

Kinetic Studies of Certain Anti-Gibberellins^{1, 2}

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A number of chemical compounds which have the specific effect of retarding stem elongation have been described in recent years (11, 13, 15). Compounds of several rather distinct chemical classes have been found which give the same general growth responses with various plant species. These compounds include 2-chloroethyl trimethyl ammonium chloride (CCC), 2,4-dichlorobenzyl-tributylphosphonium chloride (Phosfon-D), allyl trimethylammonium bromide (AMAB), and 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine-carboxylate methyl chloride (AMO-1618). The most striking symptoms shown as a result of treatment with these compounds are a marked decrease in stem and petiole elongation. These compounds exert relatively little influence on leaf expansion or root development except at high doses. The marked specificity of action of these compounds on stems and petioles not only suggests immediate practical applications but interesting physiological questions as well.

Many authors have noted that responses to these growth inhibitors tend to be just the opposite of responses to gibberellin (1, 16). Apparent interactions between gibberellin and certain of these growth inhibitors have been observed in bean internode growth (3), Ulothrix growth (2), cell division in *Chrysanthemum* (12), etc.

The present work was undertaken to establish definitely whether a functional (i.e. competitive) interaction relationship existed between gibberellin and typical growth retarders or whether gibberellin and these growth retarders act independently, although in opposite directions. An outline of the kinetic basis of these interpretations is presented in the Discussion. Further characteristics of the physiology of action of these materials are also described.

Methods & Materials

Seeds of *Phaseolus vulgaris*, L. cultivar Pinto (Pomona Feed & Seed Co., Pomona, Cal.) were sown in No. 2 tinned steel cans or in 4 inch unglazed clay pots. They were grown in a mixture of equal

parts soil, black sand, and vermiculite, in a conventional greenhouse. Treatments were generally begun when the first trifoliolate leaf was approximately one-third expanded, when the plants were 10 to 11 days old.

Ten plants were normally used per experimental treatment. Plants were randomized on the greenhouse bench and widely spaced to avoid excessive shading. For kinetic analysis the plants were moved daily to a different relative position on the bench according to a standardized program. This procedure seemed to reduce substantially the variability within treatments but this observation was not directly tested.

The growth inhibitors, Phosfon-D and CCC, were applied as soil drenches at the beginning of the experiment. The usual dose of Phosfon-D was 0.01 or 0.005 g (i.e., 0.1-0.05 g 10 % formulation) per pot in 5 to 10 ml water and that of CCC was 25 ml of a 2 % solution (made from a 50 % formulation giving a 1 % solution of active chemical). Phosfon-D was obtained through the courtesy of Dr. S. L. Felton of Virginia-Carolina Chemical Co. and CCC through the courtesy of Dr. R. E. Deems of the American Cyanamid Co. Maleic hydrazide (Nutritional Biochemicals Co.) was sprayed on the leaves at concentrations of 0.3 to 1.0 g/liter.

The gibberellin treatments were given as 4 μ l ethanolic drops of the appropriate gibberellin A₃ solutions. Gibberellin A₃ was obtained through the courtesy of Merck and Co., Rahway, N. J. and E. Lilly and Co., Indianapolis, Ind. The plants were normally treated once and growth measurements were begun at once. The height of the plants from soil level to the stem apex was measured on alternate days for 6 days. In all cases growth was linear with time between the 2nd and 6th day after treatment. Growth rates were calculated from the height of the plants on the 2nd and 6th day following treatment. The variabilities given are the standard deviations of the means.

Results

► Site of Action of Growth Inhibitors: Gibberellin is active in the plant stem and probably is synthesized in the stem tip (9) or in adjacent regions. Since it will be shown below that Phosfon-D is an anti-gibberellin, it would appear probable that Phosfon

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also must act near the stem tip or in the elongating region of the stem. However, since the site of production of gibberellin has not been reported for light-grown plants and since it may be of interest from other points of view, experiments were undertaken to determine the region of the plant in which Phosfon-D is active. The fact that these inhibitors seem most effective when applied to the soil at first suggested an effect on the root system. Assuming the roots to be the site of activity, Phosfon-D might be acting by preventing production or transport from the roots of some factor essential for stem growth or it might induce formation of some inhibitor which moves up the stem. The third possibility is, of course, that it might move up to the stem and there exert its effect.

