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Kinetics of Growth Retardant and Hormone Interactions in Affecting Cucumber Hypocotyl Elongation[1]

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Summary. The capacities of indole-3-acetic acid (IAA) and gibberellin A_3 (GA_3) to counteract the inhibitory effects of (2-chloroethyl) trimethylammonium chloride (CCC), 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride (Amo-1618), and N,N-dimethylaminosuccinamic acid (B-995) on hypocotyl elongation in light-grown cucumber (*Cucumis sativus* L.) seedlings were investigated. One μg of GA_3 applied to the shoot tip was sufficient to completely nullify the effect of 10 μg of Amo-1618 or 25 μg of B-995 applied simultaneously to the shoot tip, and 10 μg of GA_3 completely counteracted the effect of 10^{-3} M CCC added to the root medium. One μg of IAA counteracted the effect of 10^{-3} M CCC in the root medium, but IAA did not nullify the action of either Amo-1618 or B-995. Experiments were conducted using 2 growth retardants simultaneously, which indicated that Amo-1618 and CCC inhibit a common process, namely GA biosynthesis, essential to hypocotyl elongation. However, since the effect of CCC was overcome by applications of both GA and IAA, growth retardation resulting from treatment with CCC apparently is not due solely to inhibition of GA biosynthesis. B-995 did not interact additively with either Amo-1618 or CCC, which suggests that B-995 affects a process different from those affected by the other 2 retardants. Thus, while inhibition evoked by B-995 is reversible by applied GA, the action of B-995 does not appear to be inhibition of GA biosynthesis.

Several synthetic plant growth regulators characterized by their capacity to inhibit growth without evoking severe morphological abnormalties and which are termed growth retardants (4) have been investigated extensively in recent years. A feature which appears to be common to the biochemical modes of action of the growth retardants is interference with hormone metabolism. Amo-1618 (2, 6, 16, 24), Phosphons (6), and CCC (13, 16, 19, 27) all reportedly inhibit gibberellin biosynthesis, with CCC apparently acting at a different site in the pathway of gibberellin biosynthesis than Amo-1618 and the Phosphons (1, 6, 13). Considerable evidence is reported that CCC may also affect auxin metabolism (5, 10, 17, 20). The mode of action of B-995 and chemically related hydrazine growth retardants is quite incompletely understood. B-995 reportedly does not inhibit gibberellin biosynthesis (6, 19), but some evidence has been reported that B-995 and other hydrazine retardants may influence auxin metabolism (7, 10, 22, 23).

A popular method for testing the possible interference of growth retardants with endogenous auxin or gibberellin is to apply varying amounts of an auxin or a gibberellin to plants in the presence and absence of a standard dose of a retardant (18). Such experiments have revealed that the effects of several retardants on whole plants, including C-011, CCC, Phosphon, Amo-1618 and B-995 (1, 3, 4, 9, 10, 14, 18, 26, 27, 28) are readily counteracted with applied gibberellin. However, experiments with excised plant parts often have yielded evidence that only auxin, or neither auxin nor gibberellin, counteracted the effects of growth retardants (5, 17, 25). Thus additional direct biochemical evidence and evidence from investigations of the kinetics of growth retardant effects in whole plant systems will be necessary to fully elucidate the modes of action of the growth retardants.

At the present state of our knowledge, it would seem that considerable valuable information may yet be gained from investigations of growth retardant and hormone interactions in appropriate intact plants. Much of the previous work has been hindered in some respects by the use of excised plant material which may contain little auxin and gibberellin and perhaps be incapable of hormone biosynthesis. Work with intact plants

