relation to the eluant concentration is not an absolute one but depends on several factors, particularly the prepurification procedures and the length of the column. For example, partitioning of the aqueous extract against chloroform shifts the peak to considerably lower acetone concentrations (fig 1); use of shorter columns results in a shift to higher acetone concentrations. Any conclusion about the indentity of the active materials in differently processed extracts, based on the position of the activity peaks, would therefore be quite misleading. On the other hand, a given material, extracted and processed in an identical manner, yields highly reproducible results.

Experimental Results

I. *Increase in Gibberellin Activity of Limabean Extracts by Partitioning Procedures.* The main plant material was immature seeds of limabeans (*Phaseolus lunatus* L.), specifically commercial frozen butterbeans. This material (about 285 g or 570 g) was extracted with approximately twice the amount (v/w) of methanol. The methanol was evaporated under reduced pressure and the aqueous residue partioned against different organic solvents. As a result of this partitioning, higher levels of gibberellin activity could be found in the aqueous phases, as compared to the unpartitioned methanol-free extract.

In one experiment, the methanol-free extract was divided into 4 equal portions which were then treated in the following manner: a) not partitioned; b) partitioned 3 times against equal volumes of petroleum ether (B.R. 30-60 C) at pH5; c) as b, followed by 2 times partitioning against chloroform at the same pH; d) as c followed by 2 times partitioning against ethyl acetate at pH 7.

The final aqueous phase of each of these treatments was adjusted to pH 3 and run through long columns. The activity distributions are shown in figure 1 and the total activities, in relative values, in table I. It can be seen that the activity in extract d was almost 5 times as high as in a (i.e., the non-partitioned material); 2.2 times as high as in b (the material partitioned against petroleum ether only); and 1.8 times as high as in c (material partitioned against petroleum ether and chloroform).

The gain in activity brought about by partitioning against ethyl acetate at pH 7 can also be obtained by partitioning against chloroform at the same pH value. In a typical experiment, partitioning against chloroform at pH 7 after petroleum-ether partitioning at pH 5 yielded 2.6 times more activity than partitioning at pH 5.

These experiments were repeated several times, always with the same results.

II. *Evidence for Inhibitory Substances in the Chloroform Phase of Limabean Extracts.* The results described in the preceding section indicated that partition against chloroform (or ethyl acetate) at a slightly acid and to an even greater extent at a neutral pH removed from the original limabean extract substances which interefere with the activity of gibberellin-like substances in the dwarf-pea test.

In order to test this conclusion, the chloroform phase of the pH 5 partition was dried by evaporation in front of a fan, the residue was taken up with water, 3 identical dilution series were prepared, and the resulting samples were lyophilized. One sample of each dilution was taken up with 0.1 ml of a 10 μg/ml gibberellin A_3 solution, the second with 0.1 ml of 3 μg/ml gibberellin A_3 (in either case with 0.05% Tween 20) and the third with 0.1 ml 0.05% Tween 20 so that each test plant would receive 0.05 and 0.015 μg of gibberellin A_3, and no gibberellin at all, respectively. All samples were then tested on dwarf pea plants.

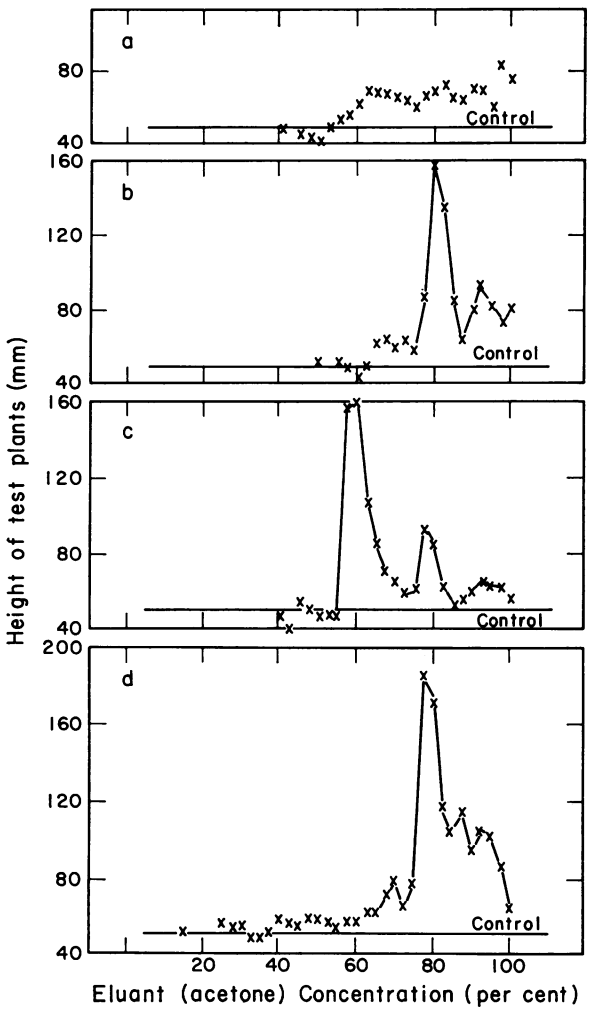
The results are shown in figure 2. (The dilutions are expressed as the fraction of the original, i.e. undiluted sample applied per test plant. Thus, 1/200 means that the original sample was diluted tenfold and 5 μl of this diluted sample were applied to a dwarf pea plant, etc.)