The first possibility was tested by growing plants with their roots divided into two pots. When normal bean seedlings were about ten days old the soil was washed from the roots, and the roots separated into two equal fractions. The two portions of the roots were transplanted into two separate pots fastened together with wire and treatments were begun after stem growth resumed. If Phosfon-D acted by reducing the formation of a stem growth factor produced in the roots, then Phosfon applied to half the roots, i.e., to only one pot, should be no more inhibitory than simply cutting off half the roots. The results of an experiment to test this possibility are presented in table I. Clearly, application of Phosfon-D to half the roots is fully as inhibitory as treatment of all the roots with the same total amount of Phosfon. Removing half the roots from the plants is far less inhibitory. It was tentatively concluded, then, that the Phosfon or some product of it moved to the stem and exerted its inhibitory effect in the stem.

Table I

Influence of Treating $\frac{1}{2}$ Roots With Growth Inhibitor (Phosfon-D) Compared to Total Root Treatment

Treatment	Avg. daily growth
Roots in one pot	
Control	1.9 \pm 0.55 cm/day
20 ml 1% PD	0.2 \pm 0.04
Divided roots	
Control	4.1 \pm 1.0
$\frac{1}{2}$ roots removed	1.9 \pm 0.5
20 ml 1% PD in one pot	0.4 \pm 0.03
10 ml 1% PD in each pot	0.4 \pm 0.12

Experiments were also undertaken to determine the duration of the effect of Phosfon in the plant and in the stem (table II). One week after Phosfon treatment a group of bean plants was transplanted into untreated soil. Every effort was taken to wash the roots clean of soil prior to transplanting. Untreated plants resumed growth almost immediately following transplanting, but treated plants remained stunted for the duration of the experiment, a matter of 16 more days. At the same time some of the

Table II
Effect of Removing Roots or Transplanting on Carry-Over of Phosfon-D Effects on Stem Elongation

	Avg. daily rate of stem growth*	
	Untreated	PD-treated
Control plants	10.2 \pm 0.25 cm	0.6 \pm 0.03 cm
Transplanted plants	6.3 \pm 0.42	0.4 \pm 0.07
Rerooted plants	7.1	0.8

* Growth rate of control and transplanted plants measured for 6 days immediately following transplanting. Growth rate of rerooted plants measured for 8 days after plants had been rerooted and transplanted back to soil.

plants were being transplanted, other treated and untreated plants were cut off well above the soil line, and were then rooted in one-fourth strength Hoagland's solution, transplanted back to untreated soil and subsequent growth observed. These treated plants, then, were growing without any of the treated root system remaining. They continued to show growth inhibition effects for the duration of the experiment, while control plants not previously treated with Phosfon-D immediately resumed growth. Phosfon-D treated plants were observed for 16 days following termination of regular growth measurements and growth inhibition continued throughout that time.

Either the Phosfon or some product of this material apparently moves to the stem and appears to exert its inhibitory effect there. The inhibitory activity lasted throughout the duration of the experiment (about 30 days). No lessening of effect with time has been observed in the author's experience, even after inhibitor treatment was discontinued. This is consistent with the findings of other workers with these growth retarders.

Note that the growth rate of the controls increases markedly after the plants are 3 to 4 weeks old. In general, growth rate is essentially linear with time during the usual experimental period. As the plants became older an acceleration in growth rate was observed and a new, approximately linear, growth rate was established. This has been noted repeatedly with these plants and is even more striking with Pharbitis (Lockhart, unpublished). The Phosfon-D treatment appeared to prevent this increase in growth rate (table II). If Phosfon is an anti-gibberellin, this result suggests the acceleration in growth of these older plants may be due to an increased supply of endogenous gibberellin. Further work will be necessary to support this suggestion.