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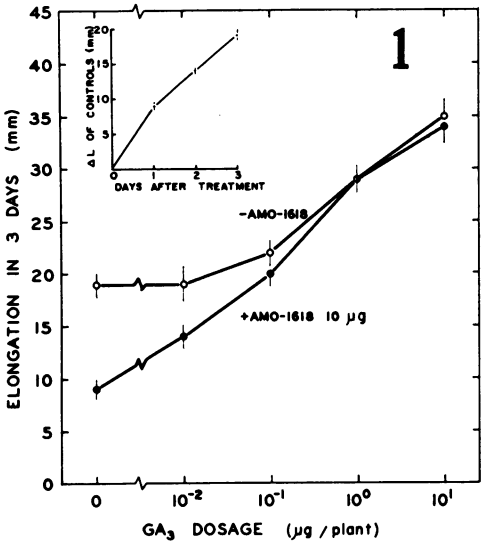
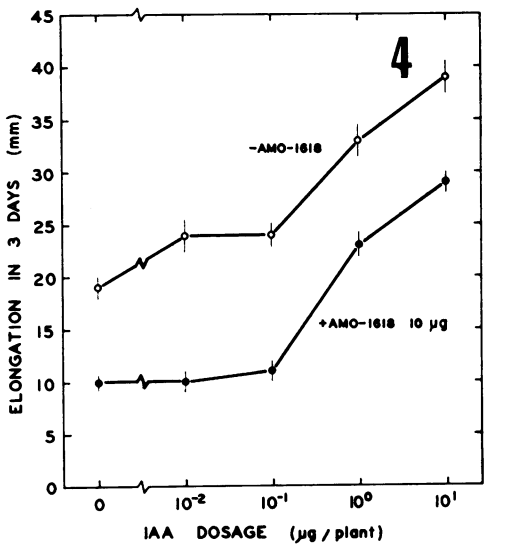
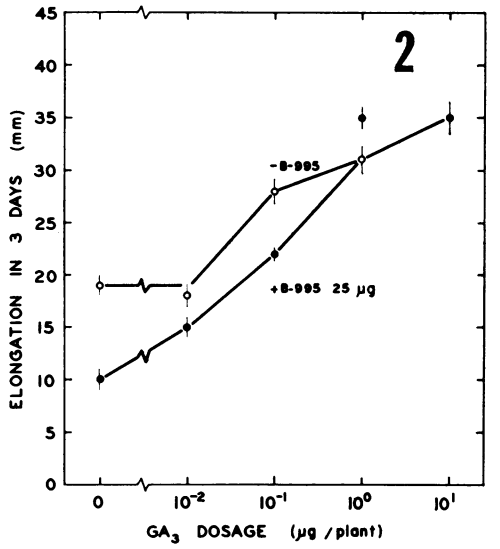
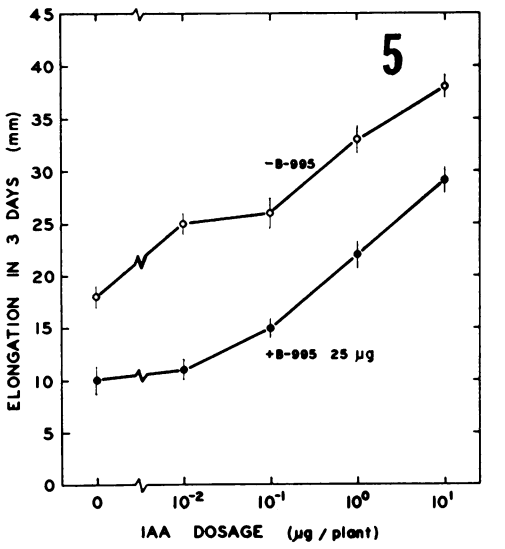
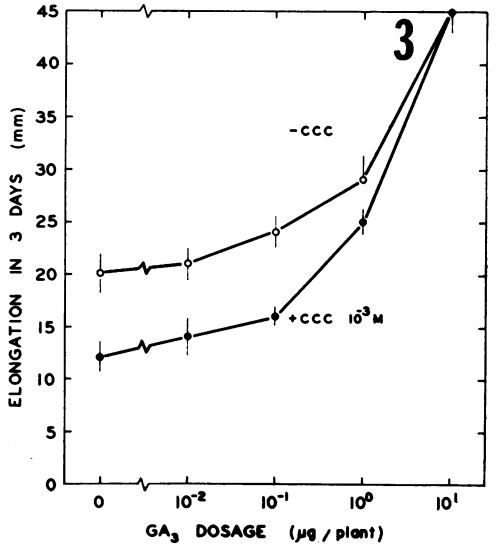
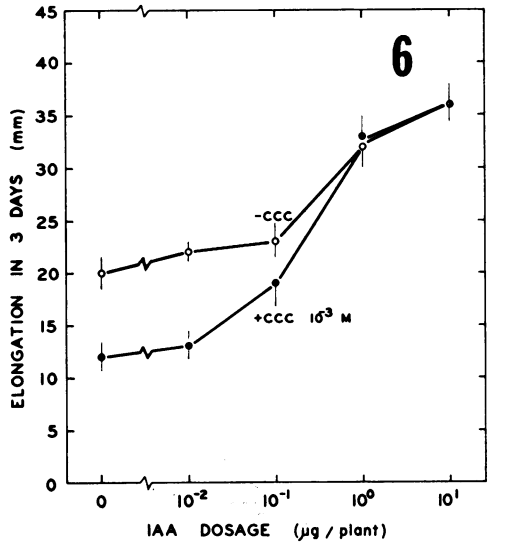