It can be seen that test plants in the series without added gibberellin did not show any growth reduction. It may be useful to point out that the height of the test plants at treatment is about 18 mm (limits, 14 to 23 mm) while the height of control plants at measurement is about 50 mm. Thus, there is an ample differential for the manifestation of a growth inhibition even in absence of gibberellin.

In contrast to the no-gibberellin series, the test plants of the series with added gibberellin A_3 showed very marked inhibition at the lower dilutions. In the series with 0.015 μg gibberellin per test plant, the reduction in growth caused by the lowest dilution, i.e. the highest concentration, corresponded to a loss of about 73% of gibberellin.

Similar results were obtained in a dilution series of the organic phase of an ethyl acetate partitioning.

Water extracts of limabean seeds contained the same levels of gibberellin activity as methanol extracts after partition against the organic media. This finding indicates that the inhibitory materials are not readily released into water. The fact that these materials pass into the chloroform phase in larger quantities at pH 7 than at pH 5, as evident from the higher gain in gibberellin activity after partitioning



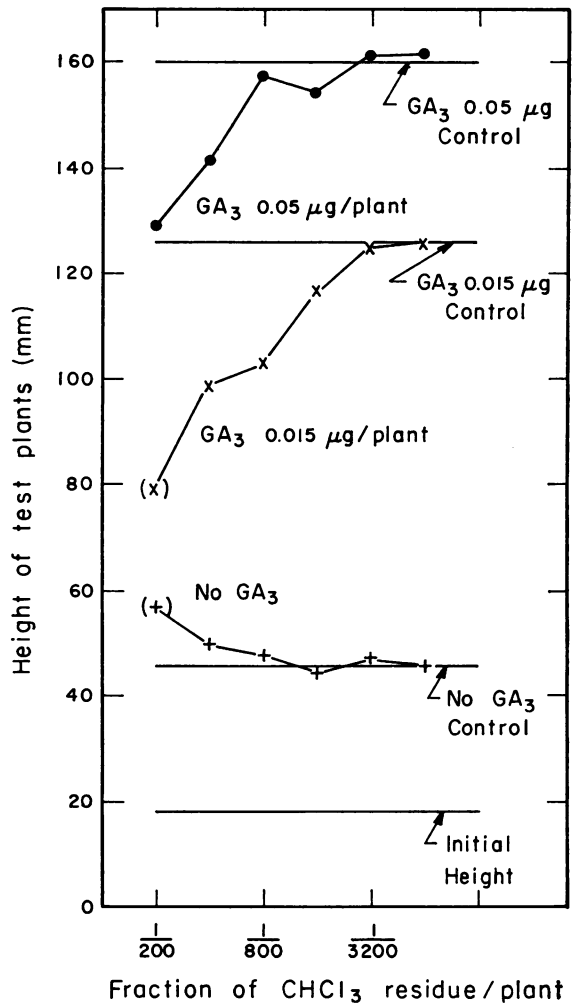


FIG. 1 (*left*). Growth response of dwarf peas (Progress No 9) to chromatographed extracts from limabeans, without partitioning (a) and after partitioning against petrol ether at pH 5 (b), petrol ether and chloroform at pH 5 (c), and petrol ether and chloroform at pH 5 and ethyl acetate at pH 7 (d).

FIG. 2 (*right*). Growth response of dwarf peas to different dilutions of a nonfractionated chloroform (pH 5) phase from a limabean extract in presence and absence of gibberellin. Points in ( ) = part of test plants injured.

at the higher pH value (table I), suggests that they have a slightly basic character.

III. *Chromatography of Inhibitor Extracts.* Chloroform phase residues which had been prepared as described in the preceding sections were run through short columns, the fractions taken up with Tween or with gibberellin A_3 solutions and applied to test plants. The results of such experiments are shown in figure 3a. No evidence for inhibition can be seen in the series without added gibberellin and little in that with the higher gibberellin level, but very marked inhibition is evident in the series with the lower gibberellin level, at the 15 to 20 % and the 80 % acetone fractions.

Experiments using a long column gave quite similar results (fig 3b) except that the second inhibition peak appeared at a lower acetone concentration (55 % and the adjacent fractions), as typical for long columns.

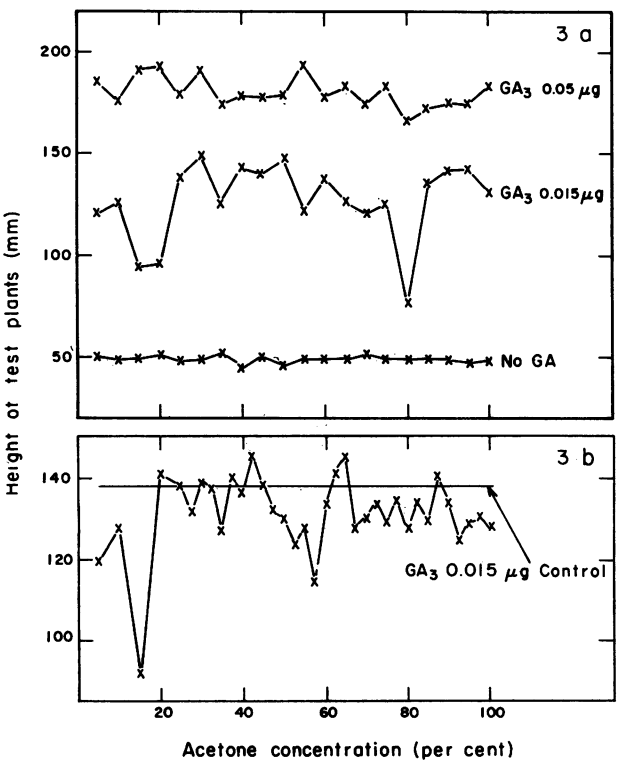
It should be noted that the second inhibitory zone in the chromatograms corresponds closely to the zone of principal gibberellin activity obtained from the same plant material and by the same fractionation procedures (compare fig 3b with 1c). This fact undoubtedly accounts for the deficit of gibberellin activity evident prior to the chloroform (and ethyl acetate) partitioning (fig 1 and table I).

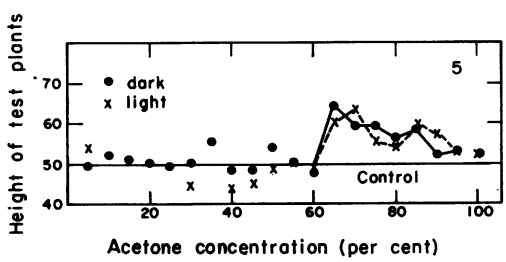
IV. *Presence of Inhibitory Substances in Other Plant Materials.* After demonstrating the presence in limabeans of inhibitory substances which interfere with the response of dwarf peas to gibberellin a preliminary search for similar substances was made in other plant materials. The materials chosen were tall and dwarf peas, and hemp (*Cannabis sativa*

Table I

Relative Gibberellin Activity in Methanol Extracts from Immature Limabeans after Removal of Methanol and Various Partitioning Procedures

Treatment of extract	Activity
(a) Not partitioned	100
(b) 3 × petroleum ether (pH 5)	210
(c) 3 × petroleum ether, 2 × chloroform (pH 5)	264
(d) 3 × petroleum ether, 2 × chloroform (pH 5) 2 × ethyl acetate (pH 7)	471





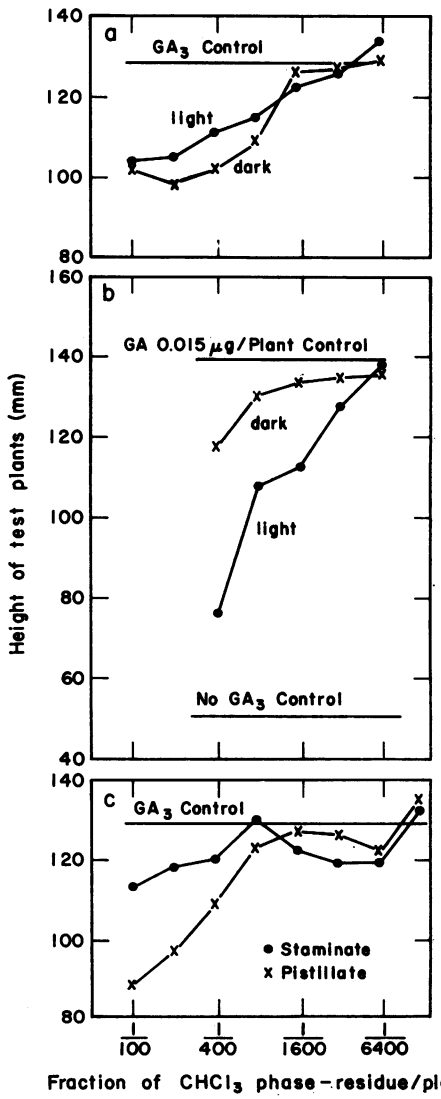


FIG. 3 (*upper left*). Growth response of dwarf peas to the chloroform phases from limabean extracts. a, Chromatographed on a short column, in the presence of 2 gibberellin levels, and in absence of gibberellin. The lower and the upper curve are from one experiment but the amount of extract represented by the upper curve was only four fifths of that represented by the lower. The center curve (0.015 μg gibberellin) is from a separate experiment, b, Fractionation on a long column; growth response in the presence of 0.015 μg gibberellin.

FIG. 4 (*right*) Growth response of dwarf peas to different dilutions of the nonfractionated chloroform phases of extracts from different plant materials in the presence of 0.015 μg gibberellin. a, Extracts from 8,000 red light- and dark-grown seedlings of a tall pea variety (Alaska). b, Extracts from 600 seedlings of a dwarf variety (Progress No 9). c, Extract from the inflorescence of male (pistillate) and female (staminate) hemp plants which had received 7 short days.

FIG. 5. (*lower left*) Growth response of dwarf peas to chromatographed extracts from red light- and dark-grown Alaska pea seedlings, showing presence of gibberellin-like substances.

L.). The reason for this choice was that these 2 plants exhibit marked growth differences, depending on genotype and environmental conditions. When grown in darkness both tall and dwarf peas exhibit approximately identical growth rates but when grown in red light of low intensity the dwarf are strongly inhibited (4) whereas tall types show only a transient growth depression (14). Under our conditions, six-day-old dwarf seedlings (Progress No 9) grown in red light (and 27 C) were half as tall as dark-grown seedlings of equal age although the fresh weights were the same. Female (pistillate) hemp plants, when transferred to short days and thus induced to flower formation, cease to grow almost completely after 7 short days whereas typical (XY) male (staminate) plants continue to grow at a rapid rate (6). XX males, which occur in hemp in addition to females and typical (XY) males (7), have a growth pattern like that of the females, indicating that the growth difference between XY males and the females is not related to sex as such.

Application of gibberellin eliminates these growth differences, raising the height of peas grown in low-intensity light to that of the dark-grown ones (4, 8), and causing female and XX-male plants of hemp to assume the inflorescence type of the XY males, without however having an effect on sex expression (6, and Köhler, unpublished data).

The peas (varieties Alaska and Progress No 9, from Ferry-Morse Seed Company, Mountain Vie, Calif. and Asgrow, resp.) were grown in darkness or in red light at 27° for periods of 6 to 8 days. Lots of plants were then extracted and the chloroform phases tested in the same manner as with limabeans. The extracts from approximately 8,000 six-day-old seedlings of the tall variety (Alaska) caused a depression equivalent to a 50 % loss of the applied gibberellin A_3 at a dilution of 1/100 or 1/200 (higher concentrations killed the test plants); there was little if any difference between the inhibitory activity of extracts from light- and dark-grown plants (fig 4a). In contrast, an extract from approximately 600 light-grown dwarf-pea seedlings (Progress No 9) caused a very marked inhibition even at a dilution of 1/1,600 at which the extract from the tall peas, even though obtained from more than 10 times as many seedlings, was inactive. The inhibitory effects of the 1/300 and 1/400 dilutions were still greater, but the inhibitory effect of extract from an equal number of dark-grown seedlings of the same age was considerably smaller (fig 4b). The loss of gibberellin activity at the 1/400 dilution was 46 %, as compared against 84 % for extract from light-grown seedlings.

Determinations of the gibberellin content showed presence of gibberellin-like materials in seedlings of tall peas and no difference between plants grown in light and in dark (fig 5), but no activity could be found in either light- or dark-grown dwarf peas. This latter result means either that the gibberellins in these seedlings, if present at all, are at such a low level that they escaped detection by our methods, or else that they are different from those of the tall seedlings, not giving a measurable response in the dwarf-pea assay. Radley (16) had found gibberellin-like materials in both tall and dwarf peas. Her data seem to indicate that the level in the former is higher than in the latter, at least in the upper part of the shoot. (The authoress herself does not draw this conclusion, but see her table 5.) The levels, as far as they can be judged from the data, are similar to those found in our experiments with tall peas. The reason why Radley did find gibberellin activity in dwarf peas while we were unable to do so is not clear. It may be due to different extraction procedures or, more probably, to the fact that Radley was extracting considerably older plants[4].

The hemp plants (variety Pelozella) were grown in long-day conditions (18 hours of light, natural day extended with fluorescent light) at a temperature of 23° from 8:00 AM to 5:00 PM and 19° from 5:00 PM to 8:00 AM for 10 weeks and then transferred to short day (8 hours natural light; same temperature regime). The inflorescences of 100 male and 100 female plants (fr wt, 94 and 146 g, resp.) were extracted seven days later, that is when the male plants were still rapidly elongating while the female ones had practically stopped growing (height of plants—males 202 cm, females 166 cm). Extraction and partitioning were the same as with limabeans and peas. The chloroform phases from female plants had a considerably higher inhibitory activity than those from the male ones. At a dilution of 1/100, the former caused a loss of 70 % of the added gibberellin A_3, the latter of 37 % (fig 4c). Extracts of XX males which, when photoinduced, grow as slowly as the females showed approximately the same, high inhibitory activity as the latter.

Chromatograms of the pea and the hemp cloroform phases appeared to be similar to those of the corresponding limabean extracts.

Discussion

Growth-inhibiting materials have been extracted from many plants, and negative correlations between their levels and the levels of growth activity have been repeatedly established (for a recent review, see 5). However, these inhibitors have a general growth-depressing action which may be unspecific; their physiological significance is therefore open to some question. Inhibitors which seem to affect selectively the action of gibberellin have been obtained by Corcoran and Phinney (3) from the seeds of *Ceratonia siliqua* and by Bünsow (2) from dormant buds and seeds of *Aesculus hippocastanum* and from vegetative plants of *Lapsana communis* and *Mycelis*

[4] Dr. H. Kende (this laboratory), using other procedures, has meanwhile been able to obtain gibberellin activity from dwarf-pea seedlings (unpublished data).

muralis. Indications for similar substances were also found by other authors, for example Radley (17) in a brown alga. However, in all these cases the correlation with growth, if any, has not been established.

Our results indicate the existence of substances which occur in quite different plants and which partition from water into chloroform at a slightly acid or, even more completely, at a neutral pH, suggesting a slightly basic character. The substances have no activity when applied to a particular, gibberellin-sensitive test system alone but they decrease the response of this system to gibberellin. The decrease can apparently be overcome by increasing the amount of gibberellin (see fig 3). The situation is thus suggestive of a competitive type of inhibition.

The quantity of these substances seems to be inversely correlated with the growth of that plant material as determined by its genotype and the environmental conditions. Tall peas, the growth rate of which is rapid both in dark and in (relatively weak) red light, seem to contain similar, relatively low amounts of the inhibitory substances. Dwarf peas, when grown in light and markedly inhibited, have high levels of the inhibitors but when grown in dark and elongating almost as rapidly as the tall ones they contain lower levels. Female hemp plants, when having stopped growing, contain more inhibitors than male ones which still continue to grow.

From the available data it is conceivable that these inhibitors participate in the growth regulation of plants, particularly by an interplay with gibberellins. High levels of the inhibitors can depress the growth rate; this depression can be overcome by gibberellins, either as present in the plant or as supplied from the outside. The results with dwarf pea seedlings indicate that the level of the inhibitors may be regulated by light. The low growth rate of these peas in light may be due either to lack of adequate amounts of gibberellin, or to the presence of such gibberellins which are inefficient in overcoming the action of the inhibitors. Simpson and Wain (18) have concluded, on indirect evidence, that the effect of light on stem elongation in dwarf peas is most simply explained by assuming the formation of a light-dependent inhibitor the action of which is reversed by applied gibberellin A_3.

Summary

Immature limabean seeds contain substances which reduce the gibberellin response of dwarf peas while they have no effect on the growth of the latter in the absence of gibberellin. Similar substances occur in pea seedlings and in hemp plants. The substances can be extracted with methanol and partitioned into chloroform and ethyl acetate at pH 5 and 7. In peas and hemp, an inverse correlation was found between growth rates and the amounts of the inhibitors extractable from the plants. The possible significance of these inhibitors in growth regulation is pointed out.

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