► Interactions Between Gibberellin & Growth Inhibitors: To determine the kinetic interrelations of two growth factors it is necessary to: A, use a steady state system, and B, vary one factor, the promoting factor, from zero to saturation at two levels (or in the presence & absence) of the inhibitory factor. Total stem elongation of the pinto bean has already been found to be linear with time (3, 10) and

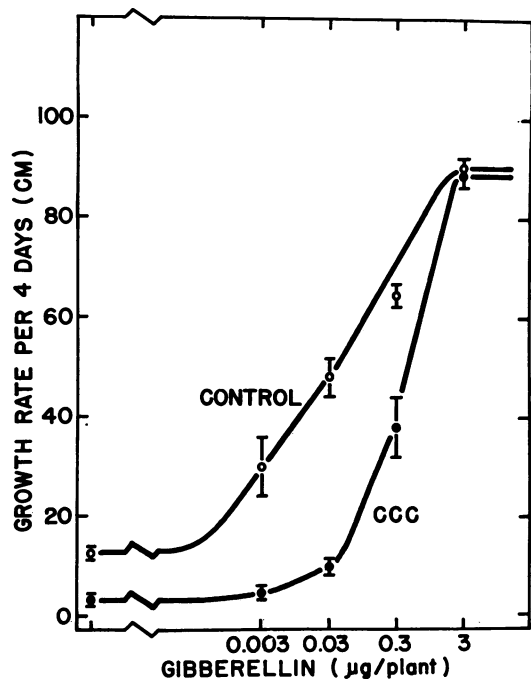
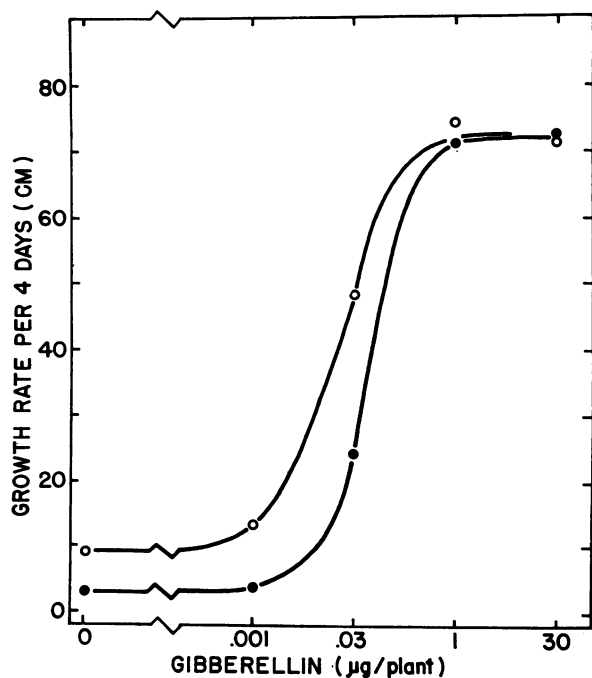


Fig. 1 (above). Interaction between gibberellin and phosphon-D. Phosphon-D applied at 0.1 g 10% formulation per pot. Stem growth of pinto bean between 2nd and 6th day following treatment. Upper curve, control, lower curve, phosphon-treated.

Fig. 2 (below). Interaction between gibberellin and chlorocholine chloride. CCC applied at 0.5 ml 50% formulation per pot. Stem growth of pinto bean between 2nd and 6th day following treatment.

steady state conditions have been confirmed by measuring the plants on alternate days in each experiment reported here.

Gibberellin doses ranging from those giving a minimum response to doses exceeding saturation were applied to bean seedlings either untreated or treated with 0.01 g Phosfon-D per plant. The results of a typical experiment are presented in figure 1. Sets of curves of identical shape have been obtained in separate experiments. The shape of these curves leaves little doubt that the Phosfon is interacting competitively with added gibberellin, that is, the Phosfon is acting as a true anti-gibberellin in this system (see Discussion).

Substantially identical experiments were run, testing for an interaction between CCC and gibberellin. Results of a typical experiment are presented in figure 2. The results are essentially the same as with Phosfon, only the detailed shape of the curve is slightly different. Thus, CCC also shows complete interaction with gibberellin and is an equally effective anti-gibberellin.

Previous studies (10) have shown that gibberellin interacts with only one environmental factor, light, of the number of environmental factors tested. Since the two stem growth inhibitors studied above both show complete interaction with gibberellin, it was considered desirable to test a third chemical growth inhibitor of a somewhat different nature for possible interaction.