1
ELONGATION IN 3 DAYS (mm)
GA3 DOSAGE (µg / plant)
-AMO-1618
+AMO-1618 10 µg
ΔL OF CONTROLS (mm)
DAYS AFTER TREATMENT


4
ELONGATION IN 3 DAYS (mm)
IAA DOSAGE (µg / plant)
-AMO-1618
+AMO-1618 10 µg


2
ELONGATION IN 3 DAYS (mm)
GA3 DOSAGE (µg / plant)
-B-995
+B-995 25 µg


5
ELONGATION IN 3 DAYS (mm)
IAA DOSAGE (µg / plant)
-B-995
+B-995 25 µg


3
ELONGATION IN 3 DAYS (mm)
GA3 DOSAGE (µg / plant)
-CCC
+CCC 10-3 M


6
ELONGATION IN 3 DAYS (mm)
IAA DOSAGE (µg / plant)
-CCC
+CCC 10-3 M

likewise has sometimes been handicapped by a lack of responsiveness of the plants to applied auxin. Hence the report by Katsumi et al. (14) that hypocotyl elongation in intact cucumber seedlings was promoted by 6 auxins as well as by 2 gibberellins brought to attention what appears to be an ideal plant material for certain types of investigations on growth retardant and hormone interactions. The utility of cucumber seedlings in growth regulator experiments had been recognized earlier (9, 10, 11, 12), but their responsiveness to applied auxin apparently had not been reported. Results of 2 types of investigations are described in this paper: A) the capacities of IAA and GA_3 to counteract the effects of CCC, Amo-1618 and B-995 on hypocotyl elongation; and B) the kinetic interactions of 2-growth retardants applied simultaneously on hypocotyl elongation.

Materials and Methods

General procedures for culturing and handling plants were patterned after those of Katsumi et al. (14). Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee Seed Company) were soaked for 2 to 3 hours in distilled water. Then seeds were planted in plastic containers filled with equal volumes of vermiculite, and the seedlings were cultured in growth chambers programmed to provide a 16-hour photoperiod at 30 ± 1 C, alternating with an 8-hour dark period at 27 ± 1 C. Cool white fluorescent and incandescent lamps provided a light intensity at plant level of approximately 600 ft-c during the photoperiod. Measured volumes of complete mineral nutrient solution were used consistently to moisten the vermiculite. When growth retardant was added to the root medium, the chemical was prepared in nutrient solution. Routinely, 500 ml of nutrient solution were added to each planter at the time the seeds were planted. When additional nutrient solution was required before the time of treatment, an equal volume of solution was added to each planter. Ordinarily it was necessary to add 100 ml of nutrient solution at 2-day intervals prior to the time of treatment.

Five days after planting, at which time the hypocotyls were 2.5 to 3.0 cm in length, the seedlings were thinned to leave 12 to 15 uniform seedlings in each container. The hypocotyl of each seedling was marked with India ink at the cotyledonary node and at a distance 2 cm below the cotyledonary node, the designated segment being hereafter called a hypocotyl unit. The plants were treated immediately and returned to the growth chambers for an additional 3 days, at the end of which time lengths of the hypocotyl units were measured.

