

Plant Growth Retardants as Inhibitors of Sterol Biosynthesis in Tobacco Seedlings^{1,2}

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ABSTRACT

Three plant-growth retardants 2'-isopropyl-4'-(trimethylammonium chloride)-5-methylphenylpiperidine carboxylate (Amo 1618), β -chloroethyltrimethylammonium chloride, and tributyl-2,4-dichlorobenzylphosphonium chloride were tested for their effects on sterol production in, and growth of tobacco (*Nicotiana tabacum*) seedlings. As the concentration of each retardant increased, there was an increased inhibition of the incorporation of DL-2-¹⁴C-mevalonic acid into sterol (particularly desmethylsterol) fractions and an increased retardation of stem growth. Growth retardation was observed with both single and repeated retardant treatments, and with Amo 1618, in particular, a close quantitative relationship between inhibition of sterol biosynthesis and stem growth was obtained. Gibberellic acid completely overcame retardant effects and application of sterols also restored normal growth. It is concluded that the concept of causality in the relationship between growth retardation and gibberellin biosynthesis is probably premature, since growth retardants have a more general inhibitory action on isoprenoid biosynthesis in plants.

Some 25 years have elapsed since the first effects of what have been called growth retardants were reported (23). The number of chemically rather diverse compounds, and their effects, has expanded considerably, but the most widely investigated retardants (Amo 1618,³ CCC, Phosfon D, and B-995) are known primarily for their "dwarfing" effects.

The fact that the decreased rate of stem growth induced by the retardants frequently could be overcome by exogenous GA₃ suggested that in higher plants (11, 16), the predominant action was an inhibition of gibberellin biosynthesis. West and co-workers (4, 19) provided the only definitive evidence for this by demonstrating, with cell-free preparations from *Echinocytosis macrocarpa* endosperm and *Ricinus communis* seed-

lings that the two-step cyclization reaction leading to kaurene formation was inhibited.

The similarity in biosynthetic sequence between sterols and gibberellins (1), and the fact that cell-free rat liver preparations could convert acetate to cholesterol, prompted Pales and colleagues (14, 17, 18) to examine the effects of growth retardants on cell-free rat liver cholesterol biosynthesis. The work indicated that all of the retardants examined, inhibited isoprenoid biosynthesis at more than one point in the cholesterol pathway, and that the different retardants inhibited the latter stages of the sequence at different points (14). Amo 1618, in particular, was a potent inhibitor of rat liver squalene-2,3-epoxide cyclase, causing an accumulation of the substrate (unpublished information), and similar results were obtained subsequently with rootless seedlings of tobacco (5). These findings demonstrated, by analogy, that retardants indeed had the capacity to inhibit isoprenoid, and hence gibberellin biosynthesis, but raised the question of the possible involvement of sterol biosynthesis as a direct or indirect requirement for plant growth.

The majority of available evidence, which indicated that retardants exerted an effect on gibberellin biosynthesis, has been accepted widely by plant physiologists and led to the concept that the action of some retardants, especially Amo 1618 and CCC, is limited to one single step in the gibberellin biosynthetic pathway (11). The thrust of this hypothesis depends in large measure on the correlation between the retardant's effect on growth and its reversal by applied gibberellin, and culminated recently in the statement that "The two effects can be related causally only if the (retardant) effect on growth can be overcome completely by applied GA." (11). The requirement for causality in this relationship caused us to examine the significance of the retardant effects on sterol biosynthesis observed with rat livers, more closely with plants.

MATERIALS AND METHODS

Growth Studies. *Nicotiana tabacum* (cv. Turkish Samson) seeds were germinated on potting compost and grown throughout these experiments under conditions of constant temperature (20 C) and illumination (2000 ft-c fluorescent lamps). At 12 days the seedlings were transplanted and grown under the same conditions for a further 5 days. The plants were randomized into groups of five and stem height and the length and width of the second leaf were determined for each. There were five plants in each treatment.

Growth retardants (at concentrations indicated) were applied as single 5- μ l drops to the apex of the seedlings in 0.05% (w/v) Tween 20. Control seedlings received 5 μ l of 0.05% (w/v) Tween 20. Measurements of each growth parameter and fur-

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² Dedicated to Solon A. Gordon whose windmills generally turned out to be dragons.

³ Abbreviations: Amo 1618: 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine carboxylate; CCC: β -chloroethyltrimethylammonium chloride; Phosfon D: tributyl-2,4-dichlorobenzylphosphonium chloride; B-995: N-dimethylaminosuccinic acid; MVA: mevalonic acid.

ther applications of growth retardants were made every 2nd day. Pots were moved around within the constant environment cabinet at each measurement, to eliminate positional effects.

In other experiments, seedlings transplanted at 12 days were allowed to develop under the above environmental conditions until 21 days old. The plants were measured for the three growth parameters (as above) and were given a single treatment of a growth retardant, GA₃, or a combination of both, at the concentrations indicated. Ten plants were used in each treatment and were measured every 2nd day.

Statistical analyses of all growth data were carried out by computer. Each individual measurement was converted to logarithms and standard analysis of the mean variance of the log values was performed by the computer. Standard errors for each mean value were also calculated by the computer.

To obtain the LSD for any one set of data, a standard formula was applied to the analysis of variance. From the calculated LSD, it was possible to determine which treatments caused significant variation from control, and all means of controls and points significantly different from them are represented in graphs by vertical bars equal to twice the standard error of the means. Points not significantly different from control values were plotted as the means only.

Where sterols were employed, the appropriate quantity of growth retardant was applied first to the plants and, when all the solution had been taken up (usually about 30 min), an aqueous emulsion of sterol was sprayed on the plants from an atomizer. Spraying was continued at approximately 2-hr intervals until an estimated 300 μ g of sterol had been administered to each plant.