Maleic hydrazide has been known for some time as a potent inhibitor of stem growth (5). However, the general physiological effects of maleic hydrazide are somewhat different than those of the anti-gibberellins studied above. Maleic hydrazide generally appears to act principally by affecting cell division. Further, maleic hydrazide readily causes plant injury, also in contrast to the anti-gibberellins studied above.

The range of concentrations of maleic hydrazide between those resulting in threshold responses and those giving essentially complete inhibition of growth was found in these experiments to be very narrow and slightly variable, depending presumably on environmental conditions. Furthermore, growth at relatively high doses of maleic hydrazide decreased with time. However, the results obtained in several experiments leave no doubt that the inhibition of stem growth by maleic hydrazide cannot be overcome by large doses of gibberellin. The average of two such experiments is plotted in figure 3. In this case gibberellin does not counteract or overcome the inhibitory effect of the inhibitor. Rather, the percentage response to gibberellin is the same in the treated and untreated plants, or, put another way, the percentage inhibition by maleic hydrazide is the same in plants saturated with gibberellin as in those with only endogenous gibberellin. Thus, the curves are characteristic of responses to two completely independent factors. These results fully confirm the earlier conclusion of Haber and White (6) that maleic hydrazide acts independently of gibberellin.

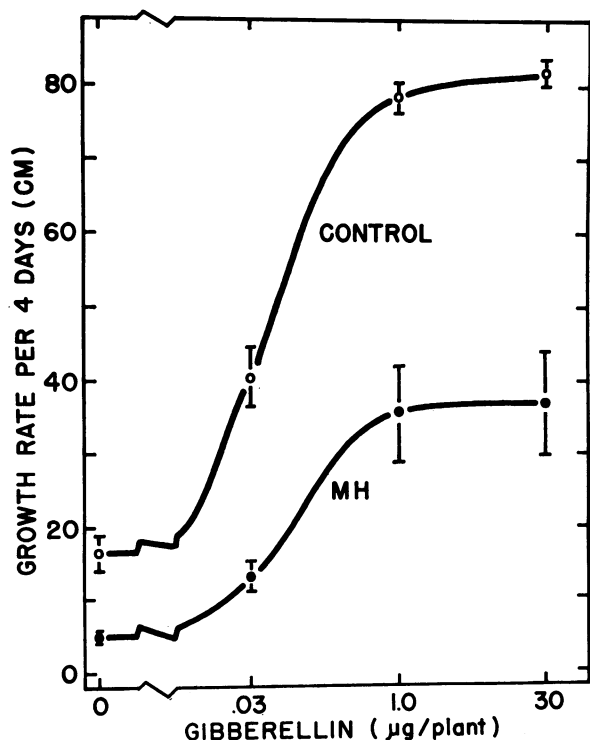


Fig. 3. Interaction between gibberellin and maleic hydrazide, average of two experiments. Maleic hydrazide sprayed on the leaves at a concentration of 0.3 g/liter.

Discussion

The results reported here are not surprising in the light of previous investigations on gibberellin and the growth inhibitors. Growth responses to Phosfon-D and CCC certainly have appeared to resemble anti-gibberellin effects, while responses to maleic hydrazide have not been those expected of an anti-gibberellin. The principle purpose of the present paper is to demonstrate appropriate kinetic analyses of these interactions.

In order that a chemical compound may be considered an antagonist of another compound and be considered an anti-whatever the other compound is, a competitive interaction must be demonstrated. Competitive interactions in simple one-enzyme systems which follow the Michaelis-Menton-Briggs-Haldane formulation may be unambiguously tested by the double reciprocal plot technique of Lineweaver and Burk (8). In order that this test may be used, at least two restrictions must be respected. First, the system must be in a steady state condition. Second, it can be shown readily that no endogenous substrate can be present. In principle it is not necessary that this test be restricted to simple two-step reactions.