In some experiments 1 growth retardant was added to the root medium while another retardant, IAA, or GA_3 was added to the shoot tips. When a growth retardant solution was added to vermiculite it was prepared immediately before use by dissolving the retardant in complete mineral nutrient solution. Then either 250 ml of growth retardant solution or 250 ml of plain nutrient solution were added to each container. No further additions to the vermiculite were needed during a period of 3 days. Growth retardants to be applied to shoot tips were prepared in distilled H_2O to which was added 0.05 % (v/v) of Tween 20, and a single 10 µl aliquot was added to each shoot tip. Ten µl of 0.05 % Tween 20 solution were applied to seedlings not receiving growth retardant. GA and IAA solutions were prepared in 25 % ethanol-0.05 % Tween 20, and 10 µl were applied to each seedling shoot tip.

Various temperature and light intensities were tested in addition to the standard conditions described above. However, the standard conditions were optimal among those tested for promoting rapid development of seedlings and a nearly constant rate of hypocotyl elongation during a 3-day period following treatment. Under the standard conditions the cotyledons were mature and the blade of the first true leaf was approximately 1 cm long at the end of the experiments.

The growth regulators used in the experiments and the sources from which they were obtained were: A) potassium salt of gibberellic acid (GA_3) ("Gibrel" of Merck and Company), which is 81 % KGA_3 containing a trace (<5 %) of GA_1; B)

FIG. 1. Interaction between gibberellin and Amo-1618 in affecting elongation of hypocotyl units. Both chemicals were applied simultaneously to shoot tips. Insert illustrates a representative growth curve for control hypocotyl units for the experimental period. Data denote the changes in length of hypocotyl units which were 2 cm long at the time of treatment.

FIG. 2. Interaction between gibberellin and B-995 in affecting elongation of hypocotyl units. Both regulators were applied simultaneously to shoot tips.

FIG. 3. Interaction between gibberellin and CCC. The vermiculite was saturated with 10^{-3} M CCC solution or mineral nutrient solution, and gibberellin was applied to the shoot tips.

FIG. 4. Interaction between auxin and Amo-1618 in affecting elongation of hypocotyl units. Both chemicals were applied to shoot tips.

FIG. 5. Interaction between auxin and B-995. Both chemicals were applied to shoot tips.

FIG. 6. Interaction between auxin and CCC. The root medium was saturated with 10^{-3} M CCC solution or mineral nutrient solution, and IAA was applied to the shoot tips.

indole-3-acetic acid (IAA) (Mann Research Laboratories); C) N,N-dimethylaminosuccinamic acid (B-995) (technical grade "Alar" of Naugatuck Chemical Division of U. S. Rubber Company); D) 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride (Amo-1618) (Enomoto and Company); and E) (2-chloroethyl) trimethylammonium chloride (CCC) ("Cycocel" of American Cyanamid Company).

The data presented in the graphs are, in each case, from single representative experiments selected from a total of more than 24 experiments. Each mean, representing the change in length of 2-cm hypocotyl units in 3 days, is based on 10 to 15 plants, and the values plotted are the means plus and minus the standard errors of the means.

Results

Interactions of IAA and GA_3 with Growth Retardants. At dosages of 1 μg or more per plant, GA_3 completely counteracted the growth-retarding action of 10 μg of Amo-1618 (fig 1) and 25 μg of B-995 (fig 2) on elongation of the hypocotyl units when standard doses of these 2 retardants were applied simultaneously with varying doses of GA_3 to the shoot tips. Ten μg of GA_3 applied to the shoot tip nullified the inhibitory influence of 10^{-3} M CCC added to the root medium (fig 3). The greater response of hypocotyl units to 10 μg of GA_3 in figure 3 than in figures 1 and 2 is attributable to a somewhat higher growth potential in the seedlings used for the experiment described in figure 3. That the relationship between the dose-response curves depicted in figure 3 is fully reproducible was confirmed in a duplicate experiment, wherein it was found that the dose-response curves again converged at a 10 μg dosage of GA_3, even though the maximum change in length of hypocotyl units was only 3 cm. The insert in figure 1 illustrates the growth curve for untreated hypocotyl units in a representative experiment. The growth rate of the control hypocotyl units was nearly constant over the 3-day period following treatment.