Examination of commercially obtained sterols by GLC on two different liquid phase columns (*viz.* 1.5% [w/w] SE-30 on Anachrom ABS, and 2.5% [w/w] OV-101 on Gaschrom Q) showed that cholesterol and stigmaterol contained less than 0.05% of contaminants of a hydrocarbon nature, and β -sitosterol consisted of a mixture of campesterol and β -sitosterol in approximately a 1:2 ratio with no more than 0.05% of contaminants of a hydrocarbon nature.

The sterol emulsions were prepared according to Stowe and Dotts (21) and a final concentration of 30 mg of sterol/l of 0.004% (w/v) Pluronic F68 (Wyandotte Chem. Corp., Wyandotte, Mich.) was obtained. All controls were sprayed with an equal volume of 0.004% (w/v) Pluronic F68 solution, and 10 plants comprised each treatment.

Incorporation Studies. Tobacco seedlings were harvested at 21 days, washed thoroughly, and the roots were excised. Seedling tops (usually four) were placed into 2 ml of a solution of 5 μ Ci of DL-2-¹⁴C-mevalonic acid lactone (Amersham Biochemical Centre, London) in 11 mM phosphate buffer (KH₂-K₂HPO₄, pH 6.5) contained in 5-ml glass Petri dishes. When required, growth retardants (at concentrations indicated) were also incorporated into the 2-ml solution.

The rootless seedlings were placed around the edge of the Petri dishes so that only the cut stems were immersed in the solution. The Petri dishes containing the seedlings were then placed under constant illumination (300 ft-c fluorescent lamps) at 23 C for 24 hr. The seedlings were removed from the dishes with forceps, blotted, and weighed. They were homogenized in 5 ml of cold ethanol-benzene (4:1; v/v) and extraction of the nonsaponifiable lipids from the plant tissue was carried out as indicated previously (5). After the 24-hr incubation period, both the amounts of solution and radioactivity taken up by the rootless seedlings, were determined, and the percentage incorporation values were based on these figures.

A one-tenth aliquot of the total nonsaponifiable lipid fraction was spotted under nitrogen onto a 0.3-mm thin layer of Silica Gel G on a glass plate (20 cm \times 5 cm) and developed

twice in 4% diethylether in methylene chloride. Standard markers of cholesterol (or β -sitosterol), lanosterol (or β -amyrin), squalene-2,3-epoxide and squalene were spotted onto, and developed on, the same plates. After chromatographing, the plates were scanned for radioactivity and the markers were located by iodine-vapor staining. Sections (0.5 cm) were scraped into scintillation vials containing 2 ml of Bray's scintillation fluid to determine the incorporation into each fraction; a Packard Tri-carb liquid scintillation spectrometer Model 3320 was used.

When whole seedlings were examined for the incorporation of DL-2-¹⁴C-MVA into sterols, 2 μ Ci ¹⁴C-mevalonate was applied to the apex when they were 21 days old. Seedlings were removed from the pots 24 hr after application of the mevalonate and, after the roots were washed thoroughly, the plants were dried, weighed, and examined for radioactive sterols as described above. Plants grown under higher light intensity were maintained at 2000 ft-c, 20 C, and those treated under lower light intensity were placed under constant fluorescent illumination at 300 ft-c, 23 C for 24 hr. Percentage incorporation figures for experiments with intact seedlings were based on the amount of radioactive mevalonate applied to each seedling apex.

In experiments with either Amo 1618 or CCC, another aliquot was taken from the 1000- μ g/ml treatment, developed on thin layer Silica Gel G plates in 25% ethylacetate in hexane, and the band of radioactivity co-chromatographing with authentic squalene-2,3-epoxide (prepared by the method of Willett *et al.* [22]), eluted from the plates. The eluted material was then chromatographed in 5% ethylacetate-hexane and eluted from the plate with methylene chloride. An aliquot of the eluate was injected into a gas chromatography glass column (1.85 m \times 0.4 cm), packed with 2% SE-30 (Applied Science Laboratories, Inc., State College, Pa.) on Anachrom ABS (Analaboratories, Inc., Hamden, Conn.), and fitted to a Shimadzu GC-1C gas liquid chromatograph. Eluate from the gas chromatograph column, fitted with a stream splitter, was collected and measured for radioactivity.

The growth retardants examined were Amo 1618 (obtained as B grade from Calbiochem., Los Angeles), CCC (obtained as a 50% [w/v] aqueous solution from Cyanamid International, Wayne, N.J.) and Phosfon D (obtained from Mobil Chem. Co., Ashland, Va.). GA₃ was obtained from Product Development Laboratories, Merck & Co. Inc., N.J. β -Sitosterol and cholesterol (A grade) were obtained from Calbiochem, La Jolla, Calif., and stigmaterol was purchased from Sigma, St. Louis, Mo. A sample of Pluronic F68 was generously supplied by Dr. B. Stowe, Yale University, New Haven, Conn.

RESULTS

Our previous report (5) illustrated the effect of one growth retardant (Amo 1618) at a single concentration (1 mg/ml) on the incorporation of MVA into sterols and sterol precursors in rootless tobacco seedlings during a 24-hr period.

The effect of Amo 1618 on MVA incorporation into rootless tobacco seedlings is concentration-dependent, and higher concentrations of retardant, induced greater inhibition of MVA incorporation into sterols, particularly the desmethylsterols (Table I, Fig. 1). Conversely, incorporation of radioactivity into squalene-2,3-epoxide increased as the concentrations of retardant increased (on both an absolute and a per g fresh weight basis), whereas the biosynthesis of squalene was apparently unaffected by the treatment. These results are in accord with those published earlier, although inhibition of incorporation into 4-methyl- and 4,4'-dimethylsterols was previously greater (5).

Table I. Effect of Amo 1618 on Incorporation of DL-2-¹⁴C-Mevalonic Acid into Tobacco Seedling Sterols and Sterol Precursors

Retardant Concentration μg/ml	Incorporation of DL-2- ¹⁴ C-Mevalonate into				
	4-Desmeth-ylsterols	4-Methyl-sterols	4,4'-Dimeth-ylsterols	Squalene-2,3-epoxide	Squalene
0	210,980	36,103	39,369	3,205	10,625
10	186,264	39,512	12,360	10,914	9,450
100	121,352	23,906	26,728	24,703	8,752
1000	45,406	23,003	31,764	87,416	8,819

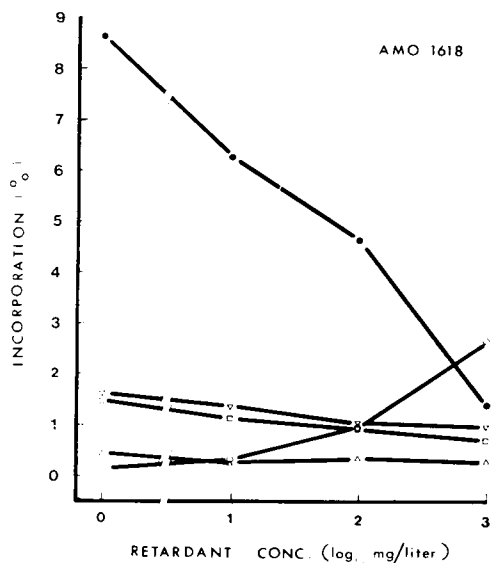


FIG. 1. Percentage of incorporation of only L-2-¹⁴C-mevalonic acid into sterols and sterol precursors of rootless *N. tabacum* seedlings in the presence of Amo 1618. Incorporation into 4-desmethylsterols (●), 4-methylsterols (□), 4,4-dimethylsterols (∇), squalene-2,3-epoxide (○), and squalene (Δ).

Identification of squalene-2,3-epoxide was reconfirmed by co-chromatography of the compound on thin layer Silica Gel G plates (25% ethylacetate in hexane and 5% ethylacetate in hexane) followed by GLC with authentic squalene-2,3-epoxide and authentic ¹⁴C-squalene-2,3-epoxide (Fig. 2). There seems little doubt that Amo 1618 effectively inhibits squalene-2,3-epoxide cyclase activity of tobacco seedlings, as it does in rat livers. (Further data on this point from experiments with cell-free preparations from tobacco seedlings will be published separately.)

The effect of CCC on ¹⁴C-MVA incorporation into sterols and sterol precursors (Table II, Fig. 3) was slightly different from that of Amo 1618. It was also most potent as an inhibitor of incorporation into desmethylsterols, but the accumulation of radioactivity in squalene-2,3-epoxide was considerably less. There was also a suggestion that, at low levels, CCC enhanced, rather than inhibited, sterol biosynthesis.

The pattern of incorporation of MVA in the presence of different levels of Phosfon D, differed slightly from that observed with the other two retardants (Table III, Fig. 4). Although desmethylsterol production was also strongly inhibited, methylsterol and dimethylsterol levels were elevated by low and middle concentrations of Phosfon D.

All of the above experiments were carried out with rootless seedlings under relatively low light intensity (300 ft-c). It was of importance, therefore, to determine whether intact seedlings

under higher light intensity would respond in a similar manner. Under conditions comparable with those used in the following growth experiments, the effect of Amo 1618 on intact seedlings (Table IV) is similar though not identical with the retardant effect on rootless seedlings (Table I). Methylsterol and dimethylsterol production is inhibited more strongly in rootless seedlings whereas squalene-2,3-epoxide accumulation is less pronounced. Higher light intensity seems to decrease the Amo 1618 effect still further. However, in spite of the quantitative differences observed, it may be concluded that, at least with Amo 1618, the absence of the roots exerted little qualitative effect on the incorporation of ¹⁴C-MVA into sterols and sterol precursors in the above ground parts of the plant. On a similar basis, it can also be assumed that Amo 1618, and probably the other retardants, will affect sterol biosynthesis in the following growth experiments (with intact seedlings grown under 2000 ft-c) in essentially the same way they did in the above experiments.

Inhibition of tobacco seedling growth by retardants was accomplished in two ways, *i.e.* either repeated relatively small doses applied every other day, or a single large dose applied at the beginning of the experiment. Results obtained with repeated dosages are illustrated in Figures 5, 6, and 7. All three

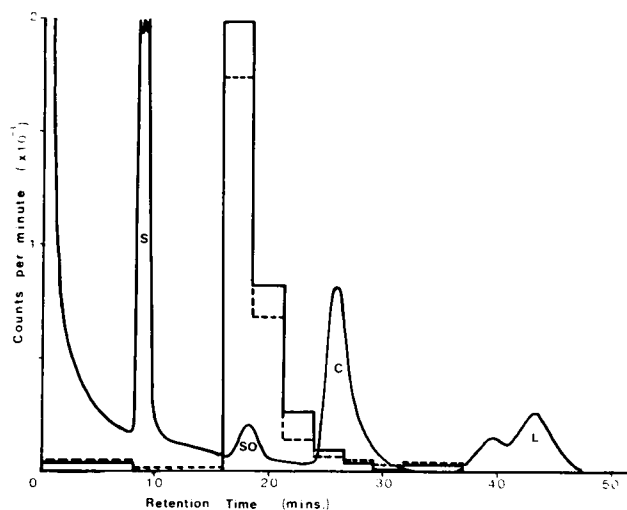


FIG. 2. Distribution of radioactivity in samples collected during gas-liquid chromatography of the eluate of the squalene-2,3-epoxide region of thin layer chromatograms from Amo 1618-treated, rootless tobacco seedlings. Solid lines (—) show the distribution of radioactivity in the eluate from TLC developed twice in 4% (v/v) diethylether in dichloromethane, and broken lines (---) show distribution of radioactivity for authentic ¹⁴C-squalene-2,3-epoxide chromatographed simultaneously. C: cholesterol; L: lanosterol; S: squalene; and SO: squalene-2,3-epoxide.

Table II. Effect of CCC on Incorporation of DL-2-¹⁴C-Mevalonic Acid into Tobacco Seedling Sterols and Sterol Precursors

Retardant Concentration μg/ml	Incorporation of DL-2- ¹⁴ C-Mevalonate into				
	4-Desmeth-ylsterols	4-Methyl-sterols	4,4'-Dimeth-ylsterols	Squalene-2,3-epoxide	Squalene
0	207,208	36,018	41,741	4,220	8,076
5	271,538	45,293	61,219	5,614	18,975
50	168,951	28,418	28,919	5,126	10,649
500	97,912	22,042	23,567	29,132	6,605
1000	65,786	15,008	17,799	29,958	6,268

retardants depressed growth and, in each case, increasing the retardant concentration increased the amount of growth inhibition. Furthermore, as with sterol-biosynthesis inhibition, the pattern of growth inhibition differs with each retardant. Phosfon D is the most potent inhibitor of both desmethylsterol biosynthesis and growth, while CCC is the least effective inhibitor of both functions. Differences in length of effectiveness were also apparent among the three retardants.

When the effect of single doses of the retardants was assessed (Figs. 8, 9, and 10) a similar picture emerged, *i.e.* Phosfon D was most active and CCC was least. Repeated dosages of even very low retardant concentrations exert strong effects on growth, *i.e.* four treatments with 1 μg of Phosfon D caused greater growth retardation in 8 days (Fig. 7) than did a single 30- μg dose (Fig. 10). The same results were observed with repeated dosages of 10 μg of Amo 1618 (Figs. 5 and 8) and 10 μg of CCC (Figs. 6 and 9). However, older seedlings seem to be somewhat less responsive to retardant treatment so it is difficult to draw firm conclusions about the efficacy of single as compared with repeated dosages. The results with a single dose of CCC (Fig. 9) are not impressive (only one point on the growth curve is significantly lower than control), but the results in Figure 6 leave little doubt that CCC, as well as Amo 1618 and Phosfon D, can cause retardation of the growth of tobacco seedlings. This confirms earlier reports of the effects of retardants on tobacco (12, 13).

Changes with time in the effects of the retardants on growth, make it impossible to correlate accurately the amount of inhi-

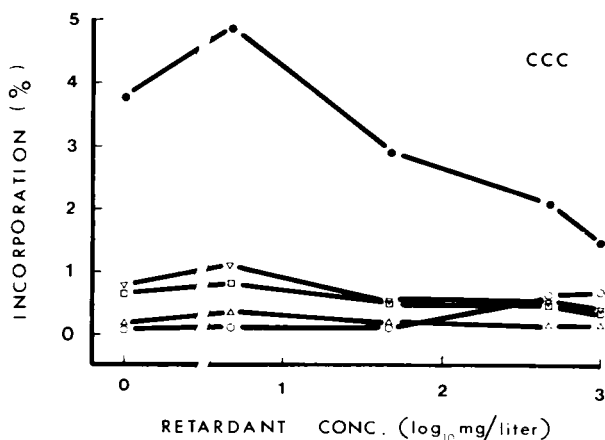


Fig. 3. Percentage of incorporation of only L-2-¹⁴C-mevalonic acid into sterols and sterol precursors of rootless *N. tabacum* seedlings in the presence of CCC. Incorporation into 4-desmethylsterols (●), 4-methylsterols (□), 4,4'-dimethylsterols (▽), squalene-2,3-epoxide (○), and squalene (△).

Table III. Effect of Phosfon D on Incorporation of DL-2-¹⁴C-Mevalonic Acid into Tobacco Seedling Sterols and Sterol Precursors

Retardant Concentration	Incorporation of DL-2- ¹⁴ C-Mevalonate into				
	4-Desmethylsterols	4-Methylsterols	4,4'-Dimethylsterols	Squalene-2,3-epoxide	Squalene
$\mu\text{g/ml}$	<i>cpm/g fresh wt</i>				
0	321,311	40,803	29,934	2,357	10,372
5	340,246	52,549	103,881	6,019	16,495
50	144,985	153,580	236,075	6,289	12,938
500	62,200	117,833	147,050	6,119	17,952
1000	22,766	37,687	77,385	2,877	7,643

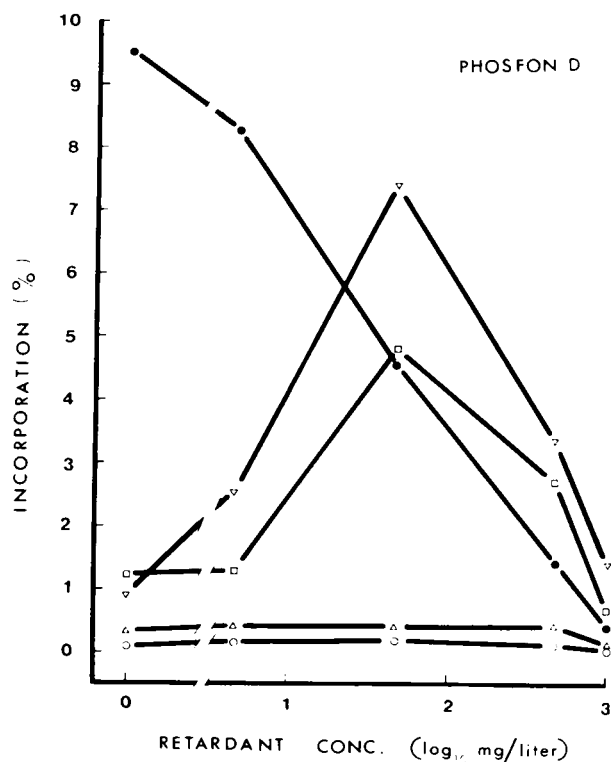


Fig. 4. Percentage of incorporation of only L-2-¹⁴C-mevalonic acid into sterols and sterol precursors of rootless *N. tabacum* seedlings in the presence of Phosfon D. Incorporation into 4-desmethylsterols (●), 4-methylsterols (□), 4,4'-dimethylsterols (▽), squalene-2,3-epoxide (○), and squalene (△).

Table IV. Effect of 300 μg of Amo 1618 and Light Intensity on Incorporation of DL-2-¹⁴C-Mevalonic Acid into Sterols and Sterol Precursors of Intact Tobacco Seedlings

Sterol Fraction	Incorporation of DL-2- ¹⁴ C-Mevalonic Acid			
	Lower light intensity (300 ft-c)		Higher light intensity (2,000 ft-c)	
	Control	Amo 1618-treated	Control	Amo 1618-treated
	<i>cpm/g fresh wt</i>			
4-Desmethylsterols	494,764	36,076	292,048	26,427
4-Methylsterols	30,403	5,839	31,661	12,875
4,4'-Dimethylsterols	42,489	6,673	30,240	21,458
Squalene-2,3-epoxide	3,399	16,057	2,232	7,002
Squalene	7,365	7,369	6,494	4,517

tion with the inhibition observed in desmethylsterol biosynthesis in 24 hr. However, an examination of this relationship was undertaken with Amo 1618 (Table V). Intact seedlings were treated in this experiment, and growth measurements were made on the same seedlings that were analyzed subsequently for desmethylsterol production. Only stems were analyzed for MVA incorporation. At the end of 24 hr there is a close quantitative relationship between the effects of Amo 1618 on stem growth and on desmethylsterol production in the stems.

An all important aspect of the current hypothesis on the mechanism of action of growth retardants is the ability of gibberellin to completely reverse the retardant-induced decreased growth rate (11). The implication of this requirement is that,

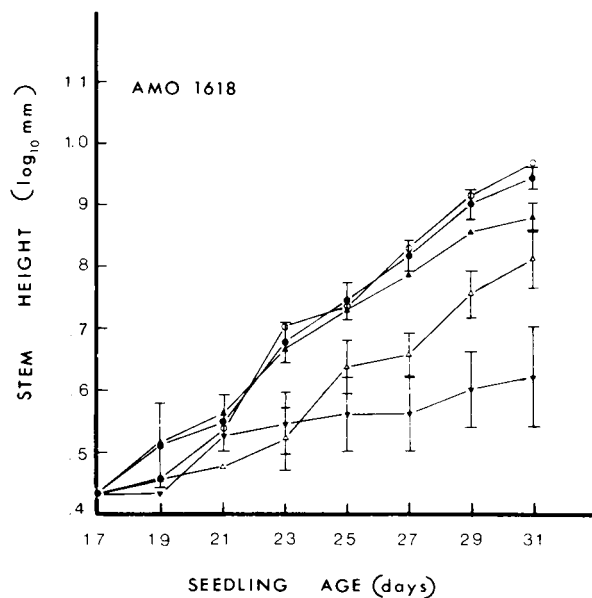


FIG. 5. Effect of Amo 1618 on stem growth of intact *N. tabacum* seedlings. Quantities of Amo 1618 applied per plant on each alternate day were 1 μg (\circ), 3 μg (\blacktriangle), 10 μg (\triangle), and 30 μg (\blacktriangledown) in 5 μl of 0.05% (w/v) Tween 20 solution. Control plants (\bullet) had 5 μl of 0.05% (w/v) Tween 20 solution added each second day.

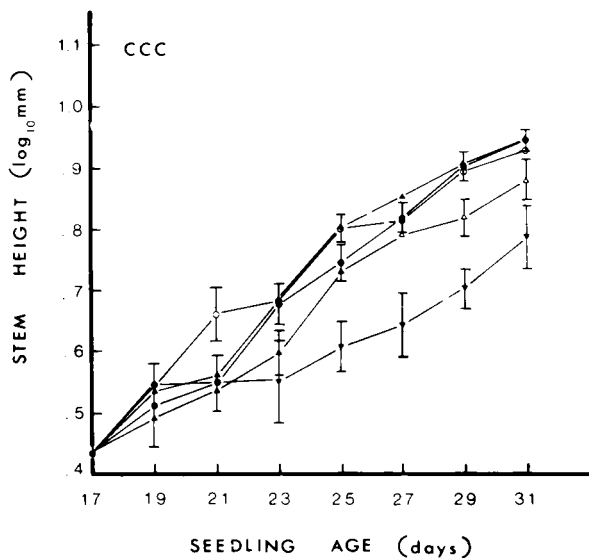


FIG. 6. Effect of CCC on stem growth of intact *N. tabacum* seedlings. Quantities of CCC applied per plant on each alternate day were 1 μg (\circ), 3 μg (\blacktriangle), 10 μg (\triangle), and 30 μg (\blacktriangledown) in 5 μl of 0.05% (w/v) Tween 20 solution. Control plants (\bullet) had 5 μl of 0.05% (w/v) Tween 20 solution added each second day.

if gibberellin can reverse the retardant effect, nothing else will be able to. The ability of GA_3 to counteract retardation of growth induced by Amo 1618, CCC and Phosfon D was examined (Figs. 8, 9, and 10), and GA_3 was fully capable of completely reversing the effects of at least Amo 1618 and Phosfon D on growth. Thus, it is clear that tobacco seedlings are "... suitable material on which to use retardants ..." (11).

In spite of the apparent similarities between retardant effects on growth and on sterol production, causality, can be questioned seriously only if sterol production can be related positively to growth. We tested this relationship in the simplest manner, by applying sterol (β -sitosterol) to Amo 1618-

treated plants. β -Sitosterol can overcome completely the effect of the retardant on the stem growth of tobacco seedlings (Fig. 11). Furthermore, not only β -sitosterol, but also stigmasterol and cholesterol can restore full growth (Fig. 12). Finally, when β -sitosterol was applied to plants treated with different retardants, Amo-1618- and CCC-treated plants all showed normal stem growth patterns (Fig. 13). Application of β -sitosterol to Phosfon D-treated plants, did not alleviate the induced

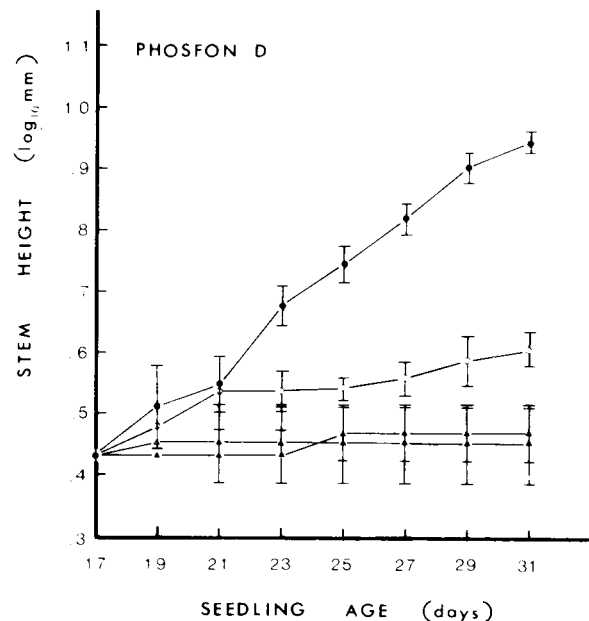


FIG. 7. Effect of Phosfon D on stem growth of intact *N. tabacum* seedlings. Quantities of Phosfon D applied per plant on each alternate day were 1 μg (\circ), 3 μg (\blacktriangle), and 10 μg (\triangle) in 5 μl of 0.05% (w/v) Tween 20 solution. Control plants (\bullet) had 5 μl of 0.05% (w/v) Tween 20 solution added each second day.

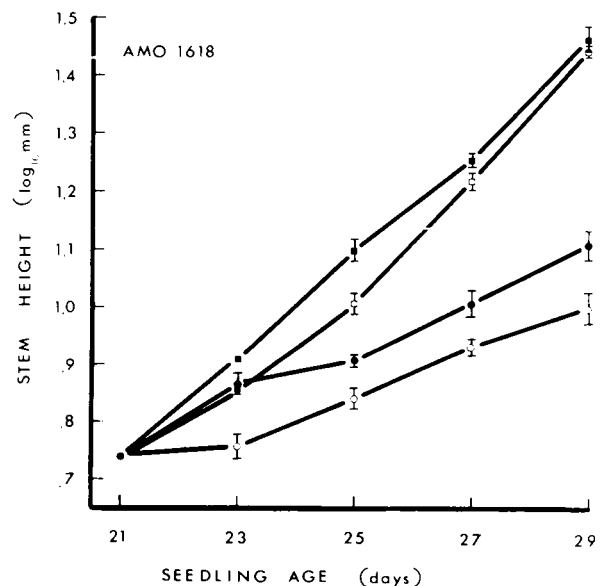


FIG. 8. Effect of GA_3 on stem elongation of intact *N. tabacum* seedlings treated with Amo 1618. A single application of 100 μg of Amo 1618 (\circ), 30 μg of GA_3 (\blacksquare), or 100 μg of Amo 1618 plus 30 μg of GA_3 (\square) in 5 μl of 0.05% (w/v) Tween 20 solution. Controls (\bullet) were treated with 5 μl of 0.05% (w/v) Tween 20 solution on day 21

growth retardation. In this connection, it is not yet clear whether other sterols would be more effective with Phosfon D.

DISCUSSION

The experiments reported here had a two-fold aim: to examine with higher plants the relevance of the retardant-induced inhibition of sterol biosynthesis observed with rat liver systems (14, 18, 20), and to assess the validity of the concept of causal-

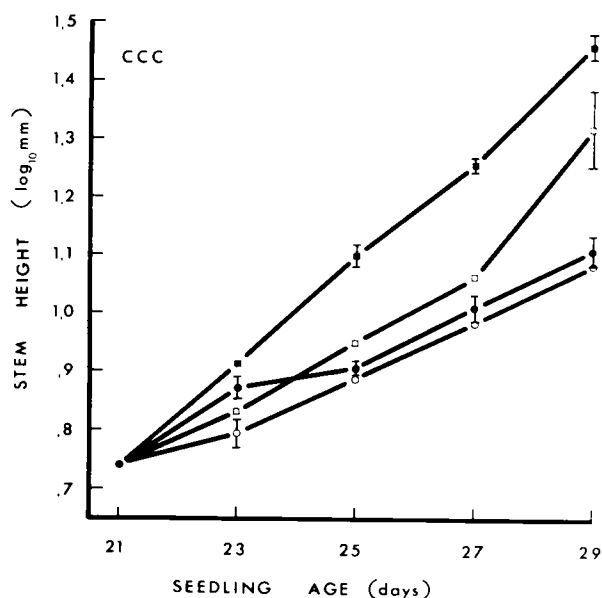


FIG. 9. Effect of GA_3 on stem elongation of intact *N. tabacum* seedlings treated with CCC. A single application of 100 μg of CCC (\circ), 30 μg of GA_3 (\blacksquare), or 100 μg of CCC plus 30 μg of GA_3 (\square) in 5 μl of 0.05% (w/v) Tween 20 solution. Controls (\bullet) were treated with 5 μl of 0.05% (w/v) Tween 20 solution on day 21.

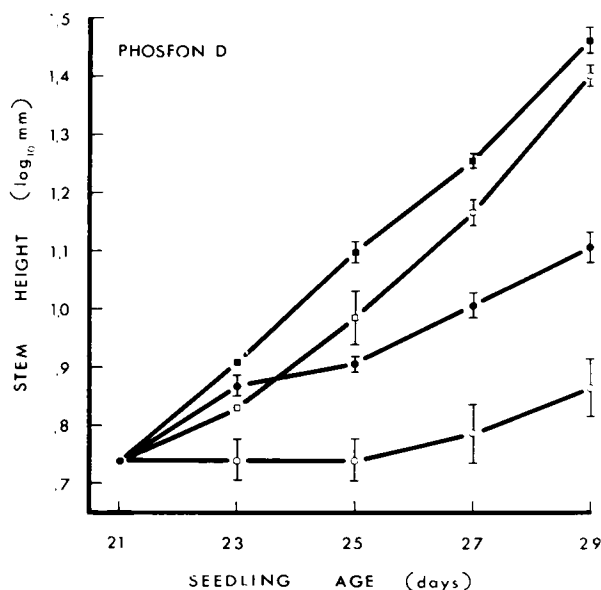


FIG. 10. Effect of GA_3 on stem elongation of intact *N. tabacum* seedlings treated with Phosfon D. A single application of 30 μg of Phosfon D (\circ), 30 μg of GA_3 (\blacksquare), or 30 μg of Phosfon D plus 30 μg of GA_3 (\square) in 5 μl of 0.05% (w/v) Tween 20 solution. Controls (\bullet) were treated with 5 μl of 0.05% (w/v) Tween 20 solution on day 21.

Table V. Simultaneous Effect of 300 μg of Amo 1618 on Incorporation of $DL-2-^{14}C$ -Mevalonic Acid into 4-Desmethylsterols in Stems of Intact Tobacco Seedlings and on Stem Growth

Growth values are means of 10 plants taken for sterol estimation.

Treatment	Incorporation		Stem Growth		
			Initial	After 24 hr	Δ
	%	cpm/g fresh wt	mm		
Control	1.40	139,735	2.2	4.2	2.0
Amo ₁₆₁₈	0.20	21,059	2.2	2.6 ¹	0.4
Percent inhibition due to Amo 1618	85.7	84.9			80

¹ Significantly different from control at $P = <0.01$.

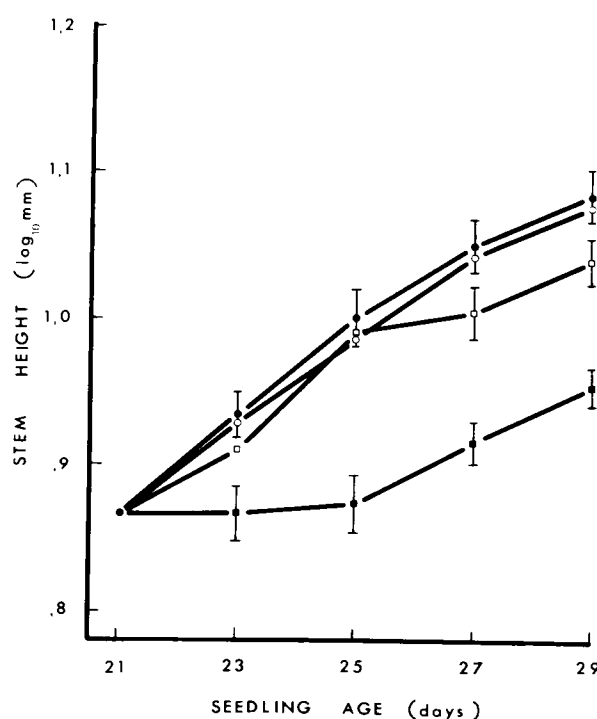


FIG. 11. Effect of β -sitosterol on the stem growth of intact *N. tabacum* seedlings treated with Amo 1618. \blacksquare : 100 μg of Amo 1618; \square : 100 μg of Amo 1618 plus β -sitosterol; \bullet : control; \circ : control plus β -sitosterol.

ity, relating growth retardation and inhibition of gibberellin biosynthesis (11).

The results with the cell-free rat liver system indicated that each of the retardants had the capacity to inhibit the incorporation of ^{14}C -MVA into sterol, and that, since the pattern of products formed from MVA was different with each retardant, it was likely that each retardant acted at a different step of the sterol biosynthetic sequence (14). Figures 1, 3, and 4 demonstrate that the results with tobacco seedlings are essentially the same in that Amo 1618, Phosfon D, and CCC all have the capacity to inhibit sterol (desmethylsterol) biosynthesis and that, at least with intermediate concentrations of retardants, the pattern of product accumulation is different with each retardant. Amo 1618 causes the accumulation of squalene-2,3-epoxide (5; Fig. 1), while Phosfon D induces the buildup of 4-methyl- and 4,4'-dimethylsterols (Fig. 4). CCC, the least potent of the

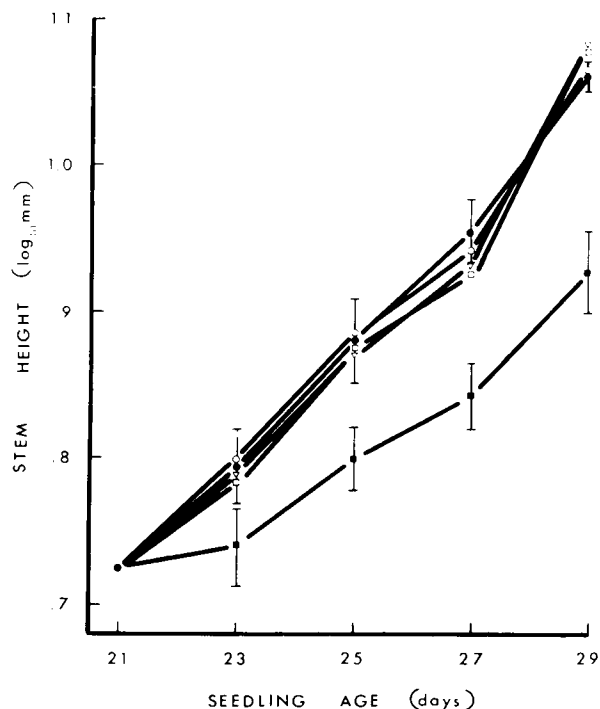


Fig. 12. Effect of various sterols on the stem growth of intact *N. tabacum* seedlings treated with Amo 1618. ■: 100 μg of Amo 1618; ○: 100 μg of Amo 1618 plus cholesterol; ▽: 100 μg of Amo 1618 plus stigmasterol; □: 100 μg of Amo 1618 plus β -sitosterol; ●: control.

retardants tested, causes inhibition of desmethylsterol production without appreciable accumulation of any particularly evident intermediate (Fig. 3). CCC seems more active on tobacco seedlings than it was on cell-free rat liver preparations (15). In general terms, however, the results indicate good correlation between retardant effects on sterol biosynthesis in cell-free rat liver preparations and tobacco seedlings.

Tables I and IV show that the presence or absence of roots has little qualitative effect on Amo 1618-induced inhibition of sterol production in tobacco seedlings. The results also seem essentially unrelated to the intensity of incident irradiation. Since several reports (3) suggest that retardants applied as root drenches are more, less, or differently effective than foliar or tip application, it seems likely that the similarity reported here is a function of the specific plant examined. Little is known of the interrelationships of the different plant parts, or of the general mechanisms, in the control of sterol biosynthesis. For the purposes of this work, it seems reasonable to assume that, qualitatively, the results, particularly with desmethylsterols, with rootless and intact seedlings are comparable, as are the data obtained on growth and incorporation of MVA in higher and lower light intensities.

The effects of repeated applications of the retardants on tobacco seedlings leave little doubt that all three compounds retard stem elongation, and that the amount of retardation is dependent upon the concentration applied (Figs. 5, 6, and 7). Each retardant is differentially effective, and the relationships between concentration and stem growth differ for the three compounds. Leaf growth was also assessed in these experiments, although the results are not presented. Single doses of retardants had no effect on either leaf length or width, while repeated doses inhibited both parameters. Contrary to the results with stem elongation, GA_3 did not reverse the inhibition of leaf growth induced by multiple application of the retardant.

It seems possible that leaf growth and stem growth are controlled in different ways.

The relationship between retardant-induced inhibition of stem growth and inhibition of gibberellin biosynthesis is widely accepted and forms the basis of interpretation of many investigations of both retardant and gibberellin effects. However, several points arise from the present work which seriously question the universality of the above hypothesis: (a) there is an over-all similarity in potency between retardant effects on growth and sterol biosynthesis; (b) there is also a similarity in pattern of retardant effects on the two parameters; (c) there is a close quantitative and temporal relationship between Amo 1618 action on both functions; (d) sterols can reverse the stem growth-retarding effects of at least Amo 1618 and CCC; and (e) Amo 1618 and CCC can exert inhibitory effects at several steps in the biosynthetic pathways of isoprenoid compounds in plants.

These findings indicate that the mechanism of growth-retardant action is still unresolved, and that there is a possible, and indeed promising alternative to complete dependence on the inhibition of gibberellin biosynthesis theory. Furthermore, an interesting and as yet unexplored aspect is the ability of added sterol to restore, but not exceed, the control rate of stem

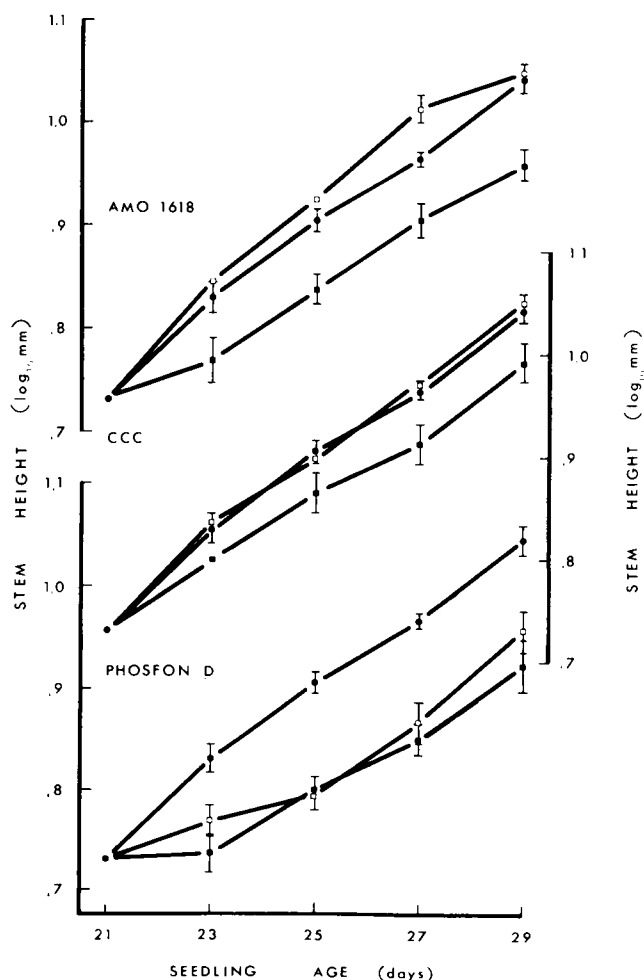


Fig. 13. Effect of β -sitosterol on the stem growth of intact *N. tabacum* seedlings treated with Amo 1618, CCC, or Phosfon D. ■: retardant alone; □: retardant plus β -sitosterol; ●: control. Quantities of retardant applied were 100 μg of Amo 1618, 200 μg of CCC, and 30 μg of Phosfon D.

growth. If this becomes a consistent finding, it raises questions concerning the relevance of amounts of gibberellins found in plants, and, indeed, of the involvement of gibberellin in the control of "normal" vegetative stem growth. The involvement of gibberellins as agents controlling the growth of dwarf varieties was also recently questioned (10).

In 1971 Heftmann (9) proposed that at least three main functions (*i.e.* as precursors of other sterols, as hormones, as membrane components) may be as common for sterols in plants as in animals. The role of sterols as structural or functional or both components of membranes is of particular importance. Not only do they play a part in controlling permeability and maintaining the integrity of plant membranes *e.g.* red beet (6) and barley root (8) tissue, but, by virtue of their wide occurrence in most, if not all, plant membranes (2, 7), they must also play an important role in protein synthesis, charge separation, and active uptake of almost all components. Certainly, it is not difficult to picture an inhibition of sterol biosynthesis retarding normal protein synthesis, and, thus, growth.

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