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THE EFFECTS OF PLANT GROWTH RETARDANTS ON STEROL BIOSYNTHESIS IN TOBACCO SEEDLINGS

by

Trevor John Douglas B.Sc. (Hons.)

Department of Plant Physiology,
Waite Agricultural Research Institute,
University of Adelaide,
South Australia

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Doctor of Philosophy

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Declaration

I hereby declare that the thesis here presented is my own work, that it contains no material previously published, except where due reference is made in the text, and that no part of it has been submitted for any other degree.

(TREVOR JOHN DOUGLAS)

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Summary

Several plant growth retardants were tested for their ability to inhibit sterol biosynthesis in, and growth of, Nicotiana tabacum (cv. Turkish Samson) seedlings. Amo 1618, Phosfon D, and CCC were shown to inhibit sterol biosynthesis in rootless tobacco seedlings and in each case the inhibition was concentration dependant. Phosfon D, the most potent inhibitor, appeared to inhibit demethylation reactions involved in the conversion of 4,4'-dimethylsterols and 4-methylsterols to 4-desmethylsterols. CCC, the least potent of the retardants, appeared to inhibit sterol biosynthesis at the squalene-2,3-epoxide cyclase step at higher concentrations. Amo 1618, the most studied retardant, was shown to act at several points in the biosynthetic sequence, the most probable major site being the squalene-2,3-epoxide cyclase step. The latter site of action of Amo 1618 was proposed to be the major site of action of the retardant on the following evidence:

1. Inhibition of 2-^{14}C-MVA incorporation into 4-desmethylsterols and sterol intermediates by Amo 1618 in intact tobacco seedlings and rootless tobacco (and digitalis) seedlings was always accompanied by an accumulation of incorporation into squalene-2,3-epoxide. A similar accumulation of radioactive label in squalene-2,3-epoxide was observed in Amo 1618-treated rootless tobacco seedlings supplied with 1-^{14}C-acetate and cell-free tobacco seedling preparations supplied with ^{14}C-squalene precursors.

2. Accumulation of radioactive label associated with squalene-2,3-epoxide increased with increasing Amo 1618 concentration.

3. The accumulation product was isolated and tentatively identified as squalene-2,3-epoxide by co-chromatography with authentic squalene-2,3-epoxide on T.L.C., using several different developing solvents, and on G.L.C., using two different column packing materials. Amo 1618 also appeared to inhibit HMG-CoA reductase activity in cell-free tobacco seedling preparations and at a second pre-squalene site in intact tobacco seedlings treated under high light intensity.

In general the retardants exerted their effects on sterol biosynthesis in tobacco seedlings in essentially the same manner that they did in rat liver homogenates.

A correlation between the effects of retardants on sterol biosynthesis in, and growth of, tobacco seedlings was established, particularly for stems of retardant-treated seedlings. Specific animal steroid biosynthesis inhibitors, known to inhibit sterol biosynthesis in higher plants, also inhibited stem growth of tobacco seedlings in a manner similar to the retardants, and further, sterol application to retardant-treated seedlings was able to completely reverse the retardant-induced inhibition of stem growth for, at least, CCC and Amo 1618.

The data are consistent with the conclusion that an inhibition of sterol biosynthesis could account for, at least in part, the stem growth retardation induced by the retardants. No comparable correlation was observed for the effects of exogeneous GA_3 on sterol biosynthesis and growth of tobacco seedlings.

Preface

Part of the work presented in this thesis has already been published. The publications are:

"Inhibition of Sterol Biosynthesis by 2-Isopropyl-4-dimethyl-amino-5-methylphenyl-1-piperidine Carboxylate Methyl Chloride in Tobacco and Rat Liver Preparations". Douglas, T.J. and Paleg, L.G. (1972) Plant Physiol: 49, 417-420,

and

"Plant Growth Retardants as Inhibitors of Sterol Biosynthesis in Tobacco Seedlings". Douglas, T.J. and Paleg, L.G. (1974) Plant Physiol: 54, (in press).

List of Abbreviations

The following abbreviations have been used in this thesis.

AMAB	allyltrimethylammonium bromide
Amo 1618	2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methyl chloride
ATP	adenosine-5'-triphosphate
AY-9944	trans-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride
B995	N-dimethylamino succinamic acid
BOH	β-hydroxyethylhydrazine
b.p.	boiling point
CCC	(2-chloroethyl)trimethylammonium chloride
C 011	N-dimethylamino maleamic acid
cv.	cultivar
2,4-DNC	1-(2,4-dichlorobenzyl)-1-methyl-2,3-pyridyl pyrrolidinium chloride
D.W.	dry weight
F.W.	fresh weight
g	gravity
GA	gibberellin
GA_3	gibberellic acid
G.L.C.	gas-liquid chromatography
gm	gram
HMDS	hexamethyldisilizane
HMG	β-hydroxy-β-methyl-glutaric acid

HMG-CoA	β-hydroxy-β-methyl-glutaryl coenzyme A
hr	hour
I.D.	internal diameter
M	molar
min	minute
ml	millilitre
μl	microlitre
μCi	microcurie
μM	micromolar
m.p.	melting point
N	normal
NAD(H)	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
Phosfon D	2,4-dichlorobenzyl-tributylphosphonium chloride
Phosfon S	2,4-dichlorobenzyl-tributylammonium chloride
dimethyl-POPOP	2-p-phenylene-bis(5-phenyloxazole)
PPO	2,5-diphenyl oxazole
p.p.m.	parts per million
R_f	(in T.L.C.) = $\frac{\text{distance of the solute from the origin}}{\text{distance of the solvent from the origin}}$
R_t	(in G.L.C.) = retention time
r.p.m.	revolutions per minute
S.D.	standard deviation
s.g.	specific gravity
SK and F 13831	diethyl[4-{3'-(4"-methylphenyl)-3-oxoprop-2'-enyl} phenoxyethyl]ammonium chloride

SK and F 525-A   β-diethylaminoethyl diphenyl propyl acetate hydrochloride

SK and F 7732-A_3 *tris*-(2-dimethylaminoethyl)-phosphate trihydrochloride

SK and F 7997-A_3 *tris*-(2-diethylaminoethyl)-phosphate hydrochloride

T.L.C.   thin-layer chromatography

tween-20   polyoxyethylene (2) sorbitan monolaurate

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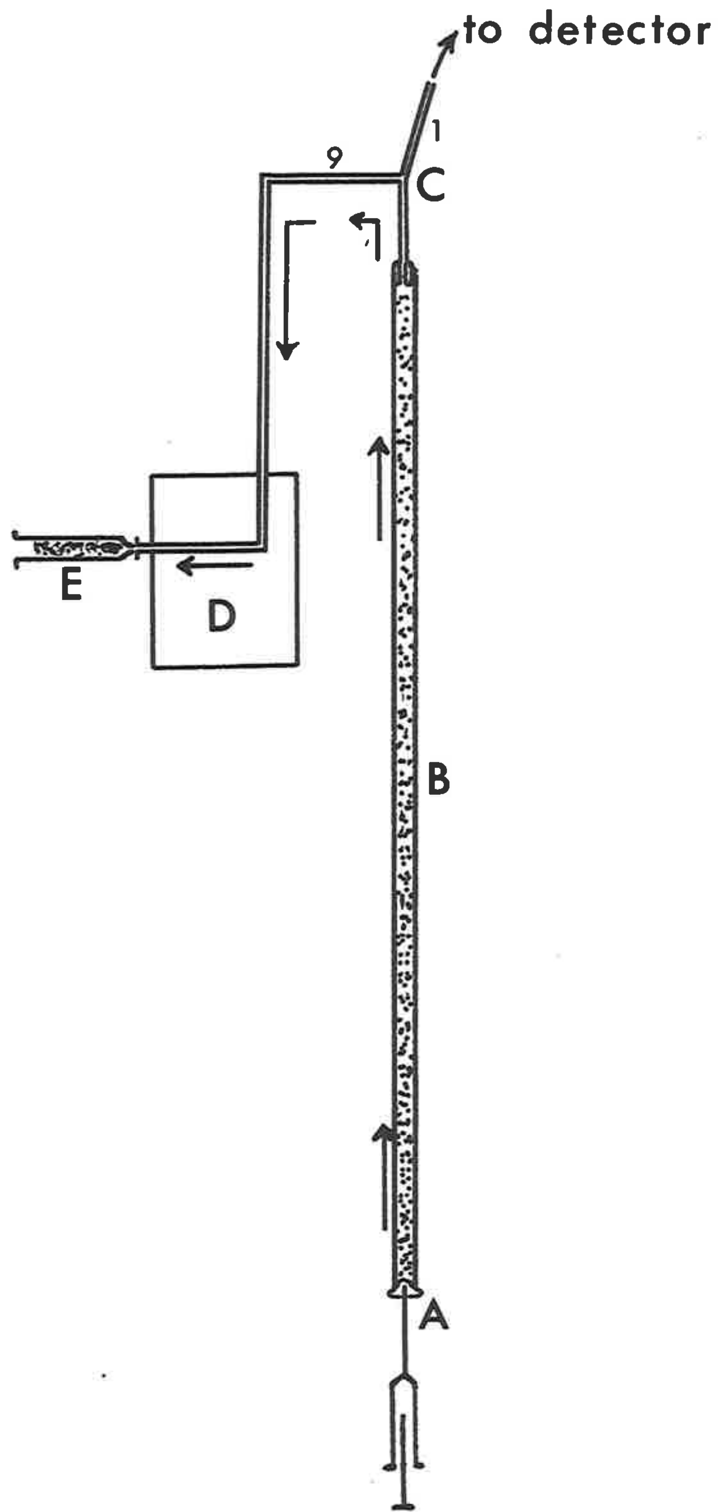
Figure 1. Flow diagram showing the method of collection of eluant from G.L.C. of sterols and sterol precursors.

The sample is injected onto the column packing material via the injection port (A), passes through the glass column (B) to the 9:1 stream splitter (C) and then either to the detector or to the collection point. The heated metal block (D) prevents condensation of eluant prior to collection in luer-lok 1 ml glass syringes packed with methanol-soaked wool (E).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I. INTRODUCTION

1. Method of Approach

This Introduction is not meant to be an in depth survey of all of the literature concerning the growth retardants, gibberellins and sterol biosynthesis. Excellent review articles on the plant growth retardants (Cathey, 1964), the gibberellins (Lang, 1970), and plant steroid biosynthesis (Heftmann, 1968; Goodwin, 1971) already exist. The aim of the Introduction is to summarize some of the general aspects of growth retardant effects, discuss proposed modes of action (updating some of the above reviews where applicable), and, it is hoped, to fully qualify the title of this Thesis as a logical extension of ideas from the literature.

2. General Aspects of Plant Growth Retardant Physiology

The plant growth retardants are a heterogeneous group of organic chemicals so named because of their ability to slow or retard the growth of higher plants. The term 'growth retardant' applies to all chemicals "that slow cell division and cell elongation in shoot tissues and regulate plant height physiologically without formative effects" (Cathey, 1964). Thus the growth retardants, in this context, are essentially dwarfing agents. This definition excludes compounds such as auxins and herbicides of the growth regulator type which cause visible malformations to stems, leaves and flowers when supplied at concentrations sufficient to cause growth inhibition. Germination inhibitors, which are active only on germinating seed or seedlings, and not on mature plants, are also excluded.

So far only synthetic growth retardants are known, and despite the common definition these compounds show an apparent lack of correlation between their specific actions and the taxonomic classification of the plants on which they act. Cathey and Marth (1960) have reported that even different cultivars of the same species (chrysanthemum) vary greatly in their responsiveness to the same growth retardant. With dahlia, B 995 causes a suppression of plant height and increases the size and quality of flowers in 10 different cultivars, whereas CCC retards plant growth in only one cultivar (Bhattacharjee _et al._, 1971) when applied as a foliar spray.

Of 44 species of plants tested, Marth, Preston and Mitchell (1953) showed only 19 to be responsive to application of the retardant Amo 1618, and of these only 7 were highly responsive. Similarly Cathey and Stuart (1961) reported that of 55 species tested 6 were responsive to Amo 1618, 19 responsive to Phosfon D, and 46 were responsive to CCC when the retardants were applied by soil application.

It is interesting to note that the method of application of the retardant can also influence the responsiveness of the plant. For example in chrysanthemum Amo 1618 is highly effective when applied either by spray or as a soil drench, and Phosfon D is effective as a soil amendment but toxic when applied as a spray (Cathey and Marth, 1960). In dahlia CCC is more effective when supplied as a soil drench than as a foliar spray (Bhattacharjee _et al._, 1971).

The only apparent indication of a taxonomic pattern of responsiveness is that the majority of sensitive species belong to the Dicotyledonae, and only a few species in the Monocotyledonae show responsiveness. Of the grass family for example, wheat seems to be most responsive to CCC (Tolbert, 1960b), whereas other cereals show variable sensitivity (Cathey, 1964). Wittwer (1971) states that all varieties of wheat respond to CCC, oats respond, barley does not, and the behaviour of rye is variable.

The types of chemicals described as plant growth retardants are summarized by Cathey (1964) and include nicotiniums, e.g. 2,4-DNC [the first retardants reported (Mitchell, Wirwille and Weil, 1949)],

quaternary ammonium carbonates, e.g. Amo 1618 (Wirwille and Mitchell, 1950), phosphoniums, e.g. Phosfon D, Phosfon S (Preston and Link, 1958), hydrazines, e.g. BOH (Gowing and Leeper, 1955), quaternary ammonium choline analogues, e.g. CCC (Tolbert, 1960a) and substituted maleamic and succinamic acids, e.g. CO11 and B 995 respectively (Riddell et al., 1962). Structures of these representative compounds are shown in figure 2.

Whereas there exists a diversity in both chemical structure and species specificity amongst the growth retardants, they nevertheless usually exhibit the same kind of qualitative effect whenever an action is exerted. The most common feature of growth retardant action seems to be a decreased rate of cell division, particularly in the sub-apical meristem regions, although the pattern of cell division in apical meristems remains unchanged (Sachs et al., 1960). Thus leaf shape and arrangement, for example, are not generally altered although stem elongation is retarded, and a rosette-like growth habit results (Sachs and Lang, 1961).

Often the suppression of stem elongation is accompanied by an expansion of the stem laterally, probably due to either greater cell proliferation in the cambium or the production of wider cells (Sachs and Kofranek, 1963). Full advantage of this action has been taken in agriculture, particularly with the application of growth retardants to cereal crops to increase lodging resistance. Tolbert's results with wheat (1960b) lead eventually to the use of CCC in areas of

Figure 2. Chemical structures of some of the growth retarding chemicals (from Cathey, 1964).

I. 2,4-DNC

II. Amo 1618

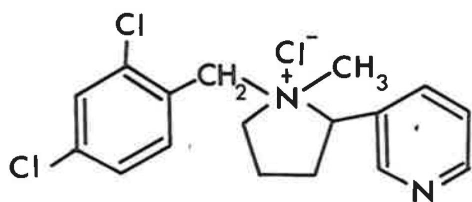
III. Phosfon D

IV. Phosfon S

V. CCC

VI. BOH

VII. B 995



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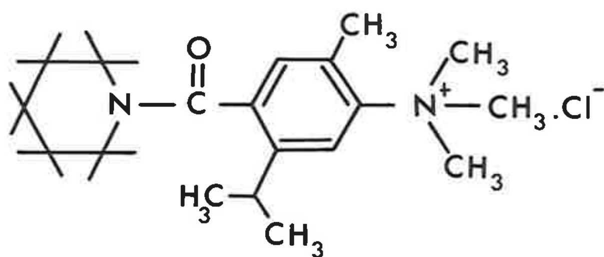

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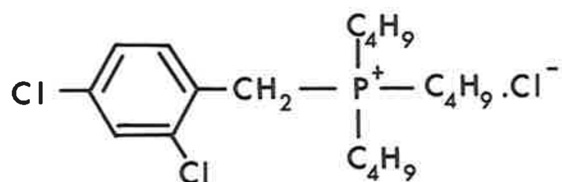

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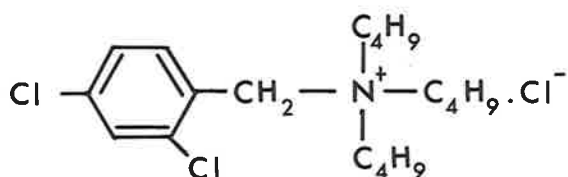

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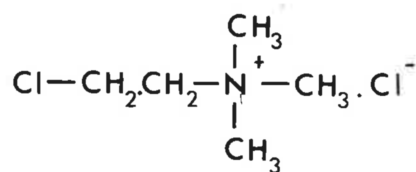

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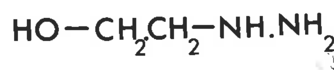

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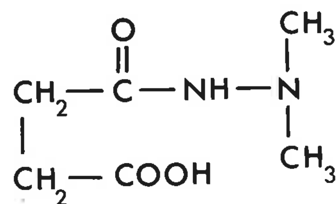


VII.

western Europe where lodging due to heavy rains or strong winds causes substantial losses in yield. The retardant-treated plants showed greener, more upright leaves and thicker, shorter and stiffer stems which resisted lodging (Humphries, 1968).

The retardation of stem elongation is not the only possible effect of the growth retardants, however, as under certain conditions and at low concentrations a growth promotion by retardants in several species including peas (Carr and Reid, 1968; Adedipe *et al.*, 1968), lemons (Monselise *et al.*, 1966), snapdragons (Halevy and Wittwer, 1965; Wünsche, 1969), gladiolus (Halevy and Shilo, 1970), and dahlia (Bhattacharjee *et al.*, 1971) has been demonstrated.

Retardant effects on leaf growth are not as common as the effects on stem elongation, and once again appear to be mostly evident in the dicotyledonous plants. Krewson *et al.* (1959) have demonstrated a restriction of leaf area and an increase in thickness of bean leaves as a result of Amo 1618 action, and CCC has been shown to exert a similar effect on tomato leaves (Laborie, 1963) and leaves of *Phaseolus vulgaris* (Felippe and Dale, 1968). Crozier *et al.* (1973) also report an inhibition of leaf area of bean seedlings (*Phaseolus coccineus*) by Amo 1618. On the other hand CCC causes an increase in leaf area in mustard plants (Humphries *et al.*, 1965) and has no effect on blade growth of wheat (Humphries *et al.*, 1965).

Retardation of root growth by retardants has been demonstrated in many plant species including chrysanthemum (Sachs and Kofranek,

1963), lentils (Gaspar *et al.*, 1971), Norway spruce (Dunberg and Eliasson, 1972), and bean seedlings (Crozier *et al.*, 1973).

The retardants have the ability to interfere with normally hormonally-controlled events in plant development such as the balance between vegetative and reproductive phases of growth. Usually when vegetative growth is arrested in plants, reproductive growth proceeds, and the retardants have been shown to suppress vegetative growth and induce flowering in some species including tomato (Wittwer and Tolbert, 1960a), *Rhododendron* (Stuart, 1961), and gladiolus (Halevy and Shilo, 1970). This effect of the retardants is not widespread, and the flowering behaviour of most species is not influenced by the retardants (Cathey and Stuart, 1961; Suge and Rappaport, 1968; Cleland and Zeevaart, 1970). However in some species (in particular the long-day plants whose flowering is promoted by added GA) flower initiation is suppressed by retardants, e.g. CCC inhibition of flowering in *Pharbitis nil* (Zeevaart, 1964) and *Lemna gibba* (Cleland and Briggs, 1969).

The ability of retardants to interfere with normal reproductive processes is not restricted to plants. CCC delays meiosis in male locusts and reduces egg-laying and fertility of cotton stainer bugs (Carlisle *et al.*, 1969).

3. Modes of Action of Retardants

Because of the great variability in both structure and action of the growth retardants, it seems unlikely that all of their many

effects on intact plants can be explained by one mode of action. For example, Heatherbell et al. (1966) showed that CCC and B 995 inhibit oxidative phosphorylation in root sections of eliolated pea seedlings, and suggested that the retardants might act as uncoupling agents, causing a reduction of mitochondrial ATP production, and thus block the flow of energy into growth processes. CCC and Phosfon D interfere with phosphorus metabolism in pea seeds (Adedipe and Ormrod, 1970) and pea plants (Adedipe and Ormrod, 1972).

Wheat shows increased salt tolerance (Naqui et al., 1970) and drought resistance (Singh et al., 1973; Plaut and Halevy, 1966) when treated with CCC, and frost-hardiness of rape plants (Kacperska-Palacz and Wcislinska, 1972) and tomato plants (Michniewicz and Kentzer, 1965) is increased by CCC treatment. Pillay and Lynn (1971) have shown high concentrations of the retardants CCC, Amo 1618, B 995 and Phosfon D to accelerate abscission in bean leaves. With the exception of CCC, low concentrations of the retardants delayed abscission.