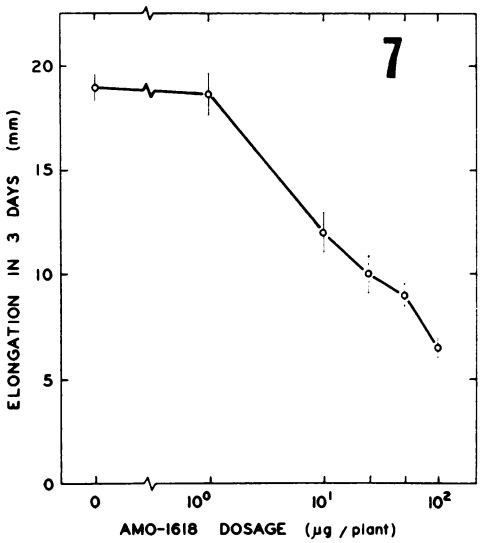
In contrast to the effect of GA_3 in counteracting the effects of all 3 growth retardants, IAA nullified only the effect of CCC (fig 6). Doses of 1 or 10 μg of IAA applied to the shoot tips completely counteracted the retarding effect of 10^{-3} M CCC in the root medium. The curves for plants treated with 10 μg of Amo-1618 with and without varying dosages of IAA (fig 4) and 25 μg B-995 with and without IAA (fig 5) show no tendency toward convergence at higher dosages of IAA.

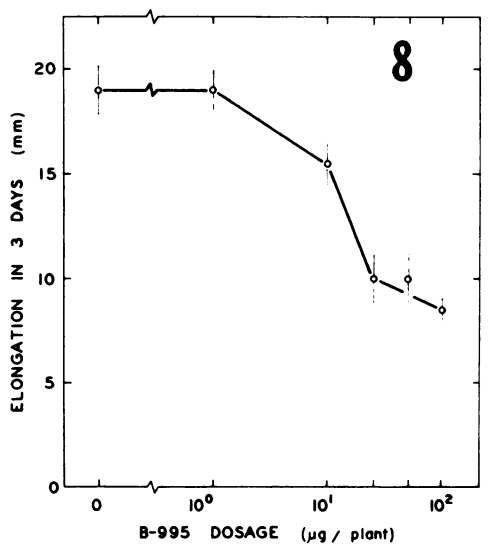
The selection of standard dosages of growth retardants to be used in the experiments described previously, as well as in those experiments to be described later, was based on preliminary dose-response experiments. The objective in selecting standard dosages was to discover dosages which would evoke approximately 50 % inhibition of hypocotyl unit elongation. From the data presented in figure 7, a standard dosage of 10 μg of Amo-1618 was selected, and in the case of B-995, a standard dosage of 25 μg was chosen (fig 8). As is apparent in figures 1 and 2, 4 and 5 and 7 through 11, these dosages of Amo-1618 and B-995 were about equally effective in causing approximately 50 % inhibition of hypocotyl elongation in the absence of GA_3 or IAA. Illustrative dose-response curves for CCC may be seen as the uppermost curves in figures 9 and 10. From such dose-response curves, 10^{-3} M CCC was selected as a standard concentration of solution of this retardant to be used in experiments where the interaction of CCC with varying amounts of another growth regulator was to be investigated.