Hearon (7) has studied the general case of a linear, reversible, catenary system, in which a catalyst

enters an early step and is regenerated in a later step. Regardless of how many sequential steps are involved, the system will reduce to the Michaelis-Menton-Briggs-Haldane formulation for velocity as a function of the amount (i.e., concentration) of a regenerated catalyst. Stem growth of an intact plant can be maintained in a steady state condition, but it seldom can be rendered free of endogenous growth factors. Thus, in an intact plant Lineweaver-Burk reciprocal plots of growth against substrates with endogenous components will not give straight lines. Hence, this technique is not generally appropriate for studying the kinetics of internal growth factor interactions in intact plants. Presumably this was the situation confronting Conrad and Saltman in their recent paper on growth of *Ulothrix* (2). Earlier, Foster et al. (4) found that the growth response of *Avena* coleoptile sections to added auxins would give a straight line when plotted as the usual double reciprocals. If the growth response to auxin can legitimately be expressed by the formulation above, this must mean the sections were without significant endogenous auxin under the experimental conditions they used.

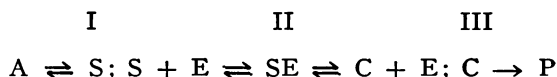
It has not yet been proven that plant growth may be expressed as a complex of catenary systems. However, both are open, steady state systems, and there appears to be no analytical property of growth which is inconsistent with the homologous property of a catenary model. Our current knowledge of biochemical processes also strongly supports the catenary system as the most appropriate for a stem growth model.

Even without using Lineweaver-Burk analysis, considerable information can be elicited in many cases through comparison of simple dose-response curves. Whenever a growth promoting factor can completely eliminate the influence of a second factor in a properly controlled system, a competitive interaction is demonstrated. At saturating doses of a promoting factor, process rate will approach the same maximum velocity in the presence or absence of an interacting factor. This, of course, compares precisely to the converging lines at infinite substrate and maximum velocity in the double reciprocal plot.

It has been suggested that the anti-gibberellins may not be acting by competing with the gibberellin molecule for the same active site, since the configurations of the various active molecules are so dissimilar (16). Results of studies with kinetic models suggest that in even a simple branched catenary system competitive inhibition (as determined by kinetic analysis) can be demonstrated without requiring that the inhibitor compete with the hormone for the same active site.

Model steady state, three step, catenary models having the same general kinetic properties as the growth system have been solved for process rate as a function of substrate concentration, concentration of the regenerated catalyst, and reaction rate constants

(Lockhart, unpublished). These models are of the general form:



Where $E_T = E + SE$

A is the initial substrate, and P is the product. The product, P, is assumed to contribute one of many factors essential for growth. A step (step II) containing a regenerated catalyst is always included to permit substrate saturation. Modifications of the model, with exogenous and endogenous sources of substrate (illustrated opposite), the presence of a competitive inhibitor, or a second substrate yield completely comparable results. Variation of the rates of any two reaction steps (but not substrate concentration) yields completely independent effects; i.e., response to one factor gives the same percentage effect regardless of the level of the other. Independent responses will not be considered further here. However, the limit of maximum process rate as substrate approaches infinite concentration is determined only by the amount of available E (i.e., E_T and rate of regeneration of free E and is independent of all other rate constants of the system. Thus, variation of substrate concentration and the rate constant of any reaction step, except the amount of available E, gives dose-response curves which converge to the same maximum process rate at saturating doses of substrate.

It has further been found that substrate-dose-response curves in which the rate constants of any one of the several reaction steps are varied separately give converging curves of two distinct shapes, depending on the position of the reaction step varied with respect to the substrate-saturated (regenerated catalyst) reaction step (fig 4). This figure illustrates a modification of the model discussed above. The model used is illustrated in the figure and the open arrows indicate the variables. In the case illustrated two alternative sources of substrate are presented. Variations in the values of reaction step constants prior to the substrate-saturated step give substrate-dose-response curves which saturate at identical substrate concentrations, while variations in the values of reaction step constants subsequent to the substrate-saturated step give dose-response curves which saturate at higher substrate concentrations, with decrease in the rate constant. Both curves attain the same maximum process rate. True competitive inhibition, in which an inhibitor reversibly competes with substrate for available E, gives substrate-dose-response curves identical to this second case, i.e., more substrate is required to achieve maximum process rate. Experimental results are not usually sufficiently precise to distinguish between these two possibilities. Thus, it is usually not yet possible to distinguish between inhibitors which A, interfere with biosynthesis, B, compete directly with, or C, interfere with the subsequent conversion of,

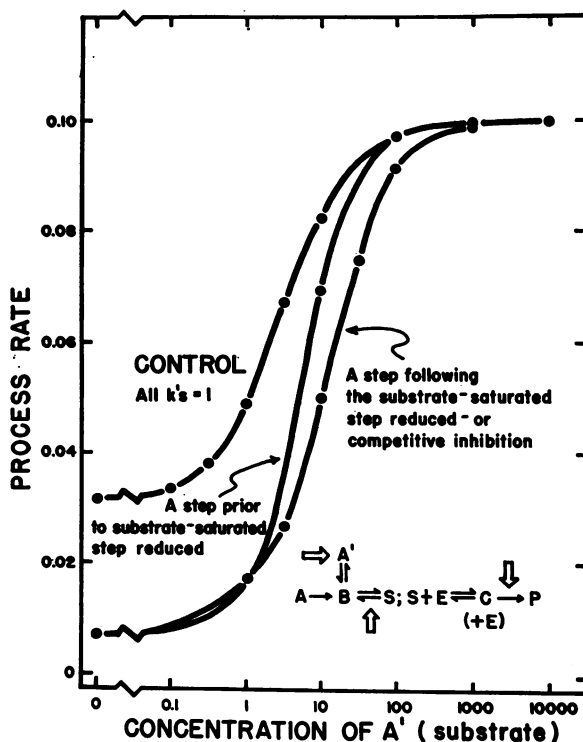


Fig. 4. Theoretical interaction between a substrate (fixed endogenous concentration & variable exogenous concentration) illustrating the influence of an inhibitor acting prior to or following the reaction step which is rate limiting for the process (when the process is substrate saturated). As indicated, true competition between substrate and inhibitor gives a curve identical to the second inhibitor system.

growth promoting factors in a growth system. To complete the model as it may apply to growth, it is assumed that the catenary sequence above contributes an active product, P, which contributes to growth by catalyzing one step of a primary catenary system.

Thus, we have for the first time in plant growth studies anti-metabolites which apparently are not analogs of the growth factor with which they compete but which nevertheless give competitive-type interactions. This brings up the question of whether these compounds are truly anti-gibberellins and whether this is truly competitive inhibition within the generally understood meaning of the term. The present author believes that these interactions should be included as competitive inhibitions, to clearly distinguish them from independent effects. Materials and treatments with precisely these relationships will be most useful in unraveling the multitude of biochemical and biophysical processes which constitute growth. Eventually it will undoubtedly be useful to distinguish by separate names the true competitive inhibitors and the inhibitors which act on the subprocesses which transform the substrate in question to the form utilized by the primary growth process or processes. At the

present time we have no way of distinguishing these three mechanisms except when the inhibitor is a chemical of such diverse structure that it can hardly be conceived to be competing with the substrate by virtue of similar molecular configurations.

It is concluded that the two compounds, Phosfon-D and chlorocholine chloride retard stem elongation by competing with the gibberellin system of the plant. Maleic hydrazide, on the other hand, inhibits growth by acting on some non-gibberellin system. These conclusions, together with the observations reported here and elsewhere that the anti-gibberellins can virtually completely inhibit stem elongation, indicate that gibberellin (endogenous or exogenous) is essential for even minimal normal cell elongation.

Summary

Evidence is presented indicating that Phosfon-D and chlorocholine chloride (CCC) exert their inhibitory effects in the stem rather than in the roots.

Kinetic analyses are presented demonstrating that Phosfon-D and CCC interact competitively with gibberellin on stem growth. It is concluded that Phosfon-D and CCC act to retard stem elongation by partially blocking the system which provides active gibberellin to the growth mechanism. The inhibition of stem growth by maleic hydrazide is shown to be independent of the promoting effect of gibberellin. The problem of interpretation and definition of growth factor and anti-metabolite action in the complex growth system is briefly discussed.

Acknowledgments

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