Cell wall, nucleic acid, and protein synthesis are inhibited by Amo 1618 (Pillay and Yu Tu, 1971) and the same retardant decreases hyperchromicity of DNA (Spang and Platt, 1972). Chlorophyll synthesis is inhibited in pumpkin cotyledons by CCC, Phosfon D and B 995 (Knypl, 1969), lettuce cotyledons by CCC (Knypl and Chylinska, 1972), and barley leaves by CCC (Shewry et al., 1971). The inhibition of chlorophyll synthesis by CCC may be the result of an

inhibition of plastid protein synthesis by the retardant, as CCC has been shown to inhibit protein synthesis (Pillay and Yu Tu, 1971; Knypl and Chylinska, 1972), and its effects on chlorophyll synthesis are paralleled by the protein synthesis inhibitor chloramphenicol (Shewry et al., 1971). The possibility of a direct inhibition of the chlorophyll biosynthetic pathway will be discussed later in this Introduction.

The growth retardants inhibit red-light induced germination of lettuce (Cathey and Stuart, 1961) and hazel (Ross and Bradbeer, 1971) seeds, and spores of Anaemia (Weinberger and Voeller, 1969), but exhibit no direct interaction with phytochrome systems (Cathey, 1964).

The most frequently assigned mode of action of the retardants has been as antagonists of gibberellin action or biosynthesis, as many of the actions of the retardants are virtually the reverse of those of the gibberellins. For example, the retardants are suppressors of stem elongation, and in some cases leaf expansion, whereas gibberellins stimulate stem elongation and leaf expansion. Also, in some flowering plants where gibberellins promote flowering, the retardants act as inhibitors of floral induction.

An interaction between gibberellin and growth retardant action has been demonstrated in many different plants and plant processes. The retardant AMAB inhibits growth of the unicellular filamentous green alga (Ulothrix subtillisima) when applied at high concentration,

and this inhibition is completely overcome by addition of GA_3 to the medium (Conrad and Saltman, 1961). Other examples are the growth of wheat (Tolbert, 1960a), red kidney bean hypocotyl elongation (Downs and Cathey, 1960), stem elongation of chrysanthemum (Sachs and Kofranek, 1963), shoot growth of Norway spruce (Dunberg and Eliasson, 1972), fruit set in Vitis vinifera (Coombe, 1965), leaf expansion of Poa pratensis L. (Van andel, 1973), DNA hyperchromicity of corn (Spang and Platt, 1972), invertase activity of Jerusalem artichoke tuber tissue (Bradshaw and Edelman, 1971), peroxidase activity of soybean seeds (Seth and Pillay, 1971), and growth, cell wall, protein and nucleic acid synthesis in tall peas (Pillay and Yu Tu, 1971). In all of the above examples the addition of exogenous GA_3 to retardant-treated plants or plant material completely reversed the retardant effect.

Lockhart (1962) tested the relationship between GA and growth retardant actions by applying kinetic analyses to the observed interactions of Phosfon D, CCC and GA_3 on the stem growth of intact pinto beans. The data demonstrated a competitive interaction between retardants and GA_3. The growth inhibitor maleic hydrazide exhibited a gibberellin-independent inhibition of growth. This finding led the author to propose that a direct antagonism existed between the retardants and gibberellins in stem growth.

The demonstration of complete reversibility of retardant effects by exogenous GA_3 led to the retardants being named "anti-gibberellins".

This classification now appears invalid as the growth retardants do not act competitively with the gibberellins at their site of action in the cell. Cleland (1965) tested the ability of five growth retardants (viz. Phosfon D, Amo 1618, CCC, CO11 and B 995) to inhibit the GA_3-induced growth of Avena leaf sections. This system only responds to exogenous GA, hence the Avena leaf system tested the ability of growth retardants to act at the site of action of GA, not GA-biosynthesis. Amo 1618 (at levels above 3×10^{-4} M), and Phosfon D (at levels above 3×10^{-5} M), were able to effectively inhibit the elongation of GA-treated sections, and at 10^{-4} M Phosfon D saturating dosages of GA_3 were unable to completely reverse the inhibition caused by the retardant. Similar results were reportedly obtained with Amo 1618 but no data was presented. This implies that at least Phosfon D and Amo 1618 do not act competitively with GA at its site of action.

Similar results were obtained for the retardants CCC and Phosfon D on leaf discs of Raphanus sativus L. by Kuraishi and Muir (1963), who found that inhibition of expansion of the leaf discs caused by 10 g/l CCC and 10 and 30 mg/l Phosfon D was not reversed by saturating doses of exogenous GA_3.

Paleg et al. (1965) reported no effect, either inhibitory or stimulatory, of five growth retardants on the GA_3-induced release of reducing sugars from excised barley endosperm tissue.

Weinberger and Voeller (1969) showed that although Amo 1618 was able to inhibit light-induced germination of Anaemia spores, it was unable to inhibit the GA_3-induced germination of spores held in the dark, implying that the retardant did not interfere with or alter the action of GA_3.

It is thus apparent that the retardant-gibberellin opposing actions are not at the site of GA-action and must therefore be either at some other level of GA function, or possibly involve other growth promoting hormones. For example there are reports which implicate the growth retardants in auxin metabolism. Kuraishi and Muir (1963) found that where the retardation of growth of CCC-treated Avena colyoptiles and pea stem segments was not reversed by GA_3, IAA was able to reverse the effect. Also CCC reduced the levels of diffusible auxins in growing tips of pea stems. Cleland (1965) demonstrated a reversibility of the effects of Phosfon D and Amo 1618 on Avena leaf sections by added IAA.

Halevy (1963) provided evidence for an increase in IAA-oxidase activity in cucumber seedlings treated with retardants, suggesting that a possible mode of action might be to reduce the levels of available IAA by increasing its oxidation. The inhibition resulting from such a mechanism would be reversed by exogenously applied IAA.

Reed et al. (1965) proposed another mechanism for the antagonism of auxin metabolism by retardants. They suggested that B 995 was hydrolysed in plants to produce the diamine oxidase

inhibitor unsymmetrical dimethylhydrazine (UDMH), which reduced the conversion of tryptamine to indoleacetaldehyde, and hence the levels of IAA and ultimately growth. Ryugo and Sachs (1969) put this hypothesis to the test in several ways. They examined the effect of B 995, UDMH, and the structurally related retardant BOH, on the growth of mung bean (Phaseolus aurius) and on flower initiation of Fuschia hybrida. At minimal concentrations where UDMH and BOH no longer inhibited the growth of mung bean, B 995 was still very active. In Fuschia only B 995 induced flower initiation. Further, when plants were supplied with ^{14}C-labelled B 995, the radioactive retardant was recovered from the plant unchanged. They concluded that the postulated mechanism for B 995 action by Reed et al. (1965) was not consistent with these data.

The most popular mode of action of the growth retardants is as inhibitors of gibberellin biosynthesis. The evidence for such a mode of action stems from three main approaches to the problem: firstly, the proof of the ability of retardants to inhibit gibberellin biosynthesis in Gibberella fujikuroi; secondly, the demonstration of a reduction in endogenous, extractable gibberellins caused by retardant treatment of several plant species; and thirdly, the localization of a specific point of inhibition in the gibberellin pathway in enzyme and cell-free systems from higher plants.

Kende et al. (1963) reported an inhibition of GA-production but not growth by Amo 1618 and CCC in cultures of Fusarium moniliforme.

Phosfon D caused no inhibition of GA-production and increased dry weight of the fungus by 50%. Ninneman et al. (1964) extended this work on the fungus and showed that CCC was able to effectively block GA-production, and that the decreased gibberellin levels could not be accounted for by either destruction of GA or failure of its release from the mycelium.

Schechter and West (1969) showed Phosfon D to be a potent inhibitor of GA production in cell-free preparations of the fungus, and the lack of effectiveness of the retardant in cultures of Fusarium was concluded to be due to the very effective destruction of the retardant by the organism (Harada and Lang, 1965).

B 995 showed no ability to inhibit GA-production in Fusarium, and this inability was not due to break-down of the retardant as it could be readily recovered from the medium (Harada and Lang, 1965).

The first chemical evidence for an inhibition of gibberellin biosynthesis in Gibberella was obtained by following the incorporation of 2-^{14}C-mevalonic acid into gibberellins in the presence and absence of retardants. Ruddat et al. (1965) demonstrated that 100 mg/l Amo 1618 caused complete inhibition of incorporation into extractable gibberellins.

Cross and Meyers (1969) presented similar evidence for the inhibition of gibberellin biosynthesis by Amo 1618 and CCC in Gibberella, and showed the retardants to inhibit the incorporation of 2-^{14}C-mevalonic acid into (-)-kaurene, an intermediate in the

gibberellin pathway.

Barnes et al. (1969), as an extension of this finding, located the point of inhibition of Amo 1618 and CCC as the cyclization of trans-geranylgeranyl pyrophosphate into (-)-kaurene.

It is therefore evident that in the fungus Fusarium moniliforme, Amo 1618, CCC and Phosfon D are all able to inhibit gibberellin biosynthesis, but B 995 is ineffective.

The effects of the retardants on gibberellin biosynthesis in plants have been studied in some detail, but such studies have always met with the problem that so far there is still a lack of knowledge and understanding of the complete biosynthetic pathway leading to gibberellin formation. Despite this fact strong chemical evidence exists that the gibberellin pathways in plants and the fungus are identical up to, at least, the formation of (-)-kaurene and its oxidation products (e.g. kaurenol, kaurenal and kaurenoic acid). It has been argued that several unrelated lines of evidence would suggest that the biosynthetic pathway from (-)-kaurene to gibberellin might well follow the same broad outlines as that in the fungus (Faull, 1973).

Several plant systems have been able to convert the labelled precursor 2-^{14}C-mevalonic acid into intermediates in the gibberellin biosynthetic pathway up to (-)-kaurene. Such systems include cell-free preparations from pea seeds (Coolbaugh and Moore, 1971; Anderson and Moore, 1967), pea fruits (Graebe, 1968), nucellar and

endosperm preparations of *Echinocystis macrocarpa* (Dennis *et al.*, 1965; Oster and West, 1968), castor bean seedlings (Robinson and West, 1970), and immature seed of *Curcurbita pepo* (Graebe, 1969). The retardant Amo 1618 was shown to inhibit the biosynthesis of (-)-kaurene in all of the above systems except *Curcurbita pepo*, where it was not tested. Dennis and West (1967) studied the oxidation of (-)-kaurene through kaurenol and kaurenal to kaurenoic acid in *Echinocystis macrocarpa*. Graebe *et al.* (1972) reported the conversion of 2-^{14}C-mevalonic acid into *ent*-gibberellane (GA_{12}-aldehyde) in a cell-free preparation from *Curcurbita pepo*. Barendse and Kok (1971) reported the conversion of ^{14}C-kaurene (synthesized from 2-^{14}C mevalonic acid in *Curcurbita pepo*) into a compound tentatively identified as a gibberellin and possibly GA_3.

The first extensions of the fungus work to the effects of retardants on higher plant gibberellin biosynthesis were conducted in the 1960's using mainly gibberellin levels, as determined by bioassay, as a measure of gibberellin biosynthesis. Baldev *et al.* (1965) showed that Amo 1618 reduced the levels of gibberellin in developing pea seeds, and that the depression of gibberellin levels was increased with increasing concentrations of retardant.

The levels of gibberellin and gibberellin-like substances are reduced by continuous application of CCC to root systems of *Impatiens glandulifera* (Reid and Carr, 1967), Amo 1618 and CCC treatment of red-irradiated barley (Reid *et al.*, 1968), Amo 1618 application to

the long-day plant *Silene armeria* (Cleland and Zeevaart, 1970), and by treatment of germinating hazel seeds with Phosfon D, B 995 and CO11 (Ross and Bradbeer, 1971).

The first chemical evidence for an inhibition of gibberellin biosynthesis by growth retardants in higher plants was obtained by Dennis *et al.* (1965). Using a nucellar and endosperm preparation from *Echinocystis macrocarpa*, they were able to pin-point the site of action of the retardants Amo 1618, Phosfon D and Phosfon S as the cyclization step in the pathway in which *trans*-geranylgeranyl pyrophosphate is converted to (-)-kaurene. In this system CCC was ineffective at both concentrations (10 and 100 μg/ml) tested, and B 995 caused only 41% inhibition at the high concentration. Dennis and West (1967), using the same system, failed to demonstrate any inhibition in the conversion of (-)-kaurene to kaurenoic acid by the retardants Amo 1618, Phosfon D, Phosfon S, CCC and B 995.

Using an enzyme system from pea seeds, Anderson and Moore (1967) demonstrated an inhibition in the incorporation of 2-^{14}C-mevalonic acid into (-)-kaurene by Amo 1618 and CCC. The inhibition by CCC only occurred at very high concentration (approximately 1,000 fold that of Amo 1618 to produce the same inhibition). Similar results were obtained in *Curcurbita pepo* (Barendse and Kok, 1971). Amo 1618 has also been demonstrated to inhibit the formation of (-)-kaurene in pea fruits (Graebe, 1968).

Robinson and West (1970) showed that CCC, Amo 1618 and Phosfon D were all able to inhibit the formation of beyerene, sandaracopimaradiene, (-) kaurene and trachylobane from *trans*-geranylgeranyl pyrophosphate in an enzyme system from castor bean (*Riccinus communis* L.) seedlings. Only Phosfon D inhibited the cyclization of *trans*-geranylgeranyl pyrophosphate to casbene in the same system.

It therefore appears that retardants may act as inhibitors of cyclization reactions in diterpene biosynthetic pathways, in particular the gibberellin biosynthetic pathway, and as such should more properly be considered enzyme inhibitors rather than anti-metabolites.

The only reported effect of the retardant B 995 on gibberellin biosynthesis comes from Ryugo and Sachs (1969), who demonstrated the inhibition of incorporation of 2-^{14}C-mevalonic acid into a product which they tentatively identified as kauren-19-ol in immature peach ovules.

A correlation between the effects of retardants on growth and gibberellin production in higher plants has been established in some cases where a depressed production of gibberellin is paralleled, to some extent, by a decreased growth rate. For example, in the long-day plant *Silene armeria*, where gibberellins are required for stem elongation but not flowering, Amo 1618 causes an inhibition of stem elongation, has no effect on flowering, and reduces markedly the levels of extractable gibberellins (Cleland and Zeevaart, 1970). A comparison of the effects of CCC and several of its analogues on

plant growth retardation (Tolbert, 1960a; Tolbert, 1960b) and their ability to inhibit gibberellin biosynthesis in *Fusarium moniliforme* was made by Harada and Lang (1965), and a close parallel was shown to exist between the two effects. Similar parallels have been demonstrated for growth and gibberellin production in barley seedlings treated with Amo 1618 and CCC (Reid *et al*., 1968), CCC-treated sunflower plants (Jones and Phillips, 1967), Amo 1618-treated *Silene armeria* (Van den Ende and Zeevaart, 1971), and seeds of CCC-treated *Pharbitis nil* plants (Zeevaart, 1966; Barendse, 1971). A parallel also exists for the effects of retardants on gibberellin production in, and germination of, chilled seeds of *Corylus avellana* (Ross and Bradbeer, 1971).

Not all of the retardant effects can be explained by an inhibition of GA-biosynthesis, however. Earlier in this Introduction it was pointed out that the application of GA_3 to retardant-treated plants would not completely reverse the retardant effect in many cases. In crown gall tissue of *Helianthus annuus* it was also shown that GA_3 exerts an inhibitory effect of its own (Bristow and Simmonds, 1968). There are also a number of reports which show increased gibberellin production as a result of CCC treatment (Reid and Crozier, 1970; Halevy and Shilo, 1970; Carr and Reid, 1968).

Sachs and Wohlers (1964) showed that GA_3 at levels up to 150×10^{-5} M was unable to reverse the inhibition of cell division and expansion by Amo 1618, Phosfon D and CCC in carrot root tissues

cultured *in vitro*. Not only did the exogenous GA_3 fail to reverse this inhibition by the retardants, but it enhanced the inhibitory effect of both CCC and Phosfon.

Crozier *et al*. (1973) examined the effects of Amo 1618 on the endogenous gibberellin levels and growth of leaves, stems and roots of *Phaseolus coccineus* seedlings. Studies on growth revealed that the retardant inhibited stem elongation, leaf area and root growth, and that application of saturating levels of GA_3 was effective in complete reversal of only the retardation of stem growth caused by the lowest (10 mg/l) level of Amo 1618. An examination of endogenous levels of GA showed that although 50 mg/l Amo 1618 caused severe dwarfing, it was only able to reduce the levels of GA by about 30%. The authors state that most of this 30% reduction could be accounted for by experimental latitude associated with bioassays, but do not discount the possibility of one specific gibberellin being strongly inhibited without an appreciable decrease in total gibberellin in the plant. This data suggests that inhibition of gibberellin biosynthesis is not the sole action of the retardant.

Gaspar *et al*. (1971) examined several processes in plants and the effects of Amo 1618 and GA_3 either separately, or in combination, on these processes. Peroxidase activity in lentil stems was stimulated by both Amo 1618 and GA_3; in combination they produced an additive effect on the stimulation of peroxidase activity. An examination of the growth of lentils showed strong inhibition of both

stem and root growth by Amo 1618, and although able to completely reverse the retardation of stem growth, added GA_3 could only partially reverse the root growth inhibition.

It therefore seems clear that, with the possible exception of stem elongation in some species, the retardants' effects on plant and cellular processes cannot be explained entirely by an inhibition of gibberellin biosynthesis.

One further suggested mode of action of the retardants involves the inhibition of cholinesterase activity in plants. Tanaka and Tolbert (1966) demonstrated that the retardant CCC interfered with choline metabolism in spinach leaves by stimulating choline kinase activity. Gibberellic acid caused an inhibition of this enzyme activity.

Newhall and Pieringer (1966) reported the synthesis of a number of quaternary ammonium derivatives of (+)-limolene, which, when tested on grapefruit and bean seedlings, showed growth retarding activity (Pieringer and Newhall, 1967; Pieringer and Newhall, 1970). The most important feature of these compounds which determined their growth retarding ability was shown to be the length of the n-alkyl carbon chain (Pieringer and Newhall, 1968). Because of the structural similarities of these quaternary ammonium compounds to certain inhibitors of animal cholinesterases, Newhall (1969) tested their effects on the activity of pseudocholinesterase from human blood serum and demonstrated (by kinetic analysis) a direct correlation

between the plant growth retarding ability of these compounds and their effectiveness in inhibiting the activity of this enzyme. During the study the growth retardants B 995 and CCC were found to have no inhibitory effect on pseudocholinesterase activity at concentrations as high as 0.01 M, whereas Amo 1618 was a potent inhibitor. As a result of this work Newhall concluded that the growth retarding ability of the chemicals tested may be mediated through inactivation of a hydrolase enzyme which is essential to gibberellin or auxin biosynthesis.

The occurrence of cholinesterases in plant tissue has been reported (Riov and Jaffe, 1973a), and although the properties of the isolated enzymes show remarkable similarity to animal cholinesterases, they are not identical. The mung bean root cholinesterases purified by Riov and Jaffe showed relative insensitivity to eserine, a potent inhibitor of animal cholinesterases. The growth retardants Amo 1618, CCC, Phosfon D and some quaternary ammonium derivatives of (+)-limolene were all tested in the mung bean root system and all caused an inhibition of cholinesterase activity at concentrations which cause growth retardation (Riov and Jaffe, 1973b). A more thorough examination of the effect of Amo 1618 on mung bean cholinesterase activity demonstrated that the retardant was a non-competitive inhibitor of the enzyme and was apparently specific for choline esterases, as all non-cholinesterases tested were unaffected (Riov and Jaffe, 1973c).

All of these findings taken as a whole present a possible point of action of growth retardants in root systems. It is difficult to envisage a direct interaction between growth in above-ground parts of the plant and cholinesterase activity, but the possibility exists that acetylcholine is itself an inhibitor of growth, or that acetylcholinesterase plays an important role in processes essential to growth. Acetylcholine has been shown to retard the growth of dark-grown cucumber seedlings at levels lower than Amo 1618 (Jaffe, 1971), and to reduce the number of secondary roots of mung bean seedlings (Jaffe, 1970).

As mentioned earlier in this Introduction, the retardants are also able to cause reduced chlorophyll levels in higher plants. Such a reduction was proposed to be due to an inhibition of plastid protein synthesis which in turn led to an inhibition of chlorophyll synthesis. Another possible explanation of this action is afforded by the work of Simpson et al. (1974) who were able to show an inhibition by CCC of carotenoid biosynthesis in excised pumpkin cotyledons, accompanied by an accumulation of lycopene. The animal sterol biosynthesis inhibitor SK and F 13831, which bears a structural similarity to CCC, also inhibited carotenoid biosynthesis, resulting in lycopene accumulation. It is therefore possible that inhibition of carotenoid synthesis would lead to reduced β-carotene levels, and consequently an increased photodestruction of chlorophyll and chloroplasts (see Walles, 1967). It is of interest that the inhibition by a retardant of yet another cyclization step (lycopene to carotenes) in an isoprenoid pathway has

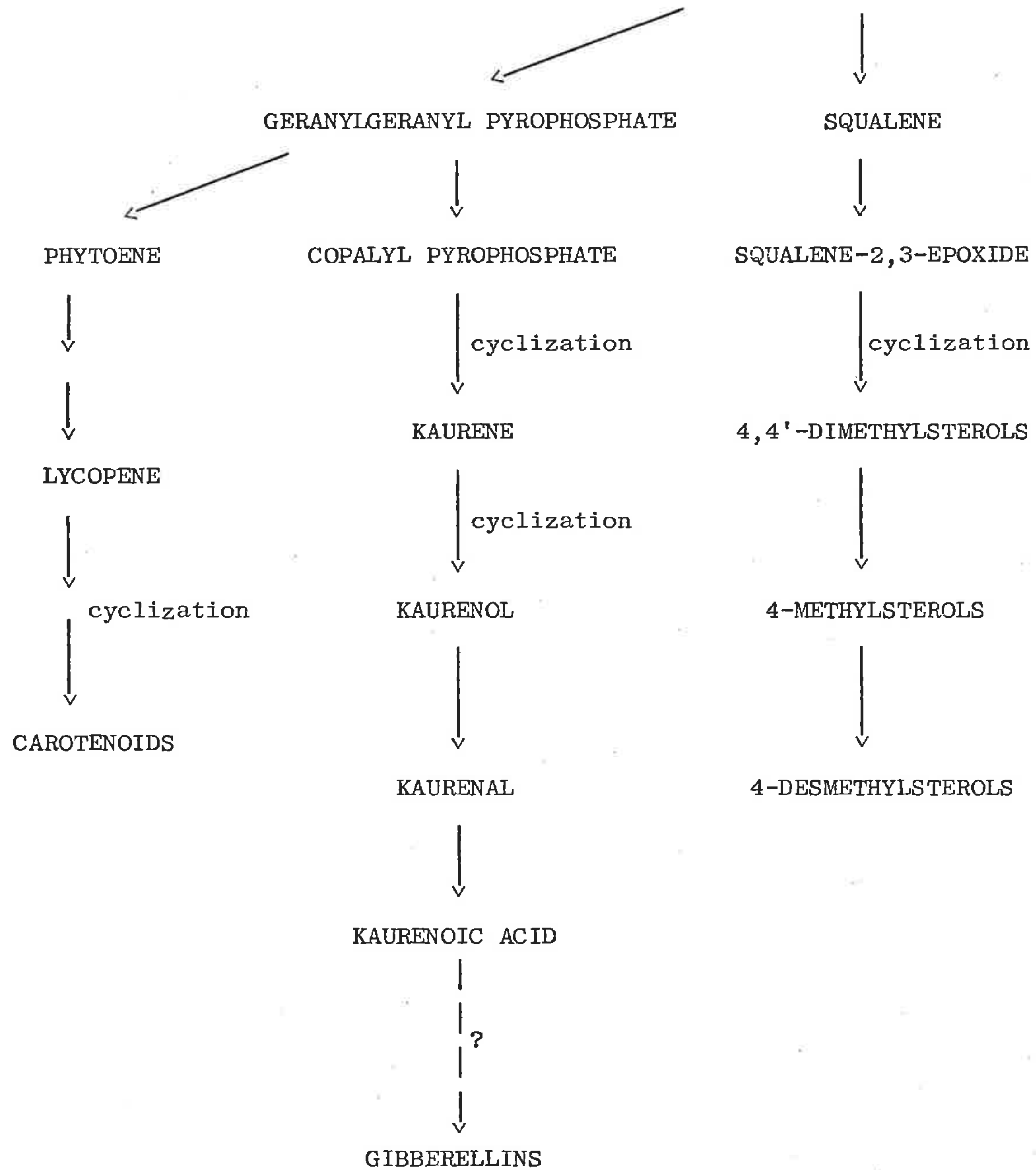
been established.

4. Effects on Animal Cholesterol Biosynthesis

An examination of isoprenoid biosynthetic pathways leading to formation of gibberellins and sterols shows a common route up to the formation of farnesyl pyrophosphate, and then a branching to geranyl-geranyl pyrophosphate (the hydrocarbon precursor of gibberellins, carotenoids and diterpenes) and squalene, the hydrocarbon precursor of sterols (see figure 3). Observing the marked similarities in the two pathways, Paleg and Seamark (1968) proposed that the growth retardants which inhibit any step in the sequence leading to gibberellin formation might also inhibit a similar step in the cholesterol biosynthetic pathway in animal systems. Using cell-free rat liver preparations as the test system Paleg and Seamark showed the retardants Phosfon D and CCC to inhibit incorporation of radioactive precursors acetate and mevalonate into cholesterol. Phosfon D was a potent inhibitor of the incorporation of both precursors, but CCC inhibited the incorporation of acetate quite markedly while not showing a strong effect with mevalonate.

As an extension of this work Paleg (1970a) examined the effects of a single concentration (1,000 μg/ml) of five retardants on the incorporation of ^{14}C-acetate and 2-^{14}C-mevalonate into cholesterol in rat liver homogenates. Phosfon D, Phosfon S, Amo 1618 and CCC inhibited the incorporation of ^{14}C-acetate into cholesterol, but B 995

Figure 3. An outline of the isoprenoid biosynthetic pathways which lead to the formation of gibberellins, sterols and carotenoids.


ACETATE
MEVALONATE
ISOPENTENYL PYROPHOSPHATE
GERANYL PYROPHOSPHATE
FARNESYL PYROPHOSPHATE
GERANYLGERANYL PYROPHOSPHATE
SQUALENE
PHYTOENE
COPALYL PYROPHOSPHATE
SQUALENE-2,3-EPOXIDE
cyclization
cyclization
KAURENE
4,4'-DIMETHYLSTEROLS
LYCOPENE
cyclization
cyclization
KAURENOL
4-METHYLSTEROLS
CAROTENOIDS
KAURENAL
4-DESMETHYLSTEROLS
KAURENOIC ACID
?
GIBBERELLINS

was ineffective. Both B 995 and CCC were ineffective in inhibiting the incorporation of 2-^{14}C-mevalonate into cholesterol. When double (2,000 μg/ml) the concentration of B 995 was applied to this system an inhibition of incorporation of both precursors was observed.

Paleg (1970b) used the ability of the plant steroid digitonin to specifically combine with and precipitate 3β-hydroxysterols from aqueous solution (see Fieser and Fieser, 1950) in order to examine the effects of the retardants on the production of sterols and cyclic intermediates collectively. The five retardants tested were Phosfon D, Phosfon S, CO11, Amo 1618 and B 995. The results showed that each of the retardants, at concentrations which inhibited sterol biosynthesis, exerted their effects in slightly different ways, suggesting that they might differ from each other in their sites of inhibition. Of particular interest was the finding that Amo 1618 caused a pronounced accumulation of radioactivity in the hydrocarbon fraction which coincided with the inhibition of mevalonate incorporation into cholesterol. The accumulation product was later isolated from the digitonin filtrate, purified in several thin-layer chromatography systems and identified by G.L.C. mass spectrometry as the intermediate squalene-2,3-epoxide (unpublished data).

It was clear that the growth retardants were cholesterol biosynthesis inhibitors, at least in rat liver preparations, and that their effects, to some extent, paralleled those of the structurally similar animal sterol inhibitors, the SK and F compounds (see Holmes

and Di Tullio, 1962). Certain of these animal sterol inhibitors have been shown to retard the growth of both tall and dwarf varieties of pea (Moore and Anderson, 1966), inhibit floral induction in Lolium temulentum (Evans, 1964), Xanthium (Bonner et al., 1963; Kimura et al., 1973), and Pharbitis nil (Bonner et al., 1963; Sachs, 1966), inhibit the biosynthesis of gibberellin in Gibberella fujikuroi (Reid, 1969), and in endosperm of Echinocystis macrocarpa (Dennis and West, 1967), and inhibit sterol biosyntnesis in tobacco (Reid, 1968a), Pisum sativum (Reid, 1968b), Petasites hybridus (Zabkiewicz et al., 1969) and Xanthium (Kimura et al., 1973).

Douglas and Paleg (1972) tested the effect of 1,000 µg/ml Amo 1618 on the incorporation of 2-^{14}C-mevalonic acid into sterols and their precursors in rootless tobacco seedlings and the results paralleled exactly those found in cell-free rat liver preparations, i.e. a marked inhibition in the incorporation of precursor into sterols accompanied by an accumulation of squalene-2,3-epoxide. These results clearly showed that the retardant Amo 1618 was a powerful inhibitor of squalene-2,3-epoxide cyclase in plants as well as in animals. Kimura et al. (1973) reported that Amo 1618 also inhibited the biosynthesis of sterols in Pharbitis in much the same way as reported for Nicotiana tabacum (Douglas and Paleg, 1972).

Reid (1968a) located the site of inhibition of the animal sterol biosynthesis inhibitor SK and F 7997-A_3 in Nicotiana tabacum as the enzymic cyclization of squalene-2,3-epoxide. Similar findings were

reported by Zabkiewicz *et al.* (1969) in *Petasites hybridus*, although Kimura *et al.* (1973) argued that the inhibition came one step later as in animal systems, i.e. at the demethylation of 4,4'-dimethylsterols to 4-methylsterols.

The retardants therefore behave in a very similar manner to the animal sterol inhibitors in both plants and animals, and must be considered as inhibitors of sterol biosynthesis in plants.

5. Plant Sterols : Biosynthesis and Functions

The occurrence of sterols in higher plants is well established and ubiquitous throughout the plant kingdom. Even that previously considered 'typical zoosterol', cholesterol, has been reported to occur in several plant species including *Solanum tuberosum* and *Dioscorea spiculiflora* (Johnson *et al.*, 1963), and *Nicotiana tabacum* (Bolt and Clarke, 1970; Bush and Grunwald, 1970). Because of their widespread occurrence and high levels in plants, and their structural similarity to functionally important sterols in animal systems, the plant sterols can no longer be considered as secondary metabolites in the plant kingdom. Rather, they must be considered to have at least the potential to carry out the same functional roles in plants as have been assigned to animal sterols (Heftman, 1971).

Such an increasing awareness of the importance of sterols in plants can only accentuate the necessity for a more detailed study of the effects of growth retardants on sterol biosynthesis in plants.

The biosynthetic pathway leading to the formation of sterols in plants has been studied in some detail during the last decade or so. The outcome of these studies has been the establishment of a complete scheme of events leading to the synthesis of such well-known sterols as cholesterol, β-sitosterol, stigmasterol and campesterol.

The first half of the pathway (prior to cyclization of squalene-2,3-epoxide) is identical with that in animals. Evidence for the pathway in higher plants comes from the following data:

(a) the conversion of radioactively labelled precursors (acetate and/or mevalonate) into squalene has been demonstrated *in vitro* using tissue cultures of tobacco cells (Benveniste *et al.*, 1964), isolated tomato and carrot plastids (Beeler *et al.*, 1963), and cell-free systems from pea shoots (Graebe, 1967), tobacco tissues (Benveniste *et al.*, 1967), pea seeds (Capstack *et al.*, 1962), and *Mentha piperata* (Croteau and Loomis, 1973).

(b) geraniol, another intermediate in the isoprenoid pathways is phosphorylated by germinating peas *in vitro* (van Aller and Nes, 1968) and converted to squalene *in vitro* as well as *in vivo* (Baisted, 1967).

(c) farnesyl pyrophosphate -4,8,12-^{14}C is converted to squalene by enzymes from carrot and tomato (Beeler *et al.*, 1963).

(d) the labelling pattern for squalene synthesized from 2-^{14}C-mevalonic acid by germinating pea seeds is identical to that obtained in rat liver (Capstack *et al.*, 1965).

(e) squalene-2,3-epoxide is synthesized from acetate-1-^{14}C in tobacco tissue culture (Benveniste and Massey-Westropp, 1967) and the latex of *Euphorbia cyparissias* (Ponsinet and Ourisson, 1968), and accumulates as a result of treatment with SK and F 7997-A_3 in *Nicotiana tabacum* (Reid, 1968a) and *Xanthium* (Kimura *et al*., 1973).

A biosynthetic scheme showing all of the major intermediates formed in plants from acetyl CoA up to and including squalene-2,3-epoxide is shown in figure 4. In this figure the carbon atoms represented by dots show the labelling pattern from 2-^{14}C-mevalonic acid as demonstrated by Capstack *et al*. (1965).

The next step in the sterol biosynthetic pathway involves the cyclization of squalene-2,3-epoxide to the first cyclic sterol intermediate. It is here that the animal and plant systems differ. The first cyclic intermediate in animal cholesterol biosynthesis is lanosterol, whereas in plants it is cycloartenol. The divergence in the pathways is now well established.

^{14}C-squalene-2,3-epoxide is cyclized only to cycloartenol and 14-methylenecycloartenol in cell-free preparations of *Phaseolus vulgaris* leaves (Rees *et al*., 1968), the phytoflagellate *Ochromonas malhamensis* (Rees *et al*., 1969), and tobacco tissue cultures (Eppenberger *et al*., 1969; Heintz and Benveniste, 1970). Of all the plant species so far examined lanosterol has only been shown to occur in the latex of *Euphorbiacea* (Goad, 1970), and its presence there may be due to the ability of the latex (e.g. in *Euphorbia lathyris*) to

convert cycloartenol to lanosterol (Ponsinet and Ourisson, 1968). Williams and Goodwin (1965) tentatively identified lanosterol in tissue cultures of Paul's Scarlet Rose, but their identification was not exhaustive and by no means definitive.

Hall et al. (1969) showed that all these 4,4'-dimethylsterols (viz. lanosterol, cycloartenol and 24-methylenecycloartenol) were converted into poriferasterol in Ochromonas malhamensis.

Hewlins et al. (1969) demonstrated a slight preference for cycloartenol by tobacco tissue cultures although the tissue did utilise lanosterol to produce sterols. As lanosterol was not detected even in trace amounts in Ochromonas (Rees et al., 1969) or Nicotiana (Eppenberger et al., 1969), Hewlins et al. (1969) concluded that cycloartenol was the true 4,4'-dimethylsterol intermediate and that plants displayed a lack of enzyme substrate specificity.

The most definitive evidence for cycloartenol as the true product of squalene-2,3-epoxide cyclization in plants comes from double labelling experiments. Rees et al. (1968) supplied [2-^{14}C-(4R)-4-$^{3}H_{1}$]-mevalonic acid to leaves of Solanum tuberosum and determined the $^{3}H/^{14}C$ ratio of the 4,4'-dimethylsterols produced. If lanosterol were formed, the $^{3}H/^{14}C$ ratio would be 5/6 due to the loss of a hydrogen (in this case ^{3}H) at C-9 during the formation of the Δ^{8} double bond. The formation of cycloartenol requires no elimination of hydrogen and the ratio would therefore be 6/6, the same as for squalene (see fig. 4). The results showed a definite

Figure 4. Synthesis of squalene-2,3-epoxide from acetyl CoA in plants and animals. A rough outline of the major intermediates formed from acetyl CoA in the sterol pathway is presented.

I = acetyl CoA

II = acetoacetyl CoA

III = mevalonate

IV = isopentenyl pyrophosphate

V = dimethylallyl pyrophosphate

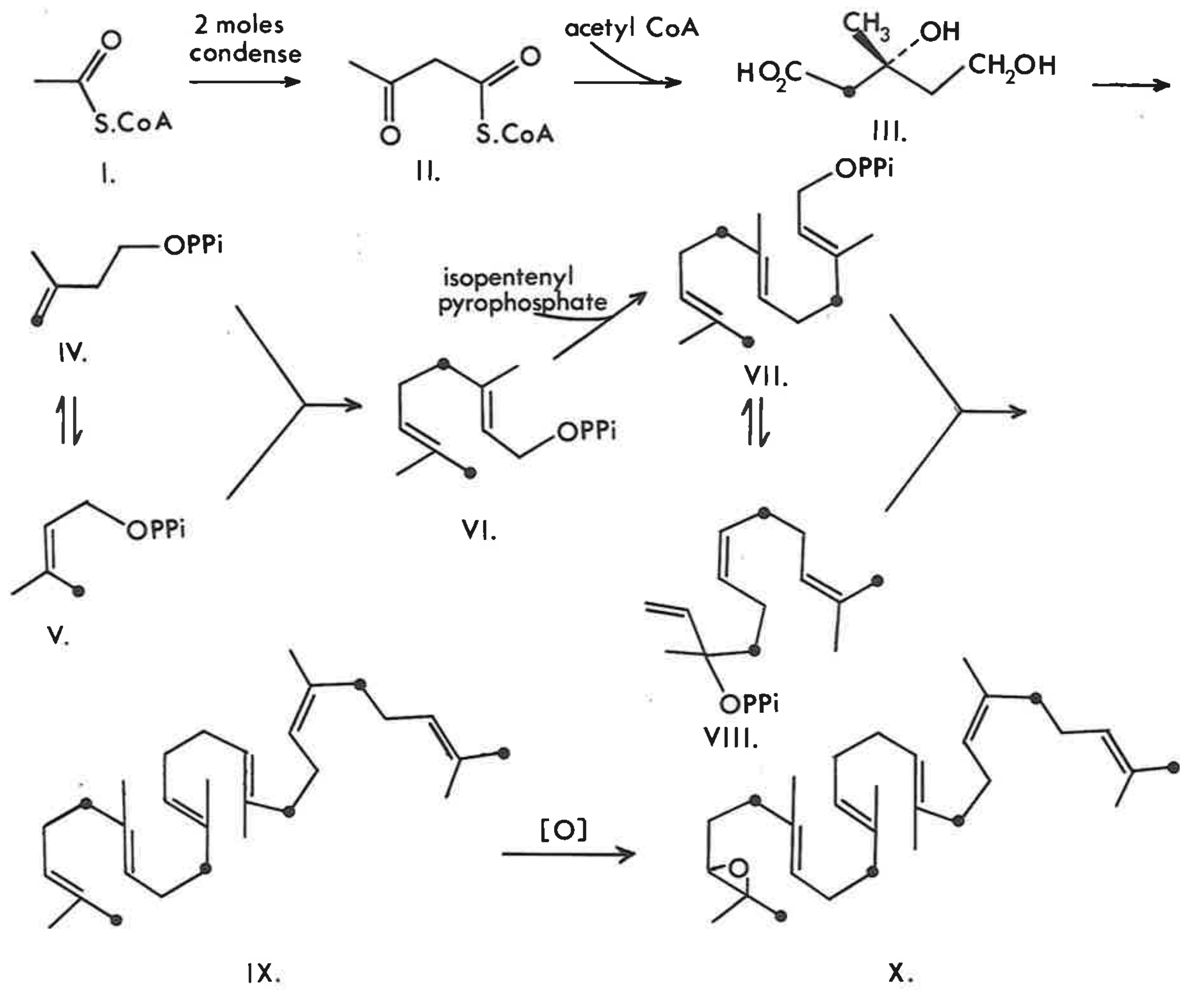
VI = geranyl pyrophosphate

VII = farnesyl pyrophosphate

VIII = nerolidyl pyrophosphate

IX = squalene

X = squalene-2,3-epoxide


2 moles
condense
acetyl CoA
S.CoA
I.
O
S.CoA
II.
CH_3
OH
HO_2C
CH_2OH
III.
OPPi
IV.
V.
isopentenyl
pyrophosphate
VI.
VII.
VIII.
IX.
[O]
X.

6/6 ratio and provided convincing evidence for the true cyclic intermediate in plants being cycloartenol and not lanosterol.

The proposed mechanism of squalene-2,3-epoxide cyclization in animals and plants (Mulheirn and Caspi, 1971) is shown in figure 5. The labelling pattern from [2-^{14}C-(4R)-4-^{3}H$_{1}$]-mevalonic acid is also shown.

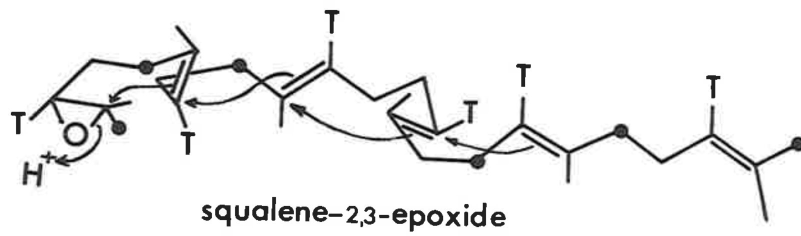
The further conversion of cycloartenol to 4-desmethylsterols such as β-sitosterol, stigmasterol, campesterol and cholesterol most likely follows the scheme shown in figure 6. This scheme was based on several points of evidence, the main one being the observation that all of the intermediates shown in the pathway have been isolated and identified as natural products (see Goodwin, 1971).

Benveniste *et al.* (1966) were able to demonstrate the conversion of acetate-1-^{14}C into highly labelled cycloartenol, which in turn was further utilised by tobacco tissue culture to form other sterols.

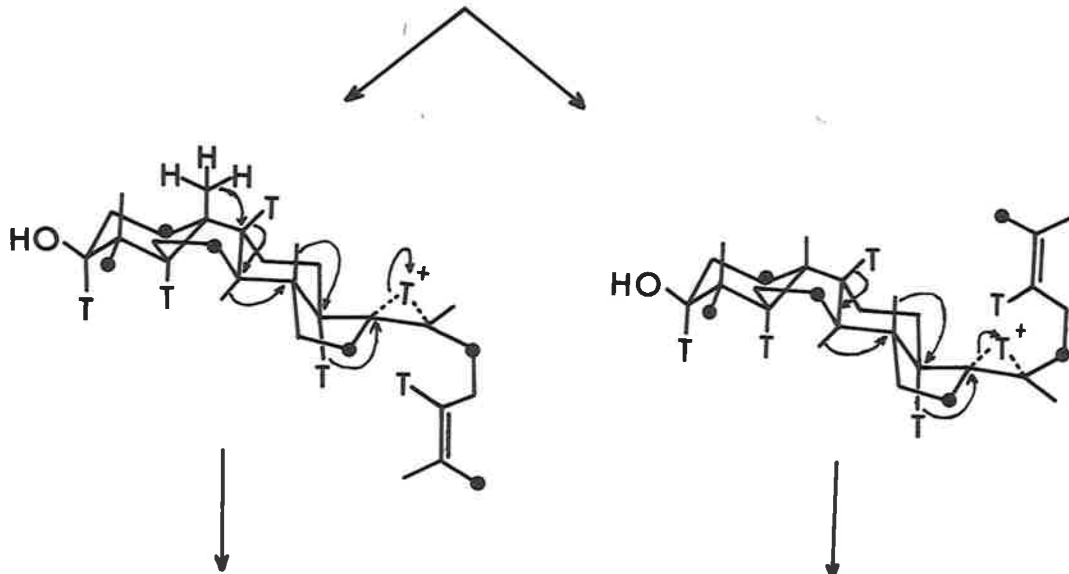
Hall *et al.* (1969) and Lenton *et al.* (1971) have demonstrated the conversion of 24-methylenecycloartenol to sterols in *Ochromonas malhamensis*.

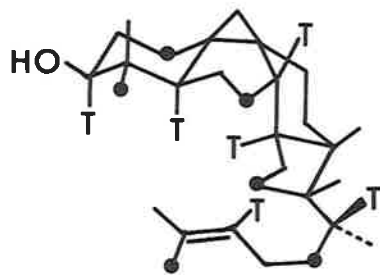
2-^{14}C-mevalonic acid is converted to, among other intermediates, cycloeucalenol and obtusifoliol in tobacco tissue cultures (Benveniste, 1968) and *Larix decidua* leaves (Goad and Goodwin, 1967). Conversion of the precursor into 28-isofucosterol has also been shown (van Aller *et al.*, 1968; Goad *et al.*, 1969) in some plant species including *Pinus pinea*, *Larix decidua*, and *Pisum sativum*. van Aller *et al.*

Figure 5. Mechanism of cyclization of squalene-2,3-epoxide. The figure shows the way in which the sterol intermediate squalene-2,3-epoxide undergoes cyclization to produce cycloartenol in plants and lanosterol in animals (from Mulheirn and Caspi, 1971). T = tritium from [2-^{14}C-(4R)-^{3}H]-mevalonic acid, and the ^{14}C atoms are denoted as black dots.

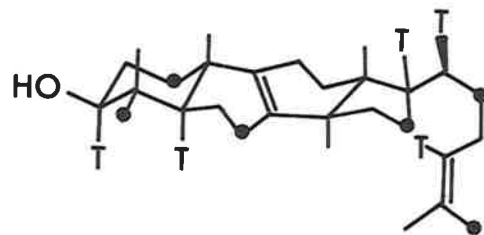


squalene–2,3-epoxide





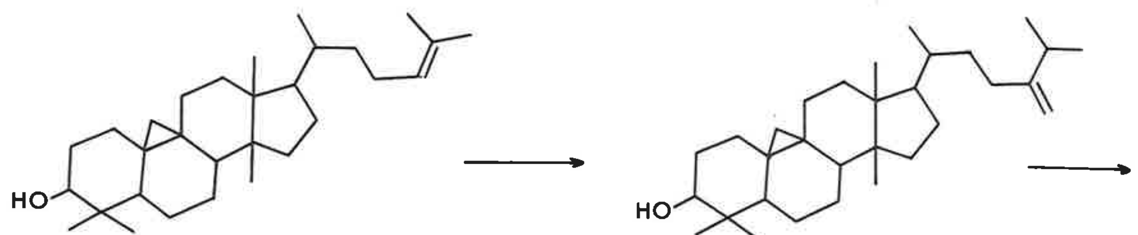
cycloartenol



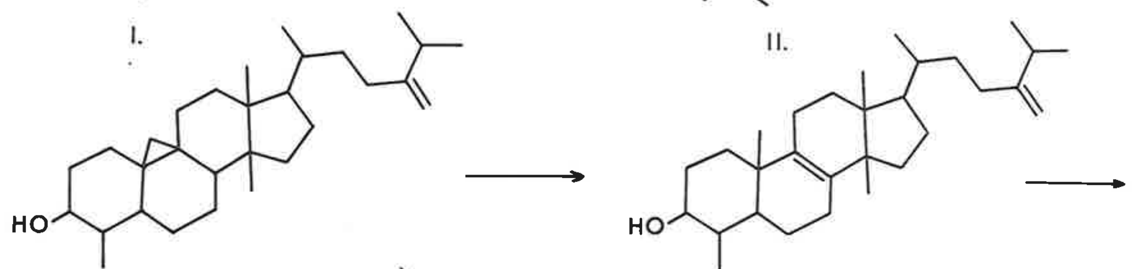
lanosterol

Figure 6. The conversion of cycloartenol to plant sterols. The figure shows the proposed pathway for the conversion of cycloartenol, the first cyclic intermediate in plant sterol biosynthesis, into some of the better known plant sterols (from Goodwin, 1971).

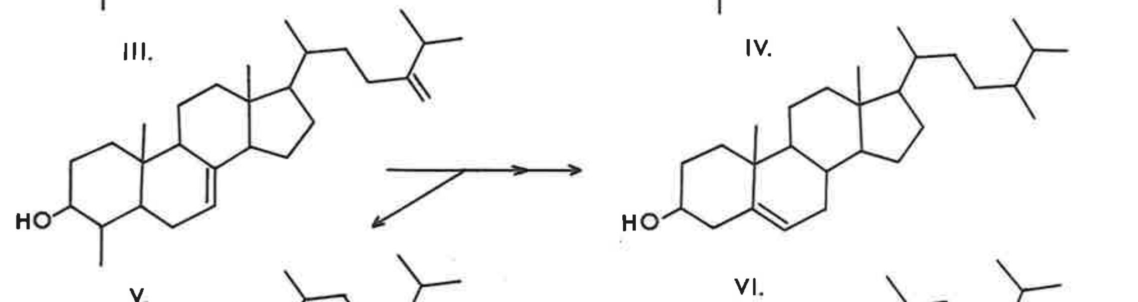
I	=	cycloartenol
II	=	24-methylenecycloartenol
III	=	cycloeucalenol
IV	=	obtusifoliol
V	=	24-methylenelophenol
VI	=	campesterol
VII	=	24-ethylidenelophenol
VIII	=	β-sitosterol
IX	=	stigmasterol
X	=	cholestatrienol
XI	=	cholesterol



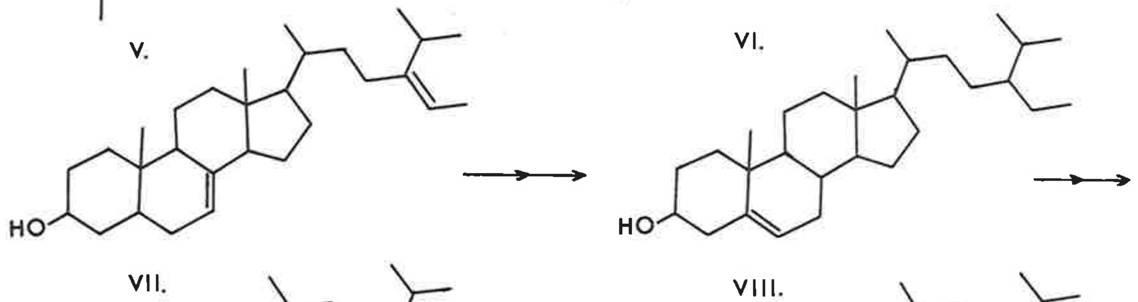
I. II.



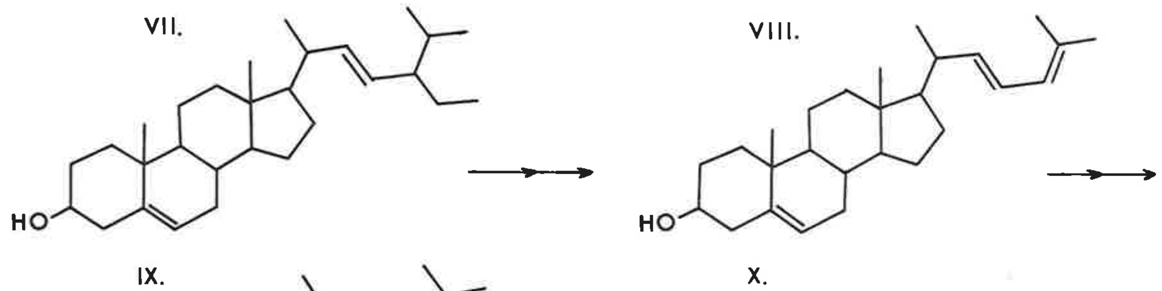
III. IV.



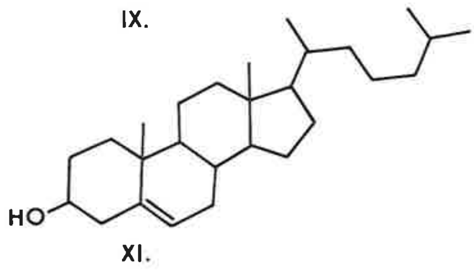
V. VI.



VII. VIII.



IX. X.



XI.

(1968) also demonstrated the conversion of 28-isofucosterol to β-sitosterol in pine.

Knapp and Nicholas (1971) measured the incorporation of 2-^{14}C-mevalonate into phytosterols and their intermediates in banana (*Musa sapientum*) peel slices. The slow turnover of phytosterol precursors in this system made it possible for the authors to establish the sequence of appearance of intermediates after cycloartenol as 24-methylenecycloartenol, then cycloeucalenol followed by the 4-desmethylsterols. The order of appearance of free 4-desmethylsterols was shown to be β-sitosterol, then stigmasterol and campesterol, confirming the observed conversion of β-sitosterol to stigmasterol in *Digitalis lanata* (Bennett and Heftmann, 1969).

A study of the labelling pattern of intermediates in poriferasterol biosynthesis from [2-^{14}C-(4R)-4-^{3}H$_1$]-mevalonic acid has allowed Smith *et al.* (1972) to establish the involvement of an 24-ethylidene sterol in the C-24 alkylation mechanism in *Ochromonas malhamensis*.

As more data is obtained, confirmation of the scheme shown in figure 6 may become more complete.

II. MATERIALS AND METHODS

A. MATERIALS

1. Chemicals and Reagents

The chemicals and reagents used throughout this project were all analytical grade unless otherwise stated. Suppliers of chemicals and reagents are listed below:

1-^{14}C-acetate, sodium salt; Radiochemical Centre, Amersham, England.

acetonitrile; British Drug Houses, Poole, England.

Amo 1618 (B grade); Calbiochem, California, U.S.A.

Anachrom ABS; Analabs Inc., Connecticut, U.S.A.

ATP; Sigma Chemical Co., Missouri, U.S.A.

B 995; United States Rubber Co., Nangatuck, Conn., U.S.A.

berberine hydrochloride; Fluka and Co., Buchs, Switzerland.

bovine serum albumin (crystalline); Sigma Chemical Co., Missouri, U.S.A.

CCC [50% (w/v) aqueous solution]; Cyanamid International, New Jersey, U.S.A.

α-cholestane; Sigma Chemical Co., Missouri, U.S.A.

cholesterol (A grade); Calbiochem, California, U.S.A.

Folin and Ciocalteu reagent; British Drug Houses, Poole, England.

gaschrom-Q; Applied Science Labs., Pennsylvania, U.S.A.

gibberellin A_3 (97.4%); Merck and Co., New Jersey, U.S.A.

glucose-6-phosphate; Sigma Chemical Co., Missouri, U.S.A.

glucose-6-phosphate dehydrogenase; Sigma Chemical Co., Missouri, U.S.A.

glutathione (reduced); L. Light Chem. Co. Ltd., Colnbrook, England.

HMDS; Applied Science Labs., Pennsylvania, U.S.A.

HMG; Sigma Chemical Co., Missouri, U.S.A.

3-^{14}C-HMG; New England Nuclear, Boston, Massachusetts, U.S.A.

lanosterol; Mann Research Labs., N.Y., U.S.A.

DL-mevalonate; Sigma Chemical Co., Missouri, U.S.A.

DL-2-^{14}C-mevalonic acid lactone; Radiochemical Centre, Amersham, England.

2-mercaptoethanol; Fluka and Co., Buchs, Switzerland.

molecular sieve (5A); British Drug Houses of Australia.

NAD; Sigma Chemical Co., Missouri, U.S.A.

NADH; Sigma Chemical Co., Missouri, U.S.A.

NADP; Sigma Chemical Co., Missouri, U.S.A.

NADPH; Sigma Chemical Co., Missouri, U.S.A.

nicotinamide; British Drug Houses, Poole, England.

nitroprusside; British Drug Houses, Poole, England.

OV-101; Applied Science Labs., Pennsylvania, U.S.A.

parafilm; Gallenkamp, England.

Phosfon D; Mobil Chemical Co., Virginia, U.S.A.

Phosfon S; Mobil Chemical Co., Virginia, U.S.A.

dimethyl POPOP; Packard Instrument Co., Illinois, U.S.A.

PPO; Packard Instrument Co., Illinois, U.S.A.

pyrogallol; Ajax Chemicals Ltd., Australia.

Q-gas; C.I.G., Australia.

SE-30; Applied Science Labs., Pennsylvania, U.S.A.

Silyl-8; Pierce Chemical Co., Illinois, U.S.A.

silica gel G; Merck and Co., New Jersey, U.S.A.

SK and F 7732-A_3; Smith Kline and French Labs., Phil., U.S.A.

SK and F 7997-A_3; Smith Kline and French Labs., Phil., U.S.A.

squalene; Sigma Chemical Co., Missouri, U.S.A.

All cylinders of gas (viz. dry air, dry nitrogen and dry hydrogen) were supplied by C.I.G., Australia.

2. Solvents

All solvents used for extraction procedures and for chromatography were distilled in a glass fractionating column before use.

Anhydrous benzene was prepared by distilling the solvent into an oven-dried (120°; 4 hr) glass container and stored over extruded sodium wire in a tightly stoppered container.

Pyridine was dried by refluxing for several hours over KOH and stored in a stoppered glass container over Molecular Sieve 5A which had been previously activated by heating to 450° for 2-3 hr.

3. Experimental Material

Nicotiana tabacum (cv. Turkish Samson) seeds were obtained by selfing of plants supplied to me by Dr. J. Randall, Dept. of Plant Pathology, Waite Agricultural Research Institute.

Digitalis (foxglove), dwarf pea (cv. Greenfeast) and dwarf bean (cv. Pioneer) seeds were all obtained from Yates, Australia.

Rats used for cell-free liver preparations were male hooded and albino Wistar rats of about 300-400 g body weight obtained from the Central Animal House, University of Adelaide.

4. Equipment

All seedlings were germinated and grown in controlled environment growth cabinets (Zankel Scientific Equipment Co.) regulated at constant temperature ($23^{\circ} \pm 2^{\circ}$) and constant light intensity (2,000 ft. c.). Light was supplied by a double bank of fluorescent tubes and light intensity at the plant level was maintained by adjusting the distance between the plants and the light bank.

Radioactivity scanning of T.L.C. plates was performed by a Nuclear Chicago Actigraph II Scanner, Model 8415.

Liquid Scintillation radioactive counting was carried out in a Packard Tricarb Liquid Scintillation Spectrometer, Model 3320.

Routine G.L.C. analysis and fraction collecting were carried out on a Shimadzu GC-1C gas-liquid chromatograph fitted with a flame ionization detector and a 9:1 stream splitter. When collecting fractions, the eluate passing from the stream splitter was allowed to move into glass luer-lock syringes packed with methanol-soaked glass wool via a heated metal Luer-lock fitting which was connected to the outlet from the stream splitter in the heated metal block (see flow

diagram, figure 1). Fractions were collected by changing glass syringes manually at regular time intervals, or when required.

High speed centrifugation was performed by an MSE Ultracentrifuge (MK 1) for spins at up to 20,000 x g and on a Beckman L-65 Ultracentrifuge using a Ti50 rotar for 200,000 x g centrifugation.

5. Scintillation Fluid

Standard toluene-based scintillation fluid prepared by dissolving dimethyl POPOP (0.8 g) and PPO (70 g) in 2 litres of toluene was employed in most cases.

Whenever the possibility of water in samples arose, Bray's scintillation fluid (Bray, 1960) was used. Bray's scintillation fluid consisted of:

naphthalene (60 g), PPO (4 g), dimethyl POPOP (0.2 g), methanol (100 ml) and ethylene glycol (20 ml) made up to a total volume of 1 litre with dioxane.

B. METHODS

1. Cultivation of Seedlings

Nicotiana tabacum (cv. Turkish Samson) seeds were germinated on damp potting compost and grown under conditions of constant temperature (23°) and illumination (2,000 ft. c.; fluorescent lamps) in a growth cabinet. During the initial germination period (5 days) the pots containing the seeds were covered with clear plastic (Glad wrap) so as to maintain high humidity around the seeds. Holes were punched in the plastic so as to allow an air supply to reach the seeds. At twelve days the seedlings were transplanted and grown individually under the same conditions for a further five days or until required for experiments.

2. Measurement of Seedlings

For all growth studies plants of the appropriate age (usually 21 days after germination) were randomized into groups of five or ten and the length and width of the second leaf and the stem height (from cotyledons to apex) was determined for each plant prior to the addition of any test substances. Leaf length was measured from the base of the petiole to the leaf tip.

3. Application of Growth Regulators, Sterols and ^{14}C-precursors to Plants

a. All growth retardants were dissolved in 0.05% (w/v) tween-20 solution and applied to seedlings as a single 5 µl drop placed on the apex.

b. GA_3 was applied to plants in an identical fashion to the retardants. Controls had 5 µl 0.05% (w/v) tween-20.

c. Sterols were applied to seedlings as a 0.003% (w/v) emulsion in 0.004% (w/v) Pluronic F68 solution. The emulsion prepared according to Stowe and Dotts (1971) was applied to the whole tops of plants as a fine spray from an atomizer. After each spray application had been absorbed by the plant, a further aliquot of emulsion was applied. This process was repeated continuously until the desired amount of sterol (usually about 300 µg/plant) had been applied. Control plants were treated with an equivalent volume of 0.004% (w/v) Pluronic F68 solution at the same time as sterol application.

d. Where required, 2-^{14}C-mevalonic acid was applied to seedlings as a single 5 µl drop of ^{14}C-precursor in 11 mM phosphate (KH_2-$K_2H\ PO_4$) buffer, pH 6.5.

4. Measurement of Seedling Growth

The three parameters of growth (viz. leaf length, leaf width and stem height) were measured routinely every second day after the

initial treatment of plants with growth substances. Recorded data was punched onto data cards and analysed by computer to obtain standard errors, mean values and L.S.D's.

5. Treatment of Rootless Seedlings

Seedlings of the required age and uniform size were harvested, washed thoroughly with distilled water and the roots excised. Roots were removed by cutting under water so as to not cause air bubbles in the vascular tissue and thus block transport of materials up the stem. A number of seedlings (usually totalling about 300-400 mg in weight) were then placed into 5 ml glass petri dishes containing 2 ml of a solution of 2-^{14}C-mevalonic acid (2.5 or 5 μCi) in 11 mM phosphate buffer, pH 6.5. Where required, growth substances (GA_3, retardants etc.) were incorporated into the 2 ml solution at the required concentration and the pH was readjusted to 6.5 prior to the immersion of the seedlings.

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 were then placed under large, ventillated glass containers and illuminated with constant light of either low intensity (300 ft. c.; fluorescent lamps) or high intensity (2,000 ft. c.; fluorescent lamps) for 24 hr at 23°. After the illumination period the seedlings were removed from the petri dishes, blotted dry, weighed and macerated in ice-cold ethanol/benzene (4:1) solution (5 ml). Sterols were

extracted from the seedlings as outlined in part 7.a.(i) of these Methods.

6. Preparation of Cell-Free Systems

a. Total cell-free homogenates

A known weight of seedlings were placed into twice their fresh weight of ice-cold phosphate buffer containing 126 mM sucrose, 28 mM nicotinamide and 7 mM $MgCl_2.6H_2O$ and homogenized by an Ultra turax (3 x 30 sec at slow speed). The crude homogenate thus prepared was filtered through 3 layers of cheese-cloth and centrifuged at 2,000 r.p.m. to remove chloroplasts and cell debris and produce a clear supernatant.

The incubation mixture consisted of 2 ml of the prepared supernatant (above) containing 1 mg each of ATP, NAD and NADPH and 0.5 μCi of DL-2-^{14}C-mevalonic acid. Where indicated, Amo 1618 was added to a final concentration of 1,000 μg/ml. The total incubation volume was 2.5 ml. Incubations were carried out in 2" x 1" glass vials plugged with cotton wool for 3 hr at 30° in a shaking water bath. After incubation the reaction mixture was treated with 5 ml of acetone; the vials were stoppered and the sterols were extracted as outlined in part 7.b. of these Methods.

b. Preparation of a cell-free system by isolation of cell components

The method employed here was exactly that of Benveniste et al. (1970) except tobacco seedlings were used instead of tobacco tissue culture.

Solution A consisted of 0.25 M sucrose, 4×10^{-3} M $MgCl_2.6H_2O$, 5×10^{-3} M glutathione in 0.10 M tris buffer, pH 7.5.

Solution B contained 2×10^{-3} M $MgCl_2.6H_2O$ and 2×10^{-3} M mercaptoethanol in 2×10^{-2} M tris buffer, pH 7.6.

Solution C contained 2×10^{-2} M mercaptoethanol, 2×10^{-2} M nicotinamide, 3.2×10^{-2} M $MgCl_2.6H_2O$, 3.2×10^{-2} M KCl, 1.6×10^{-2} M ATP, and 5×10^{-3} M NADH in 0.4 M tris buffer, pH 7.6. (ATP and NADH were added to the solution separately immediately prior to incubation with the homogenate).

Plant tissue of the appropriate age (3-4 weeks) was homogenized in twice its own weight of solution A by a motor driven glass pestle in a glass homogenizer (45 secs, 2 strokes of the pestle) at slow speed. The homogenate was filtered through 2 layers of cheese-cloth and centrifuged (1,000 x g for 5 min) to yield a pellet (P_1) and a supernatant (S_1). The supernatant was then centrifuged at 20,000 x g for 15 min in an 8 x 50 ml fixed angle rotor in an MSE MK 1 Ultra-centrifuge. The supernatant (S_{20}) was decanted and the pellet (P_{20}) collected. The remaining supernatant (S_{20}) was transferred to capped

centrifuge tubes and placed in a Ti50 rotor attached to a Beckman L-65 centrifuge and centrifuged for 90 min at 200,000 x g to produce a pellet (P_{200}) and supernatant (S_{200}) fraction.

All operations, including centrifugations, from homogenization of the tissue through to collection of P_{200} and S_{200} fractions were conducted in a cold-room at 0-4°. Half of the S_{200} supernatant fraction was subjected to 40% (w/v) ammonium sulphate fractionation to yield an insoluble enzyme fraction (E_{0}^{40}) which was pelleted by centrifugation at 10,000 x g for 15 min. The remaining supernatant was treated with 80% (w/v) ammonium sulphate solution to produce another enzyme precipitate (E_{40}^{80}) which was, in turn, pelleted by centrifugation at 10,000 x g for 15 min. The two enzyme pellets and each of the pellets produced throughout the centrifugation procedure (P_{1}, P_{20} and P_{200}) were suspended in an aliquot of solution B, transferred to dialysis bags and dialysed overnight against cold solution B (3 x 1 litre; 0-4°). Dialysis tubing was boiled in 0.01 M EDTA solution for 5 min and washed exhaustively with glass distilled de-ionized water prior to use.

The dialysed suspensions thus prepared were used in various combinations with one another in the presence of acetate-1-^{14}C, 2-^{14}C-mevalonic acid, 3-^{14}C-HMG-CoA or ^{14}C-squalene to study the effects of Amo 1618 (1,000 μg/ml) on synthesis of sterols and sterol intermediates as follows:

An estimation of protein (Lowry et al., 1951) was carried out on each particulate and enzyme fraction. Volumes of the particulate

suspensions equivalent to 0.2 mg of protein and volumes of enzyme fractions equivalent to 0.2 mg of protein were mixed with 0.125 ml of solution C containing ATP and NADH in a glass tube plugged with cotton wool and then incubated in a shaker water-bath (New Brunswick) at 30° for 1 hr. After incubation the reaction was stopped by addition of 30% (w/v) KOH (0.5 ml) to each tube, the mixture saponified overnight (16 hr; 23°) and the non-saponifiable lipids extracted as outlined in part 7.c. of Methods.

7. Extraction Procedures

a. Extraction of sterols and their intermediates from rooted or rootless seedlings

(i) total non-saponifiable lipid extraction (Reid, 1968a)

Macerated seedlings were homogenized in ethanol/benzene (4:1; 5 ml) by an Ultra turax blender and the supernatant was drawn off with a pasteur pipette and filtered through Whatman no. 1 filter papers into a 150 ml round bottomed flask. The remaining tissue was suspended in another 5 ml of the ethanol/benzene solution and re-homogenized. This process was repeated four times to ensure complete extraction of all lipid material from the tissue. The combined extracts were taken to dryness *in vacuo* on a rotary evaporator, the residue dissolved with deionized water (5 ml) and the lipids extracted with diethylether (4 x 5 ml). The ether extracts were washed with water (5 ml) and then taken to dryness *in vacuo* on a rotary evaporator.

The residue was taken up in n-hexane (20 ml) and the hexane solution was extracted with 80% (w/v) methanol (3 x 10 ml) to remove acidic and polar lipids and then the hexane was removed *in vacuo*. The residue was then saponified under reflux with 5 ml of 5% (w/v) KOH in 85% (v/v) methanol (1 hr) and the total non-saponifiable lipids extracted from the saponified mixture with diethylether (3 x 10 ml). Combined ether extracts were washed with water (3 x 5 ml) and dried over anhydrous Na_2SO_4. After filtering, the ether extract was taken to dryness (rotary evaporator, *in vacuo*) and the residue dissolved in 2 ml of anhydrous benzene ready for T.L.C. analysis.

(ii) total lipids (unsaponified) (Pryce, 1971)

Plant tissue (or organelles) to be extracted were placed into round bottomed flasks and extracted by refluxing with acetone (1 hr, 2 x 30 ml) and then chloroform/methanol (2:1) (1 hr, 1 x 30 ml). The combined organic extracts were taken to dryness *in vacuo* and the residue dissolved in 2 ml of anhydrous benzene ready for T.L.C. analysis.

The remaining plant material after organic extraction was refluxed in deionized water (2 x 30 ml) for 2½ hr and the combined aqueous extracts taken to dryness *in vacuo*. The aqueous residue was treated with $HCl/H_2O/MeOH$ (1:2:3) (30 ml) and allowed to stand at room temperature for 5 days. After dilution of the acidic mixture with 30 ml of deionized water the suspension was filtered and extracted with ether (3 x 30 ml) to extract sterols. The combined

ether extracts were washed with 5% (w/v) $NaHCO_3$ (3 x 25 ml) and water (2 x 25 ml), dried over Na_2SO_4 anhydrous and filtered. After removal of the ether *in vacuo* the residue was dissolved in anhydrous benzene (1 ml) ready for T.L.C.

The remaining aqueous extract was neutralized with 4N NaOH, taken to dryness *in vacuo* and the residue refluxed in freshly prepared alkaline pyrogallol [0.5% (w/v) methanolic pyrogallol/60% (w/v) KOH/ methanol (3:2:3); 30 ml] for 2½ hr. After refluxing the mixture was cooled and extracted with petroleum ether (b.p. 40-60°; 3 x 30 ml). The combined petroleum ether extracts were washed with 2N HCl (20 ml), 5% (w/v) $NaHCO_3$ (3 x 25 ml) and water (2 x 25 ml), dried over Na_2SO_4 anhydrous and filtered. The solvent was removed *in vacuo* and the residue dissolved in 1 ml of anhydrous benzene ready for T.L.C. analysis.

b. Extraction of sterols from cell-free homogenates

After addition of acetone to stop the reaction, the reaction mixture was extracted with ethanol/benzene (4:1; 3 x 5 ml) and the combined ethanol/benzene extract was treated exactly as in Methods 7.a.(i) to extract sterols.

c. Extraction of sterols from particulate/enzyme incubates

After saponification in 30% (w/v) KOH the non-saponifiable lipids were extracted as follows:

The alkaline mixture was made 50% (v/v) ethanolic and extracted with petroleum ether (b.p. 60-80°; 3 x 5 ml) and the combined extracts were washed with water (3 x 5 ml), dried over Na_2SO_4 anhydrous, filtered and then taken to dryness *in vacuo*. The residue was dissolved in 1 ml of anhydrous benzene ready for T.L.C. analysis.

d. Extraction of mevalonate from plant material

After incubation of the cellular fractions with acetate-1-^{14}C or 3-^{14}C-HMG CoA, the reaction was terminated by addition of 0.4 ml of 10 N KOH for 10 min. The pH was lowered to below 2 by addition of 10 N HCl and the mixture allowed to stand for 15 min. Mevalonolactone so formed was extracted into diethylether by vigorous shaking (5 extractions each with 5 ml ether was used). The combined ether extracts were taken to dryness *in vacuo* and redissolved in 0.2 ml acetone ready for T.L.C. This method of extraction gave an expected 65% recovery (Shapiro *et al.*, 1969).

8. Thin Layer Chromatography (T.L.C.)

For all thin layer chromatography 5 x 20 cm glass plates were coated with a 0.3 mm thick layer of silica gel G on a Shandon Chromatogram Spreader, activated (120°; 1 hr) and allowed to equilibrate to room conditions prior to use.

Spotting of samples onto the plates was performed in a specially designed box so that a constant stream of dry nitrogen was blown over the plate during the loading procedure.

Development of plates was performed in glass chromatography tanks sealed at the top and with solvent-saturated chromatography paper covering the sides so as to ensure a vapour-saturated atmosphere around the plates. Solvent was allowed to move to a line scratched into the silica gel exactly 15 cm from the origin.

a. T.L.C. of sterols and sterol intermediates

Unless stated otherwise all total non-saponifiable lipids and sterol extracts were developed twice in 4% (v/v) diethylether in dichloromethane.

Other solvent systems used included:

n-hexane

5% (v/v) ethylacetate in n-hexane

and 25% (v/v) ethylacetate in n-hexane.

b. T.L.C. of mevalonolactone

The solvent used for T.L.C. of mevalonolactone was benzene/acetone (1:1) and plates were developed once.

9. Thick-layer (Preparative) Chromatography

The use of preparative silica gel plates was employed in the preparation of squalene-2,3-epoxide (see Methods 13.a.). Preparative plates were made up by spreading a slurry of 38 g silica gel G in 69 ml deionized distilled water over the surface of a 20 cm x 20 cm glass plate. Gentle tapping under the plate ensured an even spread of the

slurry. The plates were left to air-dry and then dried in an oven (120°; 1 hr) to remove all water. After cooling the plates in a dry atmosphere they were ready for loading.

10. Gas-Liquid Chromatography (G.L.C.)

a. Preparation of columns for G.L.C.

(i) silanizing the columns

Prior to packing, the glass columns and glass wool used for plugging the ends of columns were silanized by rinsing thoroughly with a warm (80°) solution of 1% (v/v) hexamethyldisilazane in benzene (usually three rinses). After draining, the silanized columns and glass wool were dried in an oven (120°; 2 hr).

b. Packing material

(i) 1.5% SE-30 on Anachrom ABS

50 ml of a solution of 1.5% (w/v) SE-30 in toluene was mixed thoroughly with 12.5 g of Anachrom ABS support, the mixture degassed (*in vacuo*), allowed to stand 5 min and then filtered through a Buchner funnel until foaming had ceased. The coated material was dried on filter paper in an oven (120°; 4 hr).

(ii) 2.5% OV-101 on Gaschrom Q

4 g of OV-101 was dissolved in 160 ml of butanone and 80 ml of the solution was mixed into a slurry with 20 g of Gaschrom-Q.

After degassing (*in vacuo*) the mixture was allowed to stand (5 min), filtered through a Buchner funnel and oven dried (120°; 4 hr) on filter papers.

c. Packing the columns

One end of a clean, silanized glass column was plugged with glass wool (silanized) and then connected to a vacuum pump. Packing material was poured via a silanized glass funnel through the other end of the column and assisted in firm packing by tapping the column lightly with the flat end of a spatula. This ensured uniform packing. When packed, the other end of the column was plugged with silanized glass wool and each outlet of the column was plugged with the appropriate septum.

d. Conditioning the column

SE-30 columns were conditioned in the G.L.C. at 300° for 16 hr (overnight) with the top of the column detached from the detector and no carrier gas flow, and then at 250° with detector and carrier gas both connected for a further 6 hr.

OV-101 columns were conditioned in the G.L.C. at 250° for 4 hr with nitrogen (carrier gas) flush and the detector disconnected from the column, and then at 230° overnight (16 hr) without nitrogen flush.

e. Determination of percentage weight of packing liquid phase

A sample of packing material (usually about 2 g) was oven dried, cooled in a dessicator and weighed (W_1). The weighed amount of packing was then washed exhaustively with solvent (toluene for SE-30 and butanone for OV-101), dried in an oven (120°; 4 hr), cooled in a dessicator and reweighed (W_2). A subtraction of weights ($W_1 - W_2$) gave the approximate weight of coating material and from this and the weight of solid support (W_2), a percentage value was obtained.

f. Operating conditions

SE-30 columns were operated under the following conditions: Column temperature = 230°, flame detector temperature = 300°, and injection port temperature = 260°. Nitrogen was used as the carrier gas at a flow rate of 80 ml/min at 40 p.s.i.

OV-101 columns were operated under the same temperature conditions as above but the carrier gas flow rate was raised to 100 ml/min at 50 p.s.i.

The hydrogen and air flow rates were 65 ml/min and 1 l/min, respectively when either column packing material was employed.

Ethyl acetate was the most commonly used solvent.

11. Determination of Radioactivity associated with Sterols and their Intermediates

After localization of radioactive spots on the T.L.C. plates by radioactive scanning (see Materials 4) the developed T.L.C. plate was divided into ½-cm bands starting from ½-cm behind the origin and progressing to the solvent front. These -cm bands of silica gel were scraped from the plate into scintillation vials, covered with scintillation fluid (2 ml) and assayed for radioactivity by Liquid Scintillation Spectrometry.

Standards were located on thin layer plates by two methods, viz.

(i) Iodine-vapour staining technique, and

(ii) location by U.V. fluorescence after lightly spraying the plate with 0.1% (w/v) methanolic berberine hydrochloride solution.

The latter method was preferred when locating bands of sterol material from the plant extracts particularly when the material was to be examined by G.L.C. because the sterols were readily separated from both silica gel G and berberine hydrochloride by diethylether.

12. Collection of Fractions from G.L.C. effluent

Sterol samples to be examined by G.L.C. were washed from silica gel with diethylether.

The combined ether extracts were filtered through filter papers (Whatman No. 1) previously washed with diethylether, and taken to dryness *in vacuo*. The residue was taken up in a known volume of

ethyl acetate and an aliquot injected onto the column of the G.L.C. Effluent from the top of the column was collected at regular time intervals (as outlined in Materials 4.) and assayed for radioactivity by liquid scintillation spectrometry.

13. Preparation of Precursors and Sterol Intermediates

a. Squalene-2,3-epoxide

Squalene-2,3-epoxide was prepared essentially according to the method of Willett *et al.* (1967) after first purifying the commercially supplied squalene to obtain only the *trans*-isomer (Cornforth *et al.*, 1959). The method was as follows:

Squalene (500 mg), supplied as a mixture of *cis* and *trans*-isomers was dissolved in benzene (6 ml) and the mixture poured into a saturated methanolic thiourea solution (50 ml). The resulting mixture was cooled to 0° in an ice-salt bath and the needle-like crystals which formed were collected on a Buchner funnel, washed with petroleum ether (b.p. 40-60°) and transferred to a clean stoppered glass flask containing 5 ml of deionized distilled water. *trans*-squalene was extracted from the water-lipid mixture with petroleum ether (b.p. 40-60°; 3 x 5 ml). This procedure was repeated twice more and the squalene thus obtained was assumed to be exclusively all *trans*-squalene.

trans-Squalene (300 mg) was shaken with tetrahydrofuran (16 ml) and deionized water (2 ml) in a stoppered glass flask and the flask

placed in a beaker of ice-salt mixture to reduce the temperature to 0°. After flushing the flask with nitrogen, N-bromosuccinimide (180 mg) was added in small portions with constant stirring (sealed magnetic flea) and under an atmosphere of nitrogen while maintaining the temperature of the mixture at 0-5°. When all of the N-bromosuccinimide was added the mixture was allowed to stand at 0° (freezer compartment of a refrigerator) for 24 hr. After removal of the solvent in vacuo the residue was dissolved in benzene (2 ml) and spotted onto preparative plates under an atmosphere of nitrogen. The plates were developed once in 15% (v/v) ethylacetate in n-hexane and the fore-running band of the monobromohydrin (located by U.V.) was scraped from the plate and eluted from the silica gel with diethylether. The combined ether extracts were filtered (Whatman No. 1 paper), the solvent removed in vacuo and the residue dissolved in 2.5 ml of methanol under a stream of nitrogen. While stirring vigorously (magnetic stirrer), K_2CO_3 anhydrous (138 mg) was added to the solution in one lot. Stirring was continued for 1 hr at room temperature after which the solvent was removed in vacuo and the crude squalene-2,3-epoxide extracted with diethylether (5 x 5 ml). Combined ether extracts were washed with water (3 x 5 ml), dried over Na_2SO_4 anhydrous, filtered and the solvent removed in vacuo to yield squalene-2,3-epoxide. The squalene-2,3-epoxide thus prepared was cleaned up by T.L.C. using firstly n-hexane as solvent (to remove any squalene impurity), then 5% (v/v) ethylacetate in n-hexane (R_f = 0.8). Tentative identification was made by co-chromatography with authentic squalene-2,3-epoxide in the

above T.L.C. systems and by G.L.C.

b. ^{14}C-Squalene and ^{14}C-Squalene-2,3-epoxide

Radioactive squalene was prepared from rat liver homogenate as follows:

Rats were killed by stunning and the livers excised, weighed, minced in ice-cold 44 mM phosphate buffer (KH_2-K_2HPO_4, pH 7.4) containing 126 mM sucrose, 28 mM nicotinamide and 7 mM $MgCl_2.6H_2O$ and then homogenized in the same buffer. Homogenization was performed in a loose-fitting mechanically driven glass homogenizer at low speed and 0°. The homogenate was centrifuged at 1,500 x g for 5 min and the supernatant decanted into a chilled flask packed in ice.

Several incubation flasks (25 ml glass conical) were filled with 3 ml of phosphate buffer (pH 7.4) containing 1 mg each of ATP, NAD and NADPH and 2 ml of the supernatant, flushed with nitrogen and stoppered with tight fitting rubber injection caps fitted with hypodermic needles connected to a nitrogen cylinder. When all air was expelled from the flasks and while maintaining the nitrogen flush, 1 µCi of DL-2-^{14}C-mevalonic acid in 44 mM phosphate buffer (pH 7.4) was injected by syringe into each of the incubation mixtures. The flasks were shaken in a shaker-water bath at 35° for 3 hr while maintaining the nitrogen atmosphere.

After incubation, the reaction was stopped by the addition of 30% (w/v) KOH (2 ml) to each flask and the mixture allowed to saponify

at 35° for 16 hr. When saponification was complete the mixture was made 50% ethanolic and the non-saponifiable lipids were extracted into petroleum ether (b.p. 60-80°, 3 x 25 ml per flask). All of the petroleum extracts were combined, washed with water, dried over Na_2SO_4 anhydrous, filtered and the solvent removed to produce a residue of crude ^{14}C-squalene. The residue was dissolved in 2 ml of anhydrous benzene and spotted onto silica gel G T.L.C. plates which were developed successively in n-hexane and then 5% (v/v) ethylacetate in hexane to clean up the ^{14}C-squalene.

Tentative identification of the ^{14}C-squalene was made by co-chromatography with authentic squalene in the above T.L.C. systems and collection of fractions from the G.L.C. (95-98% of the total radioactivity applied to the column was recovered in the mass peak area of authentic squalene).

^{14}C-Squalene-2,3-epoxide was prepared on a micro scale from ^{14}C-squalene by the method outlined in Methods 13.a. The ^{14}C-squalene -2,3-epoxide was tentatively identified by co-chromatography with authentic squalene-2,3-epoxide in several T.L.C. systems and on G.L.C.

c. 3-^{14}C-HMG-CoA

3-^{14}C-HMG-CoA was prepared from ^{14}C-HMG by the method of Louw _et al_. (1969).

3-hydroxy-3-methylglutaric-3-^{14}C acid (122 mg, 60.85 μCi/mmole) was dissolved in 1.0 ml of acetic acid, and anhydrous benzene (2.8 ml)

and acetic anhydride (0.4 ml) were added in this order. The mixture was stirred at 37° in an incubator for 16 hr (overnight). The solvents were removed in vacuo and the crude crystals of the HMG-anhydride were re-crystallized twice from anhydrous benzene to yield fine white needle-like crystals (m.p. 101-102°; 89% of theoretical yield). I.R. spectrum agreed with observations of the authors (Louw et al., 1969).

Coenzyme A (31 mg) was dissolved in ice-cold water (2 ml) while nitrogen was bubbled slowly through the solution which was stirred with a magnetic stirrer. The pH was adjusted to 7.5 with 1 N KOH and 0.2 ml of a saturated solution of $KHCO_3$ was added to act as a buffer. 3-^{14}C-HMG-anhydride (5.0 mg) was added slowly to the solution while constantly stirring and stirring was continued until the nitroprusside test (Grunert and Phillips, 1951) for free sulphydryls was negative. The pH of the solution was adjusted to 5.5 (pH meter) with 2N HCl and the solution was stored at -20°.

14. Assay of HMG-CoA reductase Activity

a. 1-^{14}C-acetate, sodium salt (6×10^5 d.p.m.; μmoles) was added to 2.0 ml of solution B (see Methods 6.b.) containing fraction E_{40}^{80} (equivalent to 0.2 mg protein), fraction P_{200} (equivalent to 0.2 mg protein) and the following ingredients: 60 μmoles nicotinamide, 20 μmoles glutathione, 3 μmoles NADH, 4 μmoles $MgCl_2.6H_2O$, 30 μmoles MVA and 48 μmoles of glucose-6-phosphate. Incubation was carried out in a shaker water-bath (30°, 2 hr) and the reaction was stopped by the

addition of 0.5 ml 30% (w/v) KOH and the mixture left to stand for 15 min prior to mevalonolactone extraction.

b. 3-^{14}C-HMG-CoA (5 x 10^4 d.p.m.; 1.5 µmoles) was added to a mixture of fractions E_{40}^{80} and P_{200} (as above) and 3 µmoles NADP, 3 µmoles glucose-6-phosphate, 1 I.U. of G-6-P dehydrogenase, 100 µmoles EDTA, 10 µmoles MVA and 10 µmoles of glutathione. Incubation was at 30° for 1 hr and mevalonolactone was extracted and assayed as outlined in Methods 7.d.

Where applicable, the application of Amo 1618 to the above incubates was made from a solution of Amo 1618 in solution B, pH 7.6 to a final concentration in the incubate of 1,000 µg/ml.

15. Preparation of Sterol Emulsions

30 mg of the required sterol was dissolved in a minimal volume of acetonitrile and then injected via a 1 ml glass syringe fitted with a wide-bore (No. 18) hypodermic needle into one litre of a 0.004% (w/v) Pluronics F68 aqueous solution. Excess acetonitrile was expelled by bubbling nitrogen through the emulsion.

Control 0.004% (w/v) Pluronics F68 solutions were prepared by exactly the same method using the same volume of acetonitrile as blank.

16. Protein Estimations

50 ml Reagent A [2% (w/v) Na_2CO_3 in 0.10 N NaOH] were mixed with 1 ml Reagent B [0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) potassium tartrate] and 1 ml of this mixture was added to a tube containing 0.2 ml of test protein solution. The mixture was shaken and allowed to stand at room temperature for 10 min. Immediately prior to reading the tubes on a spectrophotometer (Shimadzu QR-50 Spectrophotometer), 0.10 ml of diluted Folin-Ciocalteu reagent was added to the tubes and the tubes were shaken vigorously and colour formation determined at 750 mμ.

A set of standards containing known amounts of bovine serum albumin were assayed at the same time and, from the standard curve, the quantity of protein in test solutions was determined.

17. Digitonin Precipitation of 3β-hydroxysterols

A known quantity (usually half) of the total non-saponifiable lipid extract was taken to dryness *in vacuo* and the residue dissolved in acetone-diethylether (1:1; 3 ml). To this solution was added 1.5 ml of an ethanolic digitonin solution [5 mg digitonin/ml of water-ethanol (8:10)], and the mixture was allowed to stand for 3 hr at room temperature. After this time 2.5 ml of aqueous digitonin (1.8 mg/ml) was added, and the mixture was allowed to stand for a further 21 hr.

The precipitate formed was filtered onto Whatman No. 1 filter paper, washed once with acetone/diethylether (1:1, 10 ml) and twice more with two 10 ml portions of diethylether. The digitonin complex thus obtained was split by heating the precipitate to 60 C for 1 hr in 3 ml of distilled pyridine. The resulting solution was washed into a centrifuge tube with six 10 ml rinses of petroleum ether (b.p. 60-80 C), and centrifuged (3,000 r.p.m. for 15 min), decanting the supernatant into round-bottomed flasks.

The solvent was removed in vacuo and the residue dissolved in 0.5 ml of chloroform/methanol (1:1) and spotted onto thin layer silica gel G plates. Chromatography was carried out in 5% (v/v) ethyl acetate in n-hexane and in 25% (v/v) ethyl acetate in n-hexane. Radioactive peaks were located by scanning and sterol standards run on the same plates were located with I_2-vapour.

III. RESULTS AND DISCUSSION

A. SITES OF ACTION OF Amo 1618 IN THE STEROL BIOSYNTHETIC PATHWAY

For the purposes of these studies the sterol pathway was divided into two sections; from acetate to mevalonate and from mevalonate to sterols, and the effects of the retardant were examined on each section.

1. A Pre-mevalonate Site of Inhibition

A single concentration (1,000 μg/ml) of Amo 1618 was tested for its effects on the incorporation of 1-^{14}C-acetate into mevalonate in a total cell-free homogenate (1,000 x g supernatant; Methods 6.a.) prepared from three week-old rootless tobacco seedlings. After incubation the ^{14}C-mevalonate product was extracted (as the lactone; Methods 7.d.) and assayed on T.L.C. silica gel G plates by the method of Shapiro *et al*. (1969).

The retardant inhibited acetate incorporation into mevalonate by 52% (Table 1A) demonstrating that Amo 1618, at one concentration at least, was able to inhibit isoprenoid and hence sterol biosynthesis in higher plants at a pre-mevalonate step in the biosynthetic sequence. This finding was in complete agreement with the work of Paleg (1970b) on the effects of plant growth retardants on rat liver cholesterol biosynthesis and points to a common mode of action of the retardants in animal and plant systems.

In rat liver preparations a prime possible site for retardant action at the pre-mevalonate stage of the cholesterol pathway might be the HMG-CoA reductase step which has been shown to be particularly inhibitor-sensitive (Siperstein and Fagan, 1966; Hamprecht *et al*., 1971). For this reason the activity of HMG-CoA reductase in cell-free preparations of tobacco seedlings was examined.

The effects of 1,000 μg/ml Amo 1618 on the incorporation of 1-^{14}C-acetate and 3-^{14}C-HMG-CoA into mevalonate were tested on a

Table 1. The effect of Amo 1618 on the biosynthesis of mevalonate in cell-free preparations of tobacco seedlings.

1A. the effect on 1-^{14}C-acetate conversion to mevalonate in a whole cell-free homogenate from tobacco seedlings.

1B. the effect on 1-^{14}C-acetate, and 3-^{14}C-HMG-CoA, conversion to mevalonate in a microsomal-rich fraction from a cell-free homogenate of rootless tobacco seedlings.

Precursor	Incorporation into mevalonate		% inhibition
	Control	Amo 1618-treated	
	c.p.m.		
A.			
1-^{14}C-acetate	7,217	3,482	51.8
B.			
1-^{14}C-acetate	9,511	5,507	41.9
3-^{14}C-HMG-CoA	1,291	650	49.7

microsomal (P_{200}) plus enzyme (E_{40}^{80}) preparation from 21-day old tobacco seedlings (Methods 6.b.). The mevalonate was extracted from the cell-free preparation and assayed by T.L.C. using benzene/acetone (1:1) as developing solvent (Methods 14).

Amo 1618 inhibited the conversion of acetate to mevalonate by ca 42% and HMG-CoA to mevalonate by about 50% (Table 1B) suggesting that all of the inhibition of mevalonate biosynthesis by the retardant could be accounted for by inhibition of HMG-CoA reductase activity.

The enzyme HMG-CoA reductase has been studied in some detail in animal (Kawachi and Rudney, 1970; Hickman, 1971), yeast (Kirtley and Rudney, 1967), and bacterial (Bensch and Rodwell, 1970) systems and is thought to be responsible for physiological regulation of the hepatic cholesterogenic pathway (Bucher et al., 1960; Siperstein and Fagan, 1966). No work of a similar nature has been conducted on the enzyme in higher plants although Brooker and Russell (1973) have recently reported phytochrome regulation of HMG-CoA reductase in Pisum sativum.

Mevalonate is a precursor of all of the isoprenoid biosynthetic pathways and thus any reduction in the synthesis of this compound would lead to reduced levels of all of the terpenes and terpenoids produced in plants. The low levels of inhibition (ca 40-50%) produced by such a high concentration of retardant, however, might argue against the HMG-CoA reductase step being the major site of retardant action. Only those compounds which are rapidly turned over in plants would be greatly affected by a 50%, or less, inhibition of

synthesis. Compounds such as sterols which are relatively slowly turned over would need to be reduced by a much greater amount to produce immediate effects on plant growth.

Finally, a word about the relatively low activity of HMG-CoA reductase observed in the cell-free tobacco preparation. Only about 2-3% of the available 3-^{14}C-HMG-CoA was converted to mevalonate in this system and these levels of incorporation are very low for such a specific precursor. Several possibilities may explain this poor utilisation of HMG-CoA. Firstly, mitochondrial contamination of the microsomal preparation is likely and Bucher et al. (1960) showed that rat liver mitochondria contain a preponderance of the HMG-CoA-cleavage enzyme. If tobacco mitochondria also possess such an enzyme much of the available 3-^{14}C-HMG-CoA could have been split to form non-utilisable HMG.

Another, perhaps less likely possibility is that mevalonate may be synthesized by an alternative pathway such as the acyl carrier protein system which operates in pigeon liver extracts (Brodie et al., 1964) and that HMG-CoA reductase is not a regulatory enzyme in the sterol biosynthetic pathway of higher plants.

2. A Post-mevalonate Site of Inhibition

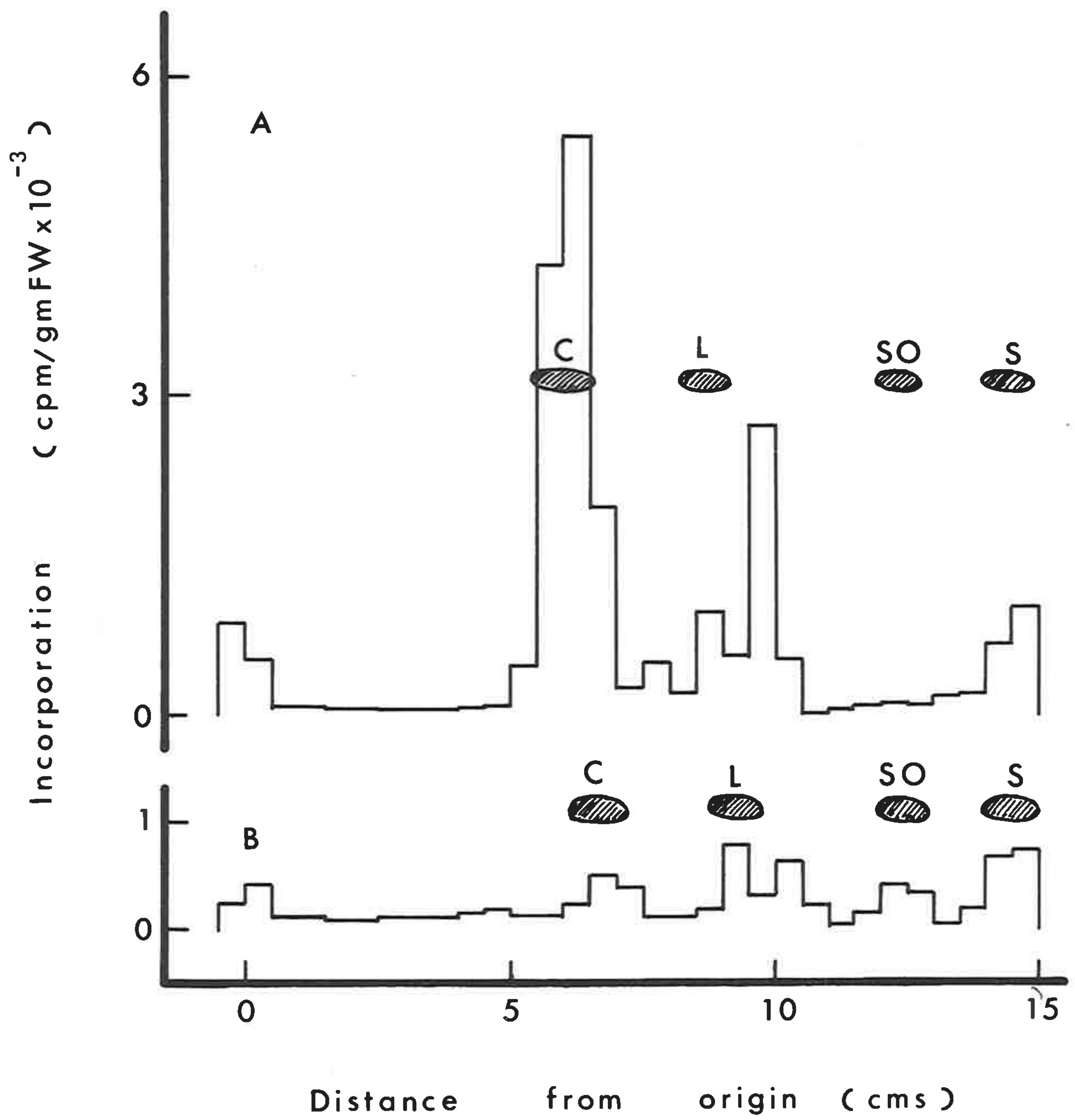
a. Effect of Amo 1618 on the incorporation of 1-^{14}C-acetate and DL-2-^{14}C-mevalonate into sterols of rootless tobacco seedlings

Three to four week-old rootless tobacco seedlings were treated with a single concentration (1,000 μg/ml) of Amo 1618 in the presence of either 10 μCi of 1-^{14}C-acetate or 5 μCi of 2-^{14}C-mevalonate (Methods 5.). After the 24 hr incorporation period sterols and sterol intermediates were extracted from the seedlings [Methods 7.a.(i)] and separated into the various sterol and hydrocarbon fractions by T.L.C. (Methods 8.a.). Each of the fractions thus obtained were assayed for radioactivity (Methods 11.).

The distribution of radioactivity along thin layer plates (figure 7) showed that Amo 1618 greatly reduced the incorporation of acetate into all sterol fractions [co-chromatographing with cholesterol (C) and lanosterol (L) standards] and had little effect on incorporation into the squalene (S) fraction. A small increase in radioactivity in the squalene-2,3-epoxide (SO) region was apparent however. The averaged data from three experiments conducted under identical conditions showed that the retardant reduced incorporation of acetate into 4-desmethylsterols (ca 90%), 4-methylsterols (ca. 82%), 4,4'-dimethylsterols (ca. 73%), and squalene (ca. 14%) and increased incorporation into squalene-2,3-epoxide by about 2-fold (Table 2). It is apparent from this data that Amo 1618 must also exert an

Figure 7. The effect of Amo 1618 on the incorporation of $1-^{14}C$-acetate into sterols and sterol intermediates in rootless tobacco seedlings.

The pattern of radioactivity from 1/10th of the total non-saponifiable lipid fraction is shown distributed along a thin-layer silica gel G plate which had been developed twice in 4% (v/v) diethylether in methylene chloride for A, control seedlings and B, seedlings treated with 1,000 μg/ml Amo 1618. C = cholesterol; L = lanosterol; SO = squalene-2,3-epoxide; S = squalene.



6
3
0
1
0
A
B
C
L
SO
S
Incorporation (cpm/gm FW x 10^{-3})
0
5
10
15
Distance from origin (cms)

Table 2. The effect of Amo 1618 on the incorporation of 1-^{14}C-acetate into sterols and sterol intermediates.

Thin layer chromatography of the non-saponifiable lipid extract from rootless tobacco seedlings treated with 1-^{14}C-acetate in the presence or absence of 1,000 μg/ml Amo 1618. Developing solvent was 4% (v/v) diethylether in dichloromethane.

Incorporation product	Incorporation of 1-^{14}C-acetate		% inhibition or stimulation
	Control*	Amo 1618-treated*	
	c.p.m./gm F.W.		
4-desmethylsterols	124,106	12,592	89.9
4-methylsterols	7,517	1,357	81.9
4,4'-dimethylsterols	36,801	9,910	73.1
squalene-2,3-epoxide	3,475	7,001	201.5
squalene	18,732	16,052	14.3

* Average of 3 experiments.

inhibitory effect on sterol biosynthesis at some site beyond mevalonate in the pathway and, because of the slight build-up in radioactivity in squalene-2,3-epoxide the squalene-2,3-epoxide cyclase step might be affected.

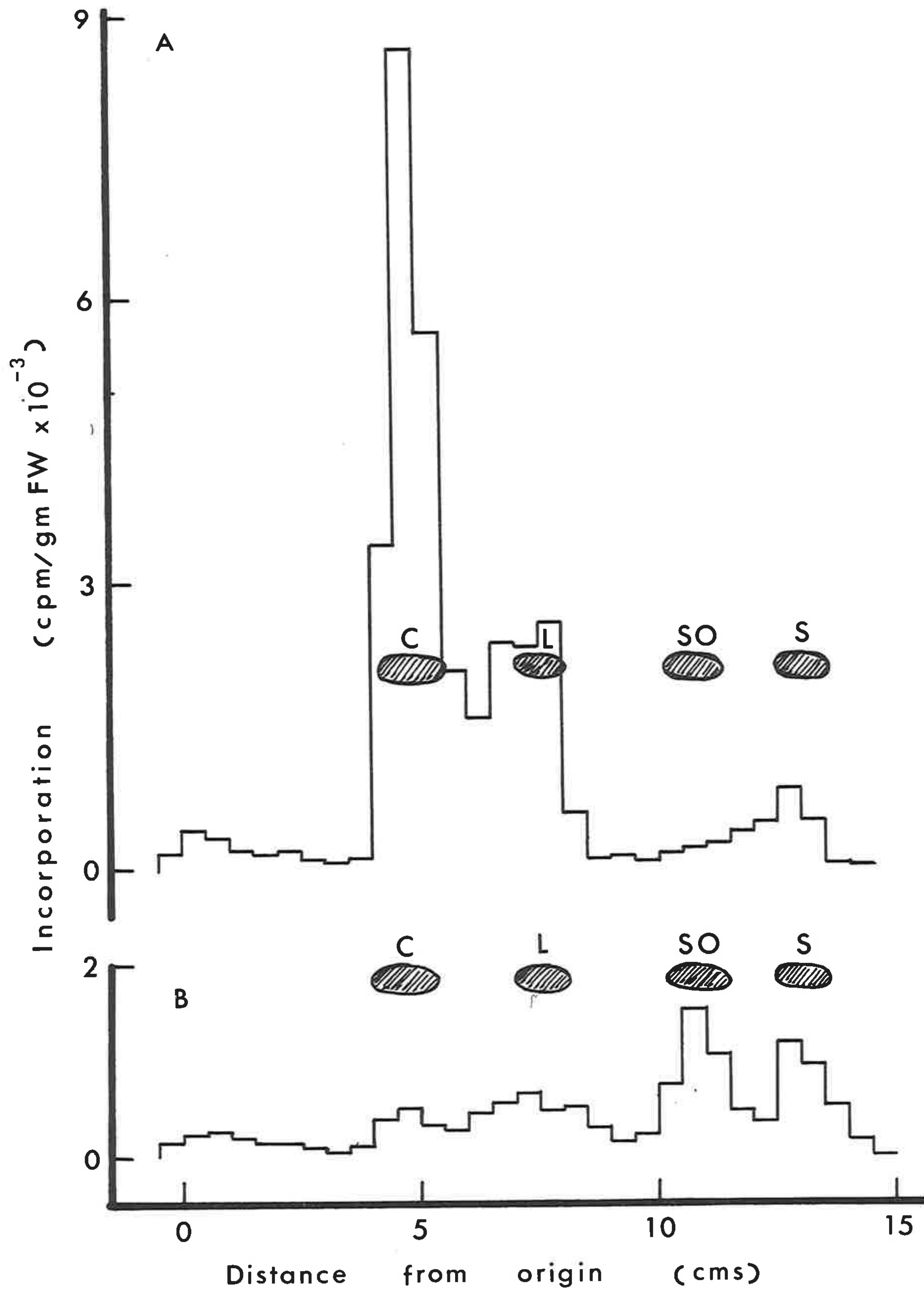
When 2-^{14}C-mevalonate was supplied, the retardant also inhibited the incorporation of this precursor into all of the sterol fractions but resulted in a much more pronounced increase in the incorporation into squalene-2,3-epoxide and a slight increase into squalene (figure 8). When expressed on a per gram fresh weight basis the actual inhibition of mevalonate incorporation into each fraction was: 4-desmethylsterols (ca. 90%), 4-methylsterols (ca. 74%), and 4,4'-dimethylsterols (ca. 46%). Squalene radioactivity increased by 27% and squalene-2,3-epoxide by 9-fold (Table 3).

Examination of digitonin-precipitable compounds (Methods 17) confirmed the marked inhibition of sterols by Amo 1618. The retardant induced better than 94% inhibition of the incorporation of mevalonate into each of the sterol fractions examined (Table 4A).

An examination of the 4-desmethylsterol, 4,4'-dimethylsterol, and total hydrocarbon fractions was undertaken by G.L.C. (Methods 12) using 1.5% (w/v) SE-30 column packing. Co-chromatography with standards was used to identify the individual compounds and collection of eluate from the column at regular intervals allowed an estimation to be made of radioactivity associated with each compound.

Figure 8. The effect of Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol intermediates in rootless tobacco seedlings.

The pattern of radioactivity is shown from 1/10th of the total non-saponifiable lipid fraction distributed along a thin-layer plate (silica gel G) which had been developed twice in 4% (v/v) diethylether in methylene chloride for A, control seedlings and B, seedlings treated with 1,000 μg/ml Amo 1618. C = cholesterol; L = lanosterol; SO = squalene-2,3-epoxide, and S = squalene.



9
6
3
0
2
0
A
B
C
L
SO
S
C
L
SO
S
Incorporation (cpm/gm FW x 10^{-3})
0
5
10
15
Distance from origin (cms)

Table 3. Effect of 1,000 μg/ml Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and their precursors in rootless tobacco seedlings.

The incorporation of precursor into sterol fractions is shown from T.L.C. of 1/10th of the total extracted non-saponifiable lipids. Developing solvent was 4% (v/v) diethylether in dichloromethane.

Incorporation product	Incorporation of L-^{14}C-mevalonate		% inhibition or stimulation
	Control*	Amo 1618-treated*	
	c.p.m./gm F.W.		
4-desmethylsterols	248,300	24,700	90.1
4-methylsterols	61,676	16,206	73.7
4,4'-dimethylsterols	41,648	22,858	45.9
squalene-2,3-epoxide	4,894	44,249	904.2
squalene	13,014	16,487	126.7

Table 4A. The effect of Amo 1618 (1,000 μg/ml) on 2-^{14}C-mevalonate incorporation into digitonin precipitable sterols from rootless tobacco seedlings.

After splitting the digitonin precipitate with pyridine and extraction with petroleum spirit, the free sterols were co-chromatographed with standards on T.L.C. silica gel G plates. Developing solvent used was 25% (v/v) ethyl acetate in n-hexane.

Table 4B. G.L.C. analysis of 4-desmethylsterols and 4,4'-dimethylsterols from Amo 1618-treated and control rootless tobacco seedlings.

Fractions of the eluate from G.L.C. on 1.5% (w/w) SE-30 on Anachrom ABS columns were assayed for radioactivity. Incorporation into each major sterol fraction is shown.

Table 4

Sterol fraction	Sterol identified	Incorporation: Control	Incorporation: Amo 1618 -treated	% Inhibition by Amo 1618
		c.p.m./gm F.W.		
A				
4-desmethylsterols	-	152,600	2,350	98.5
4-methylsterols	-	44,000	2,060	95.3
4,4'-dimethylsterols	-	45,500	2,490	94.5
		c.p.m.		
B				
4-desmethylsterols	cholesterol	575	45	92.2
	campesterol	7,145	219	96.9
	stigmasterol	4,351	498	88.6
	β-sitosterol	14,109	811	94.3
	Total	26,180	1,573	93.9
4,4'-dimethylsterols	cycloartenol	695	221	68.2
	unknown	235	47	80.0
	Total	930	268	71.2

The 4-desmethylsterol fraction consisted of four major sterols (viz. cholesterol, campesterol, stigmasterol, and β-sitosterol). Of these sterols β-sitosterol was present in greatest quantity and accounted for a major portion of the total radioactivity. The retardant reduced the radioactivity associated with each of the four sterols dramatically (figure 9). Incorporation into a fraction which eluted immediately after β-sitosterol was also greatly reduced. This peak only accounted for a small amount of radioactivity and had a very small mass compared with the major sterols and was not identified.

Identification of the 4,4'-dimethylsterols was hampered by a lack of authentic standards to use in the G.L.C. analysis. Cycloartenol and β-amyrin were used as reference standards and, not surprisingly most of the radioactivity was associated with cycloartenol. About 30% of the radioactivity co-chromatographed with the β-amyrin standard (figure 9). Amo 1618 reduced the incorporation into both of these compounds (by 68% and 80%, respectively) (Table 4B).

The hydrocarbon fraction was made up of several compounds but radioactivity was mainly associated with two, squalene and squalene-2,3-epoxide. Treatment with Amo 1618 resulted in an increase in radioactivity into both squalene (<u>ca</u>. 15% increase) and squalene-2,3-epoxide (<u>ca</u>. 6-fold increase) (figure 10).

Inhibition of mevalonate incorporation at the squalene-2,3-epoxide cyclase step showed a concentration dependence when 21-day-old rootless seedlings of tobacco were treated with several

Figure 9. The effect of Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into 4-desmethylsterols and 4,4'-dimethylsterols of rootless tobacco seedlings.

The distribution of radioactivity from G.L.C. analysis of control (solid lines) and 1,000 μg/ml Amo 1618-treated (broken lines) non-saponifiable lipid fractions is shown. The curve shows the mass peaks of sterols in the control extracts. Ch = cholesterol; Ca = campesterol; St = stigmasterol; Si = β-sitosterol; Cy = cycloartenol, and A = β-amyrin.

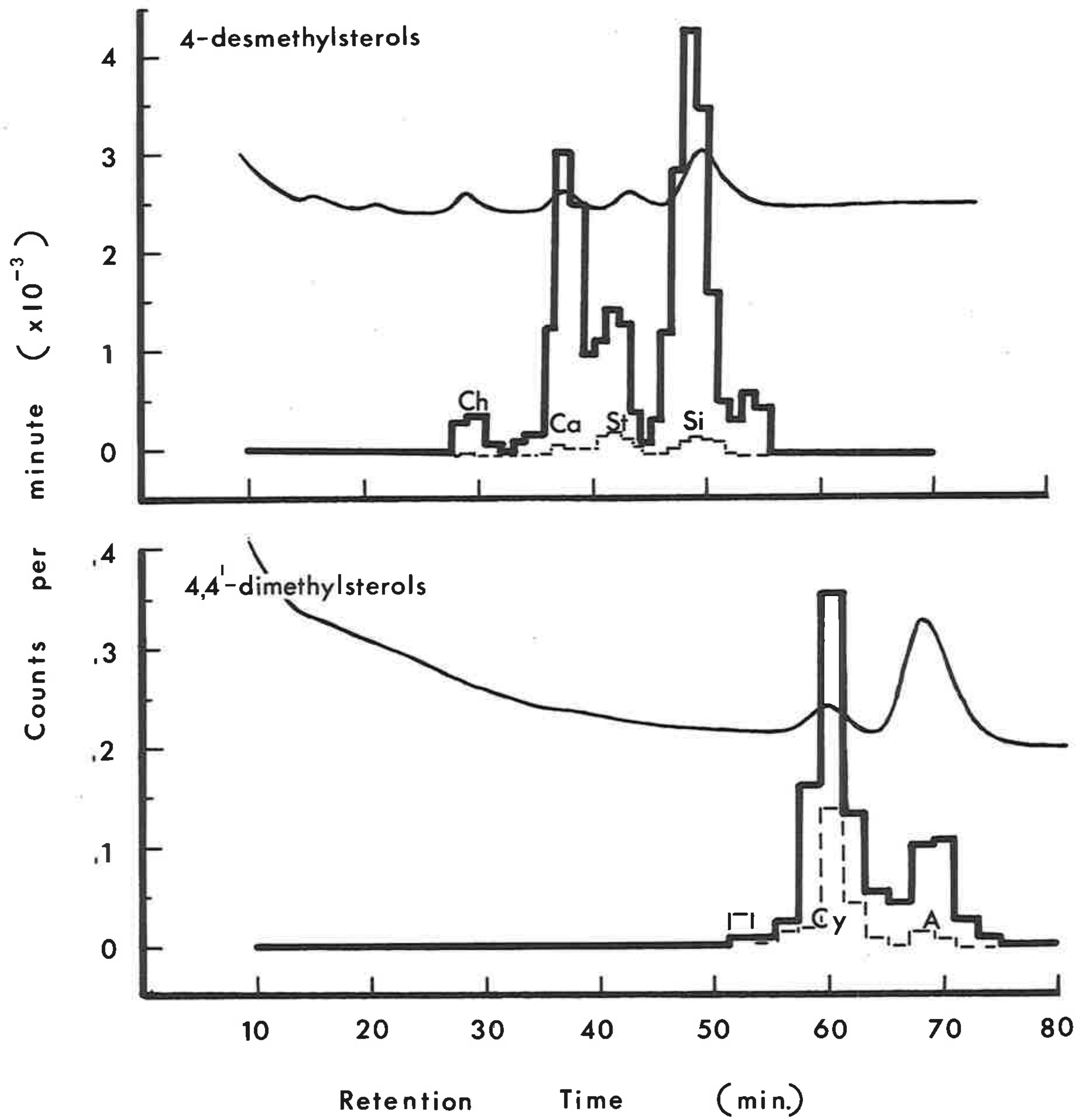
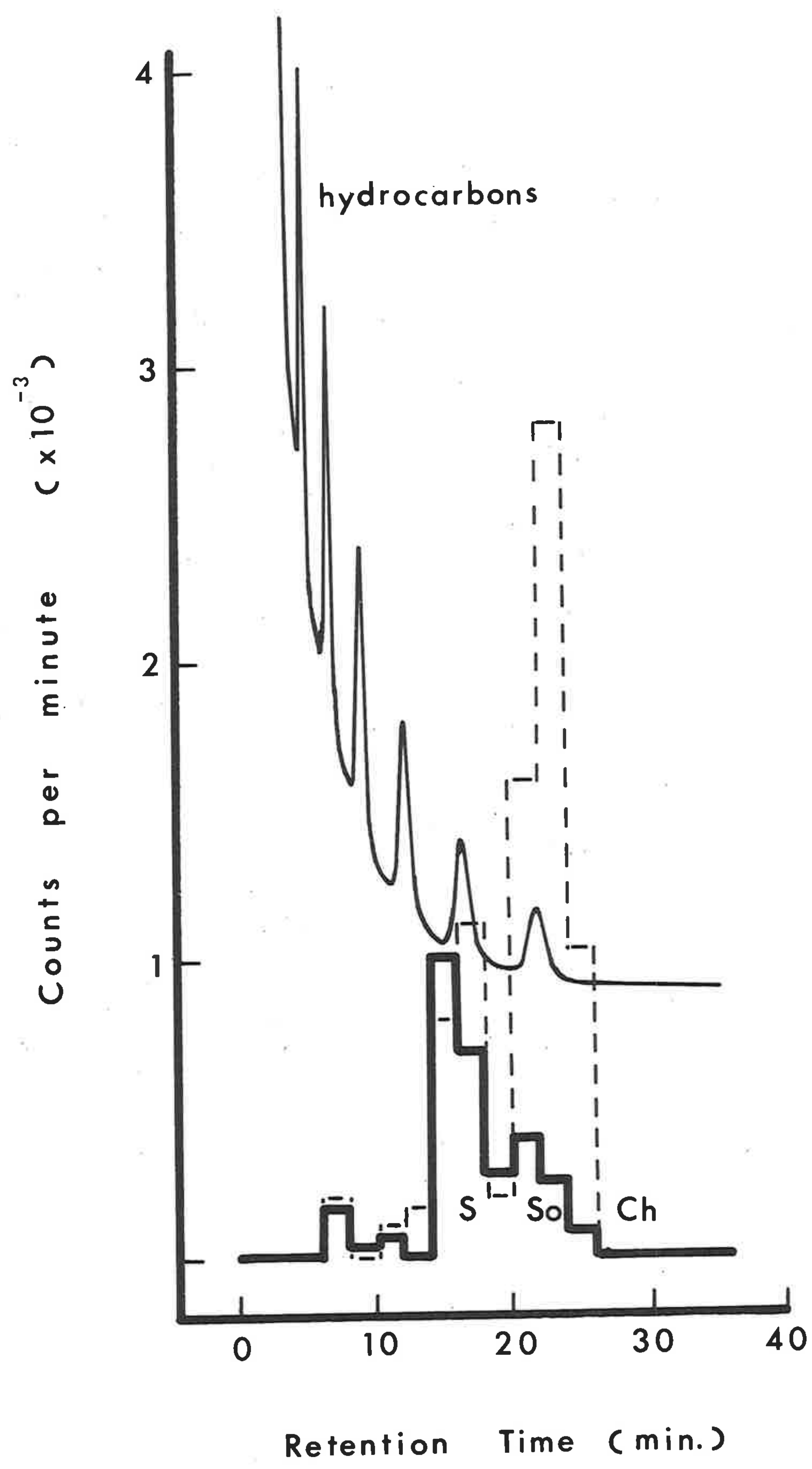

4-desmethylsterols
Ch
Ca
St
Si
4,4'-dimethylsterols
Cy
A
Counts per minute (x10^{-3})
4
3
2
1
0
.4
.3
.2
.1
0
10
20
30
40
50
60
70
80
Retention Time (min.)

Figure 10. The effect of Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into sterol hydrocarbons in rootless tobacco seedlings.

The distribution of radioactivity from G.L.C. analysis of control (solid lines) and 1,000 μg/ml Amo 1618-treated (broken lines) hydrocarbon lipid fractions is shown. The curve shows the mass peaks of hydrocarbons in the control extract.
S = squalene; SO = squalene-2,3-epoxide, and Ch = cholesterol.


4
3
2
1
Counts per minute (x 10^{-3})
hydrocarbons
S
So
Ch
0
10
20
30
40
Retention Time (min.)

concentrations of Amo 1618. An average of results from three experiments carried out under identical conditions shows that as Amo 1618 concentration is increased mevalonate incorporation into sterols (in particular the 4-desmethylsterols) decreased and incorporation into squalene-2,3-epoxide increased (Table 5).

When very low levels of Amo 1618 were supplied to rootless tobacco seedlings mevalonate incorporation was surprisingly increased at, and below 1 μg/ml of the retardant. Only at 3 μg/ml was an inhibition of squalene-2,3-epoxide cyclase activity evident (Table 6).

A combination of results obtained from several experiments in which concentrations of Amo 1618 (ranging from 0.1 μg/ml up to 1,000 μg/ml) were tested for their effects on sterol biosynthesis in rootless tobacco seedlings, under identical conditions, shows how mevalonate incorporation into 4-desmethylsterols changes as retardant concentration is increased (figure 11). Incorporation is apparently increased initially to a maximum at about 1 μg/ml of retardant and then decreased as the retardant concentration increased from 3 μg/ml to 1,000 μg/ml.

b. Amo 1618 effect on mevalonate incorporation into sterols and their precursors in <u>Digitalis</u>

A series of Amo 1618 concentrations were tested for their effects on sterol biosynthesis in 21-day-old rootless digitalis seedlings to assertain whether the findings on Amo 1618 action in tobacco

Table 5. Effect of Amo 1618 concentration on the incorporation of DL-2-^{14}C-mevalonate into sterols in rootless tobacco seedlings.

(Sterol and hydrocarbon fractions were separated by T.L.C. of the non-saponifiable lipid extract).

Retardant concen-tration	*Incorporation of L-2-^{14}C-mevalonate into				
	4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
μg/ml	c.p.m./gm F.W.				
0	266,496	31,886	37,889	2,882	7,515
10	215,977	27,639	16,474	6,588	8,062
100	122,213	19,305	14,469	13,458	9,985
1,000	41,552	15,678	13,310	46,594	12,166

* average of 3 experiments.

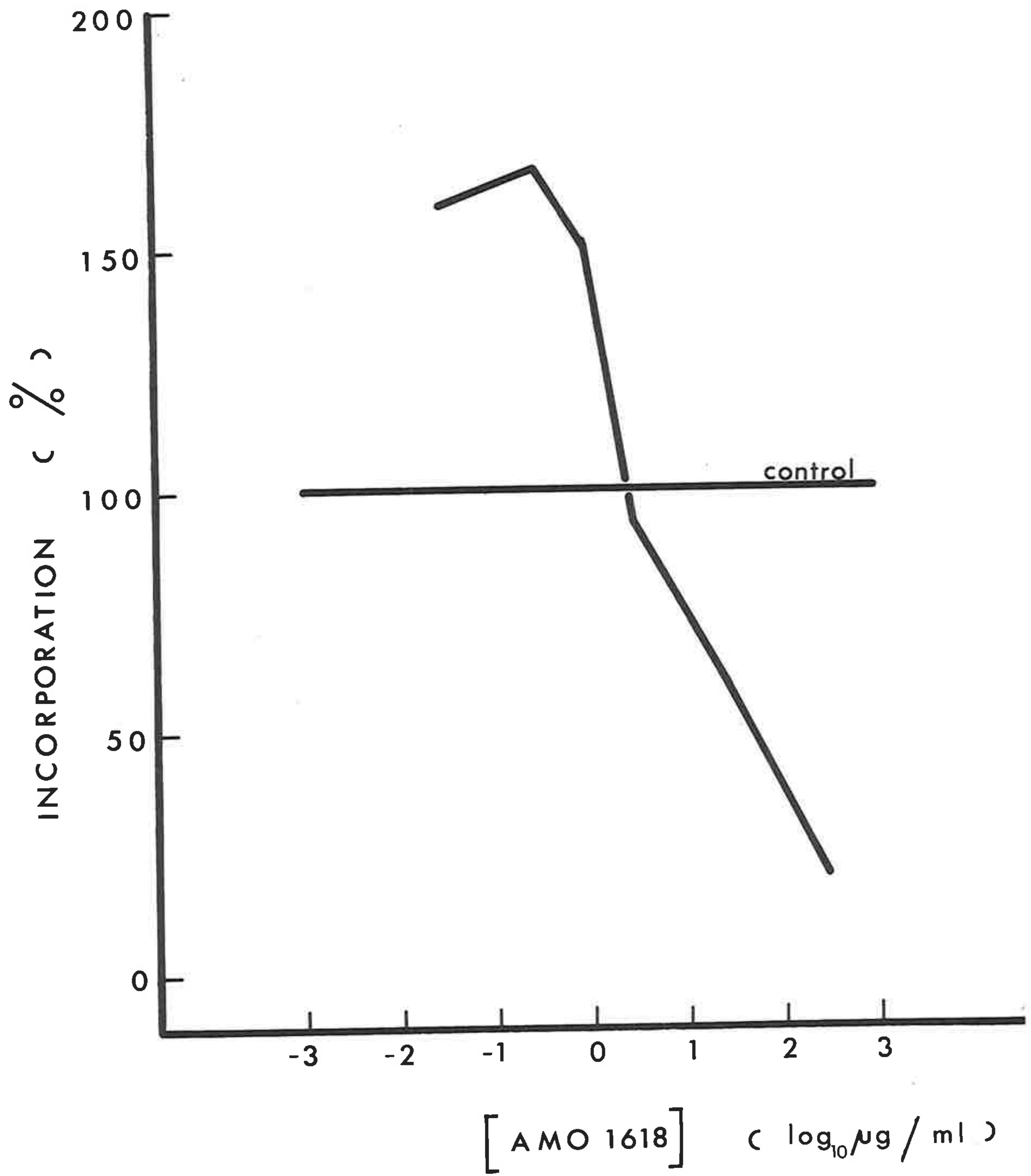
Table 6. The effects of low concentrations of Amo 1618 on sterol biosynthesis in rootless tobacco seedlings.

(Sterol and hydrocarbon fractions separated by T.L.C. of the total non-saponifiable lipid extract).

Retardant concentration	Incorporation of L-2-^{14}C-mevalonate into				
	4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
µg/ml	c.p.m./gm F.W.				
0	197,932	36,670	32,462	2,292	2,552
0.1	314,582	58,458	64,376	5,016	4,182
0.3	251,038	42,264	39,480	3,826	3,432
1.0	331,400	67,610	52,458	6,004	3,682
3.0	100,764	10,190	10,182	5,466	2,736

Figure 11. Percentage incorporation of L-2-^{14}C-mevalonic acid into 4-desmethylsterols from rootless tobacco seedlings treated with various concentrations of Amo 1618.

The radioactivity associated with the 4-desmethylsterol fraction from T.L.C. is expressed as a percentage of control for several concentrations of the retardant.



200
150
100
50
0
INCORPORATION (%)
control
-3
-2
-1
0
1
2
3
[AMO 1618] (log10 µg / ml)

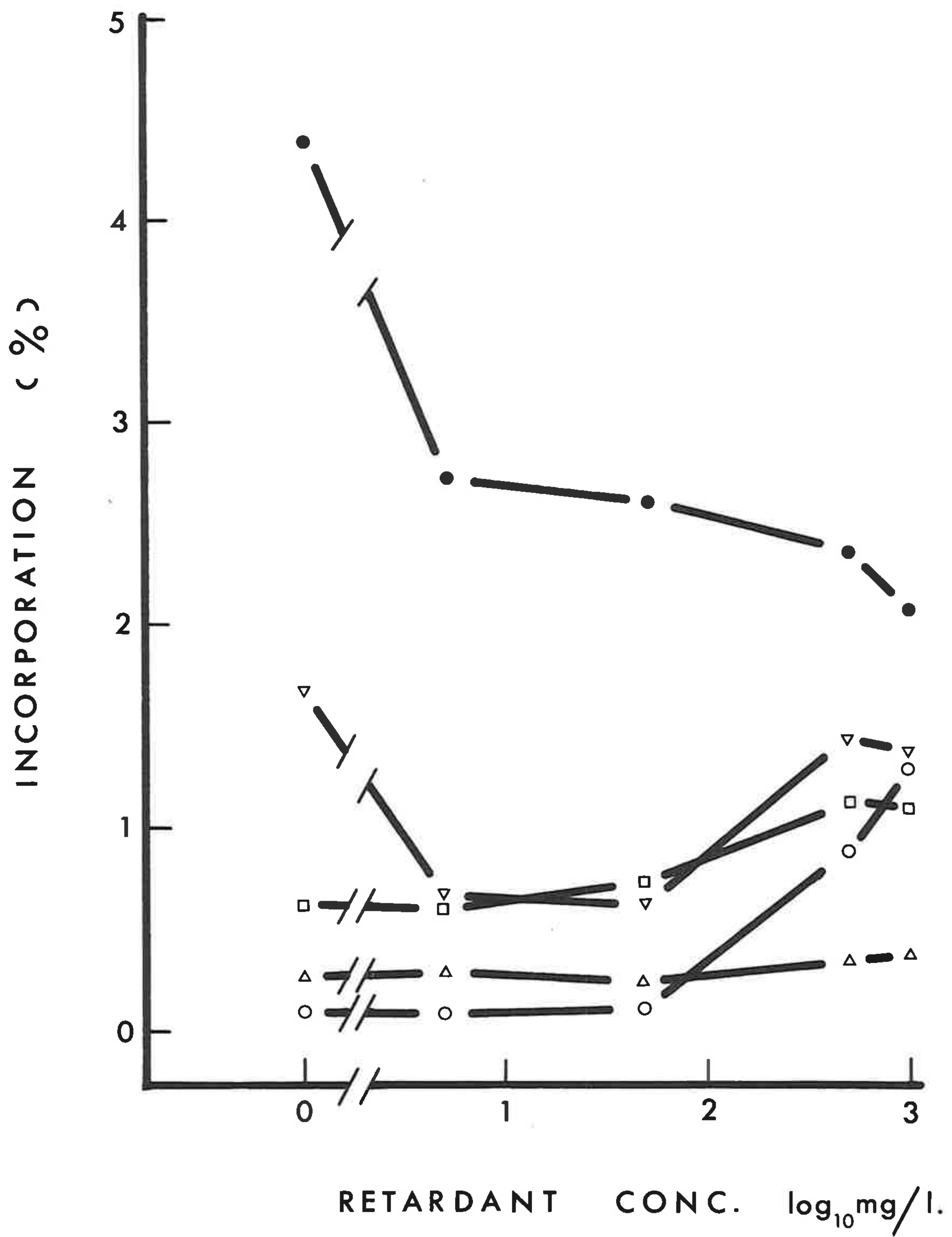
seedlings could be extended to another plant. All experimental conditions were exactly as used in studies with tobacco (Methods 5).

As Amo 1618 concentration was increased, mevalonate incorporation into 4-desmethylsterols decreased sharply at first and then leveled off to a slow decrease at 1,000 μg/ml. Radioactivity in the 4,4'-dimethylsterol region was also initially depressed but then rose almost back to control levels. Incorporation into squalene-2,3-epoxide and the 4-methylsterols increased as the retardant concentration increased (figure 12). Interpretation of this data is not easy as a more complete analysis of the sterols in digitalis (e.g. by G.L.C.) was not undertaken. The major similarity between these results and those obtained with tobacco seedlings was the decreased incorporation into 4-desmethylsterols accompanied by an increase in incorporation into squalene-2,3-epoxide. These results are not inconsistent with those obtained in tobacco but are certainly not identical to the results of the tobacco work.

One notable difference between the effects of Amo 1618 on sterol biosynthesis in rootless tobacco seedlings and digitalis seedlings was the apparent increase in mevalonate incorporation into the 4-methylsterol fraction of digitalis. Accompanying the increased incorporation was the apparent reversal of inhibition of 4,4'-dimethylsterol biosynthesis. This data suggests yet another point of inhibition of Amo 1618 in the sterol pathway viz. the demethylation of 4-methylsterols.

Figure 12. The effect of several concentrations of Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol precursors in rootless digitalis seedlings.

Percentage incorporation of L-2-^{14}C-mevalonic acid into ● 4,desmethylsterols, ▽ 4,4'-dimethylsterols, □ 4-methylsterols, ○ squalene-2,3-epoxide, and △ squalene is shown.



INCORPORATION (%)
5
4
3
2
1
0
0
1
2
3
RETARDANT CONC. log10mg/l.

c. Studies with Amo 1618 on cell-free preparations from tobacco seedlings

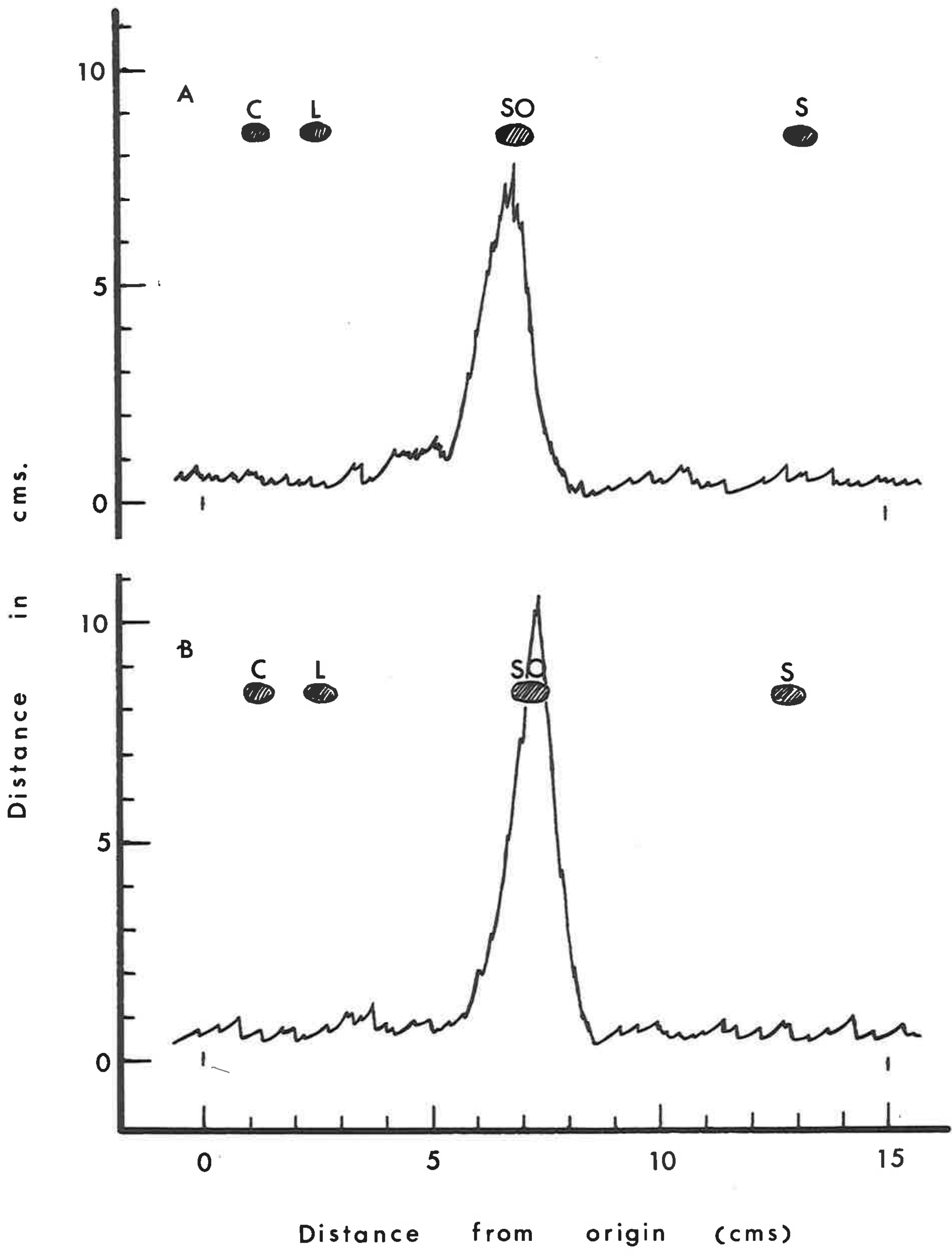
The effects of a single concentration (1,000 μg/ml) of Amo 1618 on the incorporation of 2-^{14}C-mevalonate and ^{14}C-squalene in a cell-free preparation from 21-day-old tobacco seedlings were determined. The cell-free system consisted of microsomes (P_{200}) and soluble enzymes (E_{40}^{80}) (Methods 6.b.) and ^{14}C-squalene was prepared according to Methods 13.b.

The cell-free system lacked the ability to convert 2-^{14}C-mevalonate to sterols and only synthesized squalene and squalene-2,3-epoxide from the precursor (figure 13). Other cell-free systems consisting of various combinations of particulate (chloroplastic, mitochondrial, microsomal) and soluble fractions (Methods 6.b.) were tested for mevalonate incorporation and proved to be even less able to utilise mevalonate (data not presented) than the system employed above. Amo 1618 caused no significant change in the pattern of hydrocarbon production from mevalonate (Table 7A).

When ^{14}C-squalene was supplied to the cell-free preparation some incorporation into sterols and squalene-2,3-epoxide was observed (figure 14). Addition of Amo 1618 to the incubation mixture resulted in about 79% reduction in squalene incorporation into 4,4'-dimethyl-sterols and almost a 9-fold increase in incorporation into squalene-2,3-epoxide (Table 7B). This data confirmed the earlier work and demonstrated the inhibition of squalene-2,3-epoxide cyclase activity

Figure 13. The effect of Amo 1618 on the incorporation of DL-2-^{14}C-mevalonate into non-saponifiable lipids in cell-free preparations from tobacco seedlings.

Shown are scans of radioactivity from total non-saponifiable lipids chromatographed on thin layer silica gel G plates developed in 5% (v/v) ethylacetate in n-hexane for A, control and B, 1,000 μg/ml Amo 1618-treated tobacco preparations. C = cholesterol; L = lanosterol, SO = squalene-2,3-epoxide, and S = squalene.



A
C
L
SO
S
B
C
L
SO
S
10
5
0
0
5
10
15
Distance in cms.
Distance from origin (cms)

Figure 14. The effect of Amo 1618 on the incorporation of ^{14}C-squalene into sterols in cell-free preparations from tobacco seedlings.

Shown is a scan of radioactivity from total non-saponifiable lipid extracts of A, control and B, 1,000 μg/ml Amo 1618-treated tobacco preparations which were developed on thin layer silica gel G plates in 5% (v/v) ethylacetate in n-hexane. C = cholesterol; L = lanosterol; SO = squalene-2,3-epoxide, and S = squalene.

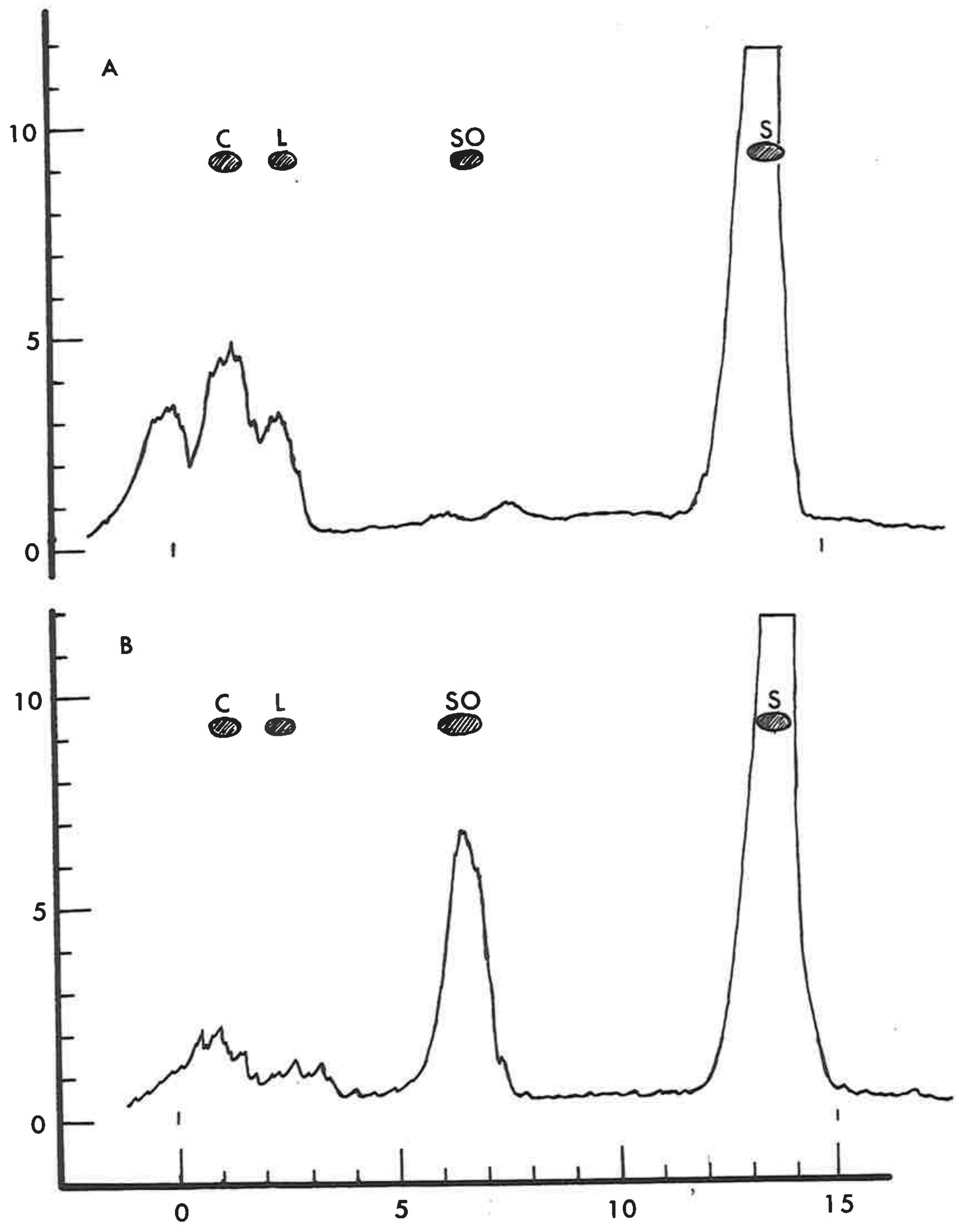

A
B
C
L
SO
S
Distance in cms.
0
5
10
15

Table 7. The effect of 1,000 µg/ml Amo 1618 on the incorporation of DL-2-^{14}C-mevalonate and ^{14}C-squalene into sterols and sterol intermediates in cell-free preparations of tobacco seedlings.

(Sterols and hydrocarbons separated by T.L.C. of the non-saponifiable lipid fraction).

7A. DL-2-^{14}C-mevalonate incorporation

7B. ^{14}C-squalene incorporation

Incorporation product	Incorporation of ^{14}C-precursor		% inhibition or stimulation
	Control	Amo 1618-treated	
A.			
4-desmethylsterols	-	-	-
4-methylsterols	-	-	-
4,4'-dimethylsterols	-	-	-
squalene-2,3-epoxide	10,216	12,392	121.3
squalene	1,020	986	3.3
B.			
4-desmethylsterols	10,721	1,112	89.6
4-methylsterols	4,443	988	77.8
4,4'-dimethylsterols	9,278	1,970	78.8
squalene-2,3-epoxide	1,079	9,112	844.5
squalene	-	-	

by Amo 1618 at the cell-free level.

The failure of cell-free tobacco preparations to utilise 2-^{14}C-mevalonate in sterol biosynthesis parallels the findings of Staby *et al.* (1973) who showed that excised shoots, but not cell-free preparations from shoots of *Wedgwood iris* converted 2-^{14}C-mevalonate to sterols. The cell-free extracts were able, however, to convert the precursor to squalene, farnesol and geranylgeraniol. Benveniste *et al.* (1970) also found that 2-^{14}C-mevalonate was converted to squalene but not sterols in a cell-free system from tobacco tissue cultures.

d. Effect of seedling age on squalene-2,3-epoxide cyclase activity

Rootless seedlings at three different ages (viz. 10, 21 and 40 days) were tested for the effect of Amo 1618 (1,000 μg/ml) on sterol biosynthesis. With increasing age, incorporation of mevalonate into sterols (in particular the 4-desmethylsterols) decreased on a per gram fresh weight (but not on an absolute) basis. Incorporation into hydrocarbons decreased with increasing age when calculated on both a per gram fresh weight (Table 8) and an absolute basis.

The retardant caused the greatest inhibition of mevalonate incorporation into 4-desmethylsterols in the 10 day-old seedlings (92.5%) and the greatest accumulation of squalene-2,3-epoxide (*ca.* 11-12 fold) in the 21 day-old seedlings. At each seedling age Amo 1618 demonstrated the ability to cause the accumulation of squalene-2,3-epoxide (and thus, presumably the inhibition of squalene-2,3-epoxide cyclase activity).

Table 8. Effect of 1,000 µg/ml Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol intermediates in rootless tobacco seedlings of different ages.

(Sterols and hydrocarbons separated by T.L.C. of the non-saponifiable lipid fraction).

Seedling age (days)	Treatment	Incorporation of L-2-^{14}C-mevalonic acid				
		4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
		c.p.m./gm F.W.				
10	Control	272,800	45,788	50,120	7,428	30,640
	1,000 µg/ml Amo 1618	20,532	15,000	16,400	26,920	33,812
% inhibition or stimulation		92.5	67.3	67.2	362	110
21	Control	246,814	64,590	49,186	3,214	5,066
	1,000 µg/ml Amo 1618	44,646	26,014	17,894	37,820	5,926
% inhibition or stimulation		81.9	59.7	63.6	1175	117
40	Control	173,250	30,280	27,520	1,430	3,300
	1,000 µg/ml Amo 1618	23,565	9,905	11,990	12,635	3,305
% inhibition or stimulation		86.4	67.3	56.4	885	0

3. Discussion

The plant growth retardant Amo 1618 has been shown to be a potent inhibitor of sterol biosynthesis in rootless tobacco seedlings and also in cell-free preparations from tobacco seedlings. Two sites of inhibition have been established for the retardant; one at the HMG-CoA reductase step in the pathway and the other presumably at the squalene-2,3-epoxide cyclase step. The most sensitive site is the latter and it is proposed as the major site of Amo 1618 action in the inhibition of sterol biosynthesis. This finding parallels other studies on the effects of the retardants on isoprenoid biosynthetic pathways which have shown both the cyclization of *trans*-geranylgeranyl pyrophosphate to (-)kaurene and other cyclic diterpenes (Robinson and West, 1970), and the cyclization of lycopene to carotenoids (Simpson *et al.*, 1974) to be inhibited by retardants. The retardants therefore seem to demonstrate the ability to inhibit cyclization reactions in isoprenoid biosynthetic pathways.

The squalene-2,3-epoxide cyclase site of Amo 1618 action has been demonstrated in rat liver preparations (Paleg, unpublished data) thus showing a close similarity between the cyclase enzymes from plant and animal sources and suggesting that the effects of other plant growth retardants on sterol biosynthesis in higher plants might also be identical to those observed in cell-free rat liver preparations.

Sterols have been shown to be associated mainly with the membranes of *Phaseolus vulgaris* (Brandt and Benveniste, 1972) and *Nicotiana*

tabacum (Grunwald, 1970) leaves and seem to be necessary to maintain the integrity of semi-permeable membranes in red-beet (Grunwald, 1968) and barley root (Grunwald, 1971) tissues. Thus sterols are structural and possibly functional components of plant membranes. It is therefore postulated that the plant growth retardants have the ability to inhibit sterol biosynthesis and thus interfere with membrane synthesis and/or function, leading ultimately to a reduction of plant growth. This postulate is further explored in experiments presented in Results and Discussion.B. which follows.

B. THE EFFECTS OF RETARDANTS ON STEROL BIOSYNTHESIS IN, AND GROWTH OF TOBACCO SEEDLINGS

In section A of these results it was demonstrated that Amo 1618 was able to strongly inhibit sterol biosynthesis at levels above 3 μg/ml when