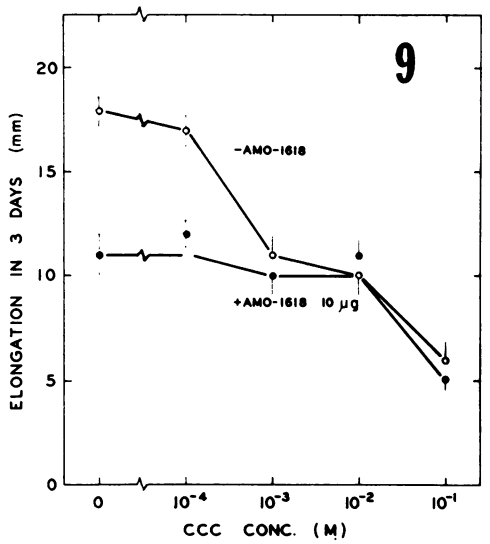
Growth Retardant Interactions. The hypothesis was formulated that it might be possible to determine whether 2 growth retardants inhibit the same or different processes essential to growth by applying 2 retardants simultaneously and observing the kinetics of their interaction in inhibiting hypocotyl elongation. Accordingly, interactions among CCC, Amo-1618 and B-995 in retarding elongation of cucumber hypocotyl units were investigated. When plants were treated with different concentrations of CCC solution added to the root medium simultaneously both with and without a standard dose of 10 μg of Amo-1618 applied to the shoot tips, it was found that the inhibition curves converged at 10^{-2} M CCC and remained essentially coincident at 10^{-1} M CCC (fig 9). Thus, according to the stated hypothesis, it would appear that Amo-1618 and CCC inhibit a common process essential to growth. It should be noted in this regard that 10^{-1} M CCC was a highly toxic concentration which caused yellowing and marginal necrosis of the cotyledons and injury to the shoot tips. Hence in the presence of 10^{-1} M CCC hypocotyl growth totally ceased within a brief time ($<$24 hours) after application of the chemical. No other concentration of CCC or any other growth retardant used evoked apparent toxicity symptoms.

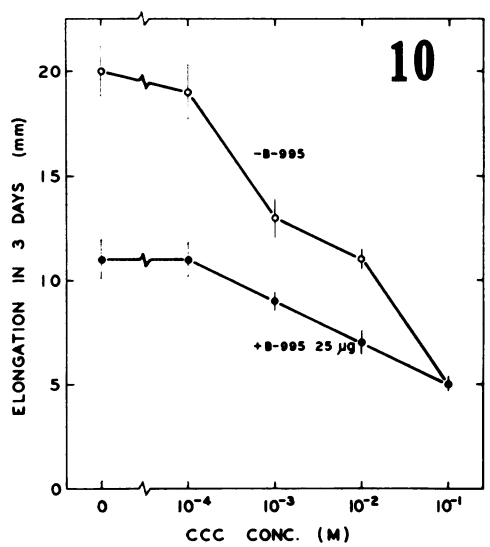
When parallel experiments were performed with a standard dosage of 25 μg of B-995 and varying concentrations of CCC, the curves converged only at the highest concentration of CCC (fig 10), which result, according to the stated hypothesis, would indicate that B-995 and CCC do not inhibit a common process essential to hypocotyl extension.

Finally, experiments using a standard dosage of Amo-1618 and variable levels of B-995 added to the root medium were performed. Convergence of the curves occurred only at 10^{-1} M B-995 which practically totally prevented extension of the hypocotyl units (fig 11). Thus, the results of experiments using 2 growth retardants simultaneously indicated that CCC and Amo-1618 probably inhibit a common process involved in hypocotyl elongation, whereas B-995 inhibits a different process.









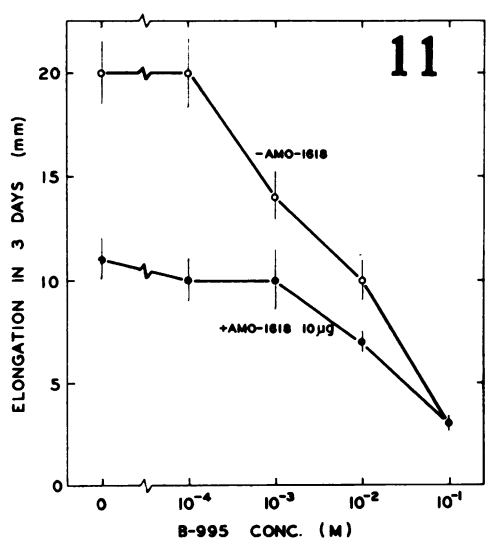


FIG. 7. Dose-response curve for cucumber seedlings treated with varying dosages of Amo-1618 on the shoot tips.

FIG. 8. Dose-response curve for cucumber seedlings treated with varying dosages of B-995 on the shoot tips.

FIG. 9. Interaction between Amo-1618 and CCC in affecting elongation of hypocotyl units. CCC was added to the root medium, and Amo-1618 was applied on the shoot tips.

FIG. 10. Interaction between B-995 and CCC. CCC was added to the root medium, and B-995 was applied on the shoot tips.

FIG. 11. Interaction of Amo-1618 and B-995 in affecting hypocotyl unit elongation when B-995 was added to the root medium and Amo-1618 was applied to the shoot tips.

Discussion

Confirming the earlier report by Katsumi et al. (14), it has been shown that hypocotyl elongation in seedlings of the National Pickling variety of cucumber (*Cucumis sativus* L.) is promoted by both IAA and GA_3. Thus cucumber seedlings are convenient for use in investigations of the capacities of auxins and gibberellins to overcome inhibitory effects of growth retardants in intact plants.

The work of Katsumi et al. (14, 15) substantiated that both auxin and gibberellin are essential for normal hypocotyl elongation in light-grown cucumber seedlings. Thus growth, a gross process, obviously is dependent upon a number of partial processes, including auxin and gibberellin biosynthesis, both of which processes conceivably may be inhibited by growth retardants. These considerations led to the formulation of the hypothesis that it might be possible to determine whether 2 growth retardants inhibit the same or different partial processes essential to growth by a type of experiment in which 2 growth retardants are applied simultaneously. To test this hypothesis, cucumber seedlings were treated with varying levels of 1 retardant in the presence and absence of a standard, sub-maximally effective dose of a second retardant. The expected result from such experiments is that the 2 dose-response curves (with and without the growth retardant used at a standard dosage) would converge at a sub-maximally effective level of the variable retardant, if the 2 retardants inhibit a common process on which growth is dependent. This would be true since the effects of the 2 chemicals would be additive. On the contrary, if the 2 retardants used in such an experiment affect different processes, the curves should only intersect at a concentration of the variable retardant which essentially stops growth. The latter would be expected since if any partial process which is truly essential to growth is totally blocked, the influence of a retardant affecting some other process would not be discernible.

The results of experiments conducted to test the above hypothesis suggest that Amo-1618 and CCC do in fact inhibit a common process essential to hypocotyl elongation, although their modes of action are not strictly identical. So far as the mode of action of Amo-1618 is concerned, the data here reported corroborate the conclusion of other authors that growth retardation evoked by Amo-1618 results from inhibition of GA biosynthesis. Clearly, Amo-1618 does inhibit gibberellin biosynthesis in *Fusarium moniliforme* (16, 24), developing pea seeds (2), and also in *Echinocystis macrocarpa* endosperm-nucellus, since it inhibits the formation of kaurene from mevalonate in the latter (6). None of the growth retardants apparently interferes with the GA stimulation of α-amylase in barley endosperm (21). In addition to the direct evidence for interference by Amo-1618 with gibberellin biosynthesis, there are several reports describing reversal of Amo-1618 inhibition by applied gibberellin in intact plants (e.g., 1, 9, 10, 14). Not to be ignored, however, are investigations with excised plant parts and callus tissues which have revealed that exogenous GA did not reverse Amo-1618 inhibition. For example, Cleland (5) found that GA did not reverse the inhibition of growth of *Avena* leaf sections caused by Amo-1618 whereas IAA partially reversed the inhibitory effect of Amo-1618. Sachs and Wohlers (25) reported that neither GA nor supplementary auxin was effective in reversing Amo-induced inhibition of growth in various callus tissues in vitro, and they concluded that the effect of Amo-1618 cannot be simply one of inhibiting gibberellin (or auxin) biosynthesis. Additional data which are difficult to reconcile with the mode of action being entirely inhibition of GA biosynthesis are reported by Halevy (8, 9, 10) who found that Amo-1618, and CCC and B-995 as well, stimulated peroxidase and IAA oxidase activity in preparations of cucumber seedlings.

CCC and Amo-1618 interacted additively in evoking retardation of cucumber hypocotyl elongation, and inhibition by CCC was readily overcome by applied GA. These findings are interpreted as evidence that CCC also inhibits GA biosynthesis. However, inhibition of hypocotyl elongation resulting from treatment with CCC apparently is not due solely to inhibition of GA biosynthesis since the effect of CCC on hypocotyl extension is overcome as readily by applied IAA as by applied GA. It has been reported previously that CCC and some analogs of the compound inhibit GA biosynthesis in *Fusarium moniliforme* (13, 16, 19) and in *Pharbitis* (27). And reversal of the effect of CCC on stem elongation in intact plants by exogenous GA has been noted (1, 18, 26, 27). The available evidence suggests that CCC and Amo-1618 act at different sites in the pathway of gibberellin biosynthesis (1, 6, 13). Yet, as in the case of Amo-1618, CCC-induced inhibition of growth of excised pea stem sections, *Avena* coleoptile sections, *Raphanus* leaf disks (17), *Avena* leaf sections (5) and callus tissues (25) was not reversed by GA. Other reported evidence, with which the present report tends to agree, suggests that CCC may interfere with endogenous auxin in some way. Auxin (IAA) was effective in overcoming the growth inhibitory effect of CCC on *Avena* coleoptile sections and pea stem sections (17), for example. Furthermore, Norris (20) has reported recently that CCC caused a reduction in the levels of both tryptophan and auxin in wheat seedlings, suggesting that CCC effects may be due to alterations of indole compound metabolism. Compatible with this hypothesis is the report of Kuraishi and Muir (17) who found that treatment of pea seedlings caused a reduction in the amount of diffusible auxin obtainable from the stem apices.

The mode of action of B-995 is perhaps even less completely understood than that of CCC, and

the results of the present investigation contribute little toward clarifying the matter. The fact that the effect of B-995 on hypocotyl extension was readily overcome by applied GA might be considered evidence that B-995 inhibits GA biosynthesis. However, this is an improbable conclusion in view of the fact that B-995 failed to interact additively with either Amo-1618 or CCC. Furthermore, Ninnemann et al. (19) reported that B-995 failed to inhibit GA biosynthesis in *Fusarium*. Dennis et al. (6) concluded that B-995 did not inhibit the formation of kaurene from mevalonate in preparations of *Echinocystis macrocarpa* endosperm-nucellus, even though their data reflect a possible inhibitory effect with the highest concentration of B-995 that they used. Significantly, the chemically related hydrazine retardant β-hydroxyethylhydrazine (BOH) was not effective in inhibiting kaurene production (6).

Some positive effects of B-995 and related retardants on the metabolism of indole compounds have been reported. These effects include enhancement of enzymatic destruction of IAA by BOH (7) and B-995 (10) and inhibition of enzymatic oxidation of tryptamine to indoleacetaldehyde by BOH and B-995 (22, 23). Thus some evidence exists that B-995 may interfere with auxin metabolism. Yet, in the present work IAA failed to reverse the effect of B-995 on cucumber hypocotyl extension, whereas GA was effective. These results agree with the report by Zeevaart (28) that GA_3, but not IAA or naphthaleneacetic acid, overcame the inhibitory effect of B-995 on stem growth and flower formation in *Pharbitis*, and also the report by Bukovac (3) that GA_3 reversed the inhibition of stem growth evoked by C-011. Additional work obviously will be required to elucidate completely the mode of action of hydrazine growth retardants. It appears from the present investigation that B-995 may affect a process essential to growth which is different from those processes affected by Amo-1618 and CCC, and that the action of applied GA in overcoming the effect of B-995 on growth may be indirect.

Obviously full understanding of the modes of action of growth retardants will require direct biochemical investigations combined with kinetic analyses of growth regulator interactions in intact plants. Cucumber seedlings would seem to be nearly ideal material for both kinds of experiments, particularly since hypocotyl growth in the intact seedlings is stimulated by both applied auxin and gibberellin. The procedure described for testing whether 2 retardants affect common or different processes also should prove useful in future preliminary attempts to determine the process essential to growth which is affected by a particular new retardant by testing its interaction with a retardant the mode of action of which is known.

Acknowledgments

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