

## Extractable and Diffusible Gibberellins from Light- and Dark-grown Pea Seedlings<sup>1</sup>

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**Abstract.** Gibberellins were obtained from light- and dark-grown peas by solvent extraction and agar diffusion. Both A<sub>3</sub>- and A<sub>1</sub>-like gibberellins were obtained by extraction; however, by diffusion only the A<sub>1</sub>-like gibberellin was found. There was no significant quantitative difference in the levels of diffusible or extractable gibberellin obtained from light- and dark-grown tall and dwarf peas. Several possible explanations for the discrepancy between diffusible and extractable gibberellin were investigated. Of these, only 1 was supported by experimental evidence, namely, that GA<sub>3</sub> can be converted to GA<sub>1</sub>.

Determinations of the gibberellin content of plants have been generally made by extraction of the active substances with solvents, followed by partial purification and separation by means of solvent partition and chromatography. More recently, a method has been developed to obtain gibberellins from plant material by diffusion into agar (9). Diffusion can be followed by partitioning and/or chromatography, using the same methods as after extraction.

In the case of auxin, where the diffusion method has been used for a long time, it is well known that extraction and diffusion may yield quite divergent results and measure different aspects of the auxin metabolism of the plant. In general, diffusion permits the measurement of auxin production while extraction measures the total hormone which is present in the tissue at a given moment, but tells little about its origin and function. In the case of gibberellin, the diffusion technique, coupled with the use of inhibitors of gibberellin biosynthesis, has permitted an unequivocal demonstration that gibberellins are synthesized in shoot buds (specifically, the young, not yet unfolded leaves) and root tips of sunflower plants (10, 11).

It appeared to us interesting to extend the examination of endogenous gibberellins by the diffusion technique to more plants, and to compare results obtained by diffusion and extraction, as such comparison may add to our understanding of the function and metabolism of endogenous gibberellins. The plant material chosen was pea seedlings, this choice being based on 2 considerations.

Firstly, the gibberellin content of pea seedlings has already been measured by several authors, using extraction techniques (13, 15, 16, 17, 22, 25), although the results have been divergent. Secondly, there are strong suggestions that gibberellins play an important part in endogenous regulation of growth in the pea shoot, and that they perform this function in some kind of interaction with light. Briefly and summarily, the essential facts are as follows (see 6, 13, 18, 19): A) Peas occur in normal (tall) and dwarf varieties, this difference being mainly determined by the alleles of 1 gene. B) Both normals and dwarfs grow at approximately the same rates in the dark. C) In either type, growth is reduced by light, but the effect is considerably greater in the dwarfs than in the normals. D) Applied gibberellin has little if any effect on growth in the dark but causes marked growth promotion in the light. E) The response of dwarfs to applied gibberellin (when grown in the light) is much greater than that of the normals, resulting in obliteration or reduction of the growth differences between the 2 types. Thus, it appears that the inhibitory effect of light on stem growth in pea plants is based on some reduction of the effective gibberellin level in the plants, and that the difference between normals and dwarfs arises because this reduction is greater in the latter than in the former. The effect of light on growth in peas is mediated by phytochrome, red light causing the inhibition of growth, far-red light negating the effect of red (18, 19).

### Materials and Methods

*Plant Material.* Normal (Alaska) and dwarf (Progress No. 9) peas (*Pisum sativum* L.) obtained from Asgrow, New Haven, Connecticut, were allowed to imbibe at room temperature for 18 hours, sown in vermiculite and grown either in darkness

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or in continuous white light (900 ft-c) at  $23^{\circ} \pm 1^{\circ}$ . They were watered with half-strength Hoagland solution. The plants were harvested for both diffusion and extraction when 8 to 10 days old. Seedlings treated with growth retardants were grown as described above except that they were transferred to plastic boxes containing half-strength Hoagland nutrient solution on the fourth day after sowing.

**Application of Gibberellins and Growth Retardants.** Both  $GA_5$  and  $GA_1$  were applied to the apical buds of seedlings in an aqueous solution containing 0.05% Tween 20. The growth retardant AMO1618 [2'-isopropyl-4'-(trimethylammonium chloride) 5'-methylphenyl piperidine-1-carboxylate] was applied as a 200 mg/liter solution in half strength Hoagland solution via the roots.

**Gibberellin Diffusion.** Agar diffusates were obtained from pea apices using the techniques described previously (9, 10). Agar blocks were prepared from a 1.5% aqueous solution of agar which was poured into 10 mm  $\times$  2 m glass tubes. After solidifying, the agar plug was removed from the tubes and cut into blocks 10 mm diameter  $\times$  5 mm height.

Following the diffusion period, the agar blocks were extracted by freezing. The frozen agar blocks were flooded with methanol and the methanolic extract obtained from the agar was evaporated on a flash evaporator until the aqueous phase remained. This was partitioned against ethyl acetate at pH 2.5 and dried over sodium sulfate prior to chromatography.

**Gibberellin Extraction.** Seedlings and seedling tips were frozen with liquid nitrogen, lyophilized, and homogenized with methanol in a Waring Blendor. The methanol was allowed to extract for 24 hours at  $2^{\circ}$ . Lipid material was removed by mixing petroleum ether (boiling range  $30-60^{\circ}$ ) with the methanol extract which had been adjusted to 80% by the addition of water. The aqueous methanol was evaporated on a flash evaporator, the remaining aqueous phase adjusted to pH 9.5 with 1 N NaOH and partitioned twice against ethyl acetate. This ethyl acetate was discarded and the remaining aqueous phase was acidified to pH 2.5 with 1 N NCL and partitioned against ethyl acetate 3 times. The acidic ethyl acetate fraction was dried over sodium sulfate prior to further purification by thin layer chromatography (TLC).

**Chromatography.** Both agar block and tissue extracts were purified by TLC prior to bioassay. Acidic fractions were reduced to dryness, redissolved in a small volume of ethyl acetate and applied as a band to the origin of a  $20 \times 20$  cm silica gel (H) thin-layer plate which was approximately  $250 \mu$  thick. The plates were developed in the following solvent systems: Chloroform/ethyl acetate/acetic acid (60:40:5 v/v); benzene/acetic acid/water (8:3:5 v/v), lower phase plus 20% ethyl acetate; isopropyl ether/acetic acid (95:5 v/v).

Following development, the plates were divided

into 10 equal zones between origin and solvent front. Each zone was scraped off and eluted 3 times with wet ethyl acetate. The eluates were reduced to dryness and redissolved in water containing 0.05% Tween 20 (polyoxyethylenesorbitan monolaurate).

**Bioassays.** Eluates were assayed with the dwarf corn (d5 mutant) seedling test, the lettuce hypocotyl test (Frankland and Wareing, 4) and the barley half-seed test; in the latter case, the activity of alpha amylase was determined directly as described by Jones and Varner (12).

## Results

When apical buds from light and dark-grown, normal and dwarf peas were excised and allowed to diffuse on agar blocks for 20 hours, bioassay with d5 corn of extracts following chromatography in a solvent mixture of benzene/acetic acid/water (8:3:5) with 20% ethyl acetate added to the lower phase indicated 1 zone of growth promotion (fig 1), corresponding to the position of  $GA_1$  and/or  $GA_5$ .

Extraction of identical apical buds, however, indicated both the presence of  $GA_1$  and  $GA_5$  (table I), confirming the observations made by Kende and Lang (13). There was no quantitative difference in the levels of diffusible or extractable gibberellin in the apices of either normal or dwarf peas grown in light or dark (fig 1, table I).

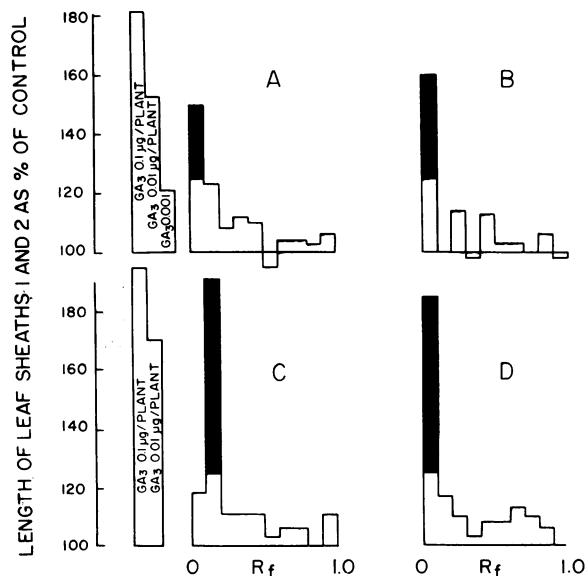


FIG. 1. Diffusates from 100 apices each of light- and dark-grown normal and dwarf pea seedlings. Extracts bioassayed with d<sub>5</sub> corn. TLC solvent; di-isopropyl ether: acetic acid (95.5 v/v). A) Normal, dark-grown; fresh weights = 6.1 g. B) Dwarf, dark-grown; fresh weights = 6.3 g. C) Normal, light-grown; fresh weights = 10.6 g. D) Dwarf, light-grown; fresh weights = 11.5 g. Base of darkened area indicates significant biological activity at the 5% level of risk.

Table I. Amount of GA Obtained from *Pisum sativum* Apical Buds by Diffusion and Extraction

		Light		Dark	
		Normal	Dwarf	Normal	Dwarf
Diffusion	GA <sub>1</sub>	1.70	2.30	1.90	2.30
	GA <sub>5</sub>	0	0	0	0
	Total	1.70	2.30	1.90	2.30
Extraction	GA <sub>1</sub>	0.90	0.60	1.00	0.80
	GA <sub>5</sub>	0.40	0.45	0.52	0.36
	Total	1.30	1.05	1.52	1.16

Because of the apparent absence of the GA<sub>5</sub>-like substance from diffusates of apical buds of peas, intensive examinations were made of agar diffusates using various chromatographic and bioassay techniques. The results are summarized in table II. It can be seen that no significant growth response was obtained by those zones of the chromatograms corresponding to the known position of GA<sub>5</sub>.

Several reasons can be visualized for the failure to find a GA<sub>5</sub>-like substance in diffusates, as opposed to extracts. The substance may be unable to move through the pea internode. This possibility was tested using conventional donor/receiver agar block experiments. Gibberellins A<sub>1</sub> and A<sub>5</sub> were incorporated into agar blocks and placed at the apical end of 15-mm-long sections from the sub-

Table II. Bioassay of Diffusates from Agar Blocks Following Chromatography in 3 Different Solvent Systems

Solvent systems: 1) Benzene/acetic acid/water (8:3:5), lower phase 20% ethyl acetate (v/v). 2) Diisopropyl ether/acetic acid (95.5 v/v). 3) Chloroform/ethyl acetate/acetic acid (60:40:5 v/v).

Solvent system	Assay	R <sub>F</sub>		Zones of growth promotion
		GA <sub>3</sub>	GA <sub>5</sub>	
1	d <sub>5</sub> Corn	0.00	0.35	0.0-0.1
	Barley	0.00	0.34	0.0-0.1
	Lettuce	0.00	0.35	0.0-0.1
2	d <sub>5</sub> Corn	0.07	0.3	0.0-0.2
	Barley	0.05	0.31	0.0-0.2
	Lettuce	0.07	0.32	0.0-0.3
3	d <sub>5</sub> Corn	0.18	0.54	0.2-0.3
	Barley	0.16	0.55	0.2-0.3
	Lettuce	0.16	0.54	0.2-0.3

Table III. Transport of GA<sub>5</sub> and GA<sub>1</sub> Through 15 mm-Long Light-Grown Pea Internode Sections

Section source	Donor block <sup>1</sup>	GA <sub>3</sub> equivalent <sup>1</sup> in receiver blocks
Normal	GA <sub>1</sub> 0.50 μg	0.045 μg
	GA <sub>5</sub> 0.50 μg	0.110 μg
Dwarf	GA <sub>1</sub> 0.50 μg	0.015 μg
	GA <sub>5</sub> 0.50 μg	0.065 μg

<sup>1</sup> 50 Donor blocks treatment.

apical internode obtained from light-grown normal and dwarf peas. The sections were then allowed to stand on receiver blocks of plain agar and following a 20-hour diffusion period the receiver blocks were removed and extracted. Bioassays of extracts indicated that both GA<sub>1</sub> and GA<sub>5</sub> diffused readily through such isolated internode sections (table III). In fact, GA<sub>5</sub> would appear to move more rapidly than GA<sub>1</sub> through pea internode sections.

An alternative explanation for the discrepancy between diffusible and extractable gibberellins may lie in the incomplete removal of the gibberellin from the extracts of the aqueous agar blocks.

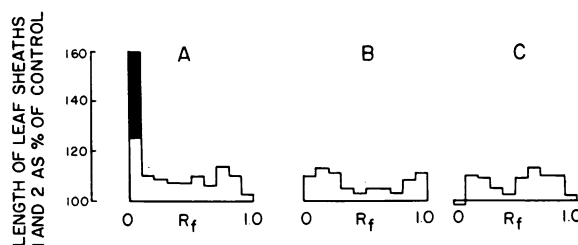


Fig. 2. Hydrolysis of aqueous extracts of the agar blocks. Bioassay, d<sub>5</sub> corn. TLC solvent, di-isopropyl ether; acetic acid (95.5 v/v). A) Acidic ethyl acetate phase from 130 light grown normal pea apices. B) Aqueous phase from 125 light-grown normal pea apices following hydrolysis with ficin. C) Aqueous phase from 130 light-grown normal pea apices following hydrolysis with 0.5 N HCl. Base of darkened area indicates significant biological activity at the 5% level of risk.

Several workers have shown that gibberellins can exist as bound complexes (7,21), the free gibberellins being obtained only after hydrolysis of the extract with acid or with certain proteases (21). It has also been shown that these 'bound' gibberellins remain in the aqueous phase during partition of extracts with conventional solvents. However, bioassay of the aqueous phase of agar block extracts following hydrolysis with dilute acid and the protease ficin (21) indicated no biological activity (fig 2). This result was not surprising as it has already been shown that GA<sub>5</sub> can be readily obtained from light and dark-grown peas by conventional extraction and partition techniques (13, table I).

It may be argued that GA<sub>5</sub> is inactivated during diffusion, either at the cut surface of the excised bud or in the agar block. This phenomenon has been encountered during the diffusion of auxin from excised organs (5), but there is no evidence for GA destruction during diffusion. Indeed, the results obtained in the transport experiments indicate that preferential destruction of GA<sub>5</sub> does not occur during diffusion. It must be pointed out, however, that much more GA<sub>5</sub> was used in the donor/receiver block experiments than would nor-

mally be found in internode sections; consequently, any inactivating mechanism present could be overwhelmed.

In an examination of the discrepancy between "free" (diffusible) and extractable auxin, van Overbeek (23) suggested that the extractable form of auxin was a precursor of diffusible auxin. Following this argument, the discrepancy between diffusible and extractable gibberellin in peas could be explained by postulating that  $GA_5$  is a non-diffusible but extractable precursor of  $GA_1$ . Phinney and West (24) have suggested that  $GA_5$  is a normal intermediate in the biosynthesis of  $GA_1$  in corn. This hypothesis has received support by circumstantial evidence obtained by Jones (8) and Brian *et al.* (2). Similarly, Macmillan *et al.* (20) suggested that  $GA_5$  was a possible intermediate in the biosynthesis of gibberellins  $A_1$ ,  $A_3$ ,  $A_8$ , and  $A_6$ .

In order to test the hypothesis that  $GA_5$  is an intermediate in the biosynthesis of  $GA_1$  in peas, the following experiments were performed. Four-day-old, light-grown normal pea seedlings were transferred to plastic boxes containing a mixture of half-strength Hoagland and AMO1618 (200 mg/l). Six days following treatment with AMO1618, 0.05  $\mu g$   $GA_5$  was applied to the apical bud of each

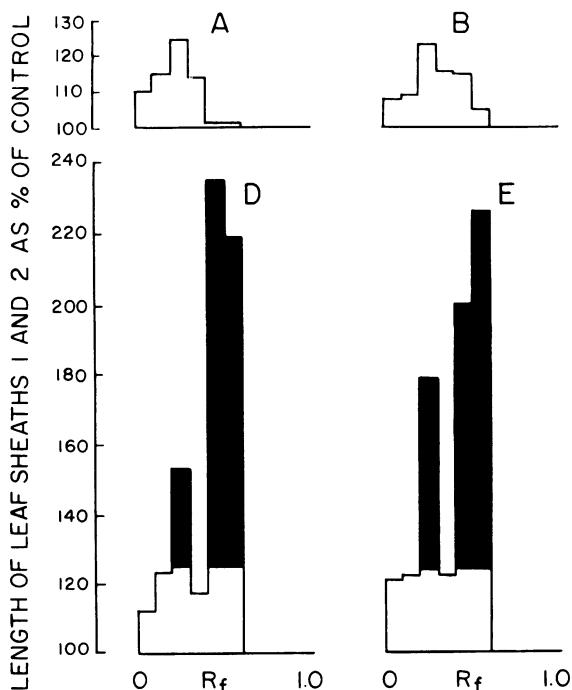


FIG. 3. Extracts from normal, light-grown pea seedlings treated with AMO1618 (A,B) and with both AMO1618 and  $GA_5$  (D,E). Samples taken 24 hours (A,D) and 48 hours (B,E) following  $GA_5$  application. Bioassay,  $d_5$  corn. TLC solvent, chloroform: ethyl acetate: acetic acid (60:40:5 v/v). Base of darkened area indicates significant biological activity at the 5% level of risk.

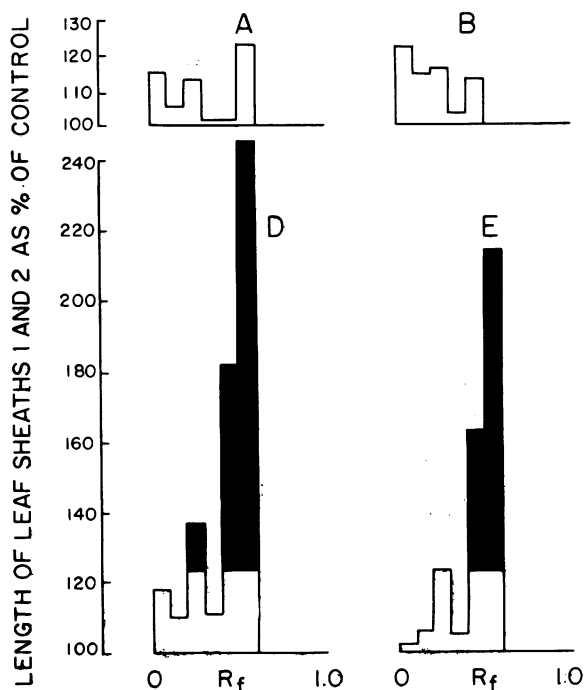


FIG. 4. Extracts from normal, dark-grown pea seedlings treated with AMO1618 (A,B) and both AMO1618 and  $GA_5$  (D,E). Samples taken 24 hours (A,D) and 48 hours (B,E) following  $GA_5$  application. Bioassay,  $d_5$  corn. TLC solvent, chloroform: ethyl acetate: acetic acid (60:40:5 v/v). Base of darkened area indicates significant biological activity at the 5% level of risk.

seedling. Plants were harvested 24 and 48 hours following the application of  $GA_5$ , frozen with liquid nitrogen, lyophilized and extracted as described in the Methods section. Bioassay of the extracts with  $d_5$  dwarf corn seedlings following chromatography in chloroform/ethyl acetate/acetic acid (60:40:5, v/v) indicated 2 distinct regions of growth promotion, 1 corresponding to the position of applied  $GA_5$ , the other to that of  $GA_1$  (fig 3). Seedlings treated with AMO1618 alone possessed no significant levels of extractable gibberellin (fig 3). Also, chromatography of the  $GA_5$  sample applied to the seedlings followed by bioassay indicated only 1 zone of growth promotion, at the  $R_F$  of  $GA_5$ .

These experiments were repeated using dark-grown seedlings and the results are shown in figure 4. Although there is no qualitative difference in the results obtained from the experiments with light or dark-grown seedlings, there appears to be a quantitative difference. This feature was consistent when the experiments were repeated, and can be best expressed in terms of the amount of  $GA_5$  converted to the  $GA_1$ -like component. In 3 experiments using light-grown seedlings the range of conversion of  $GA_5$  to  $GA_1$  was between 10 to 19%, whilst in a similar number of experiments using dark-grown seedlings the range was 4 to 8%.

## Discussion

Our experiments have yielded 2 principal results: 1) Pea seedlings contain—as has been shown before—2 kinds of extractable, acidic gibberellins, 1 similar to GA<sub>1</sub> and the other to GA<sub>5</sub>, but only 1 of these, namely, the GA<sub>1</sub>-like factor, can be obtained by diffusion; 2) there is no quantitative difference in the levels of either extractable or diffusible gibberellins obtained from seedlings of a normal (tall) and a dwarf cultivar, and in neither case was there a difference whether the plants had been grown in the dark or in the light.

The former finding is undoubtedly surprising but it underlines the experience, long known in work on auxin, that extraction procedures alone may give a 1-sided and, hence, insufficient picture of the hormone status of a plant.

Several explanations can be suggested for the absence of GA<sub>5</sub> in the diffusates from pea shoot tips. GA<sub>5</sub> may be unable to diffuse through pea stem tissue; it may be present in a bound, non-mobile form; it may be inactivated at the cut surface of the diffusing organ or in the receiver agar block; it may be a non-mobile precursor of GA<sub>1</sub>.

All of these possibilities were tested but positive evidence was found only for the latter: after application of GA<sub>5</sub> to pea seedlings a certain amount of activity was found at an R<sub>F</sub> characteristic for GA<sub>1</sub>. Thus, it is possible that GA<sub>5</sub> is a natural precursor of GA<sub>1</sub> in the pea plant. Such a conclusion would be in agreement with existing, although circumstantial evidence in literature that GA<sub>5</sub> may be a precursor of GA<sub>1</sub> (and other gibberellins) (2, 8, 20, 24). It is also supported by the fact that the observed appearance of GA<sub>1</sub> after application of GA<sub>5</sub> took place in pea seedlings treated with AMO1618. This growth retardant has been shown to inhibit gibberellin biosynthesis in the fungus *Fusarium moniliforme* (14), in developing pea seeds (1), in the endosperm of *Echinocystis macrocarpa* (3), and in seeds of *Pharbitis nil* (26). Dennis *et al.* (3) have shown that the inhibition occurs at the point of cyclization of transgeranylgeraniol pyrophosphate to (-)-kaurene. It therefore seems unlikely that GA<sub>1</sub> extracted from pea seedlings treated with both GA<sub>5</sub> and AMO is a product of the biosynthetic machinery of the plant for gibberellins, operating on residual gibberellin precursors present in the plant. Seedlings treated with AMO alone did not yield GA<sub>1</sub> or any other gibberellin-like material whatsoever.

However, it must be emphasized that the quantitative aspects of this interpretation require further investigation. The amounts of GA<sub>5</sub> applied to the plants in the conversion experiments were much higher (about 100×) than the amounts that can be extracted from the same tissue. On the other hand, the conversion factor was not very high, maximally 19% after 48 hours. It is possible that

the enzymatic system which would be responsible for the conversion was overloaded and that the conversion factor is much higher when only endogenous GA<sub>5</sub> is available as substrate. But it also cannot be ruled out that even if endogenous conversion of GA<sub>5</sub> to GA<sub>1</sub> is occurring in pea plants it is not the sole or main pathway of synthesis of the latter gibberellin. It may be possible to obtain a more precise and quantitative answer to this question by the use of labelled GA<sub>5</sub>; it is hoped that such experiments can be carried out in near future.

Our results on the quantities of extractable and diffusible gibberellins in pea seedlings are in agreement with those of the extraction experiments of Kende and Lang (13). Lockhart (18, 19) has suggested that the effect of light on growth in peas, and its reversal by applied gibberellin, may be explained in 3 ways: reduction of gibberellin synthesis in the plant; enhancement of gibberellin destruction in the plant; reduction of the responsiveness of the tissue to gibberellin. The results of Kende and Lang (13) argue against the former 2 possibilities and in favor of the third, and our results support this general conclusion.

Kende and Lang (13) had specifically shown that it is the response of the pea seedlings to GA<sub>5</sub> or the endogenous GA<sub>5</sub>-like factor that is affected by light, the response to GA<sub>1</sub> and the GA<sub>1</sub>-like endogenous gibberellin being the same in light and dark. If GA<sub>5</sub> were the sole or principal precursor of GA<sub>1</sub> in the plant and if the conversion were reduced by light this could offer an explanation for the findings of Kende and Lang. However, as already emphasized, it is premature to attribute to GA<sub>5</sub> a role as the predominant GA<sub>1</sub> precursor in pea seedlings. In this context it must be noted that the observed conversion of applied GA<sub>5</sub> to GA<sub>1</sub> was, if anything, greater in the light than in the dark (10–19 versus 4–8%). Moreover, if conversion of physiologically inactive GA<sub>5</sub> to active GA<sub>1</sub> were the critical event one would expect that the GA<sub>1</sub> content of the seedlings would be lower in the light than in the dark; this, however, was clearly not the case. Thus, our results, while supporting the general explanation proposed by Kende and Lang (13), do not provide a more specific explanation, and further studies are necessary.

Our data are in disagreement with those of Kohler (15, 16, 17) who reports that light- and dark-grown dwarf pea seedlings, dark-grown normal seedlings, and light-grown but retardant-treated normal seedlings have identical endogenous gibberellin levels but that the level in light-grown normals is more than 10 times as high. He explains the ability of the normals to make relatively good growth in light with an enhancement of gibberellin synthesis by light. We have no explanation for this discrepancy, and a comparison is difficult as Kohler expresses his results on a *per* seedling basis and does not give data on a weight

basis. It should also be borne in mind that all data of Kohler refer to the GA<sub>5</sub>-like material; he was unable to obtain significant activities in the fractions which should contain the GA<sub>1</sub>-like factor. With our methods we have never experienced such a problem. However, it may be pointed out that Kohler (15) cut his seedlings above the substrate and apparently extracted them *in toto*, that is, he determined the gibberellin content of normal seedlings that (according to our experience) were about 20 to 25 cm tall, and of dwarf seedlings only about 4 to 5 cm tall. It seems possible, assuming extractable gibberellins are rather uniformly distributed throughout the shoot of pea seedlings (see Radley, 25) that if the gibberellin content were expressed on a weight basis the difference between normals and dwarf seedlings would be greatly reduced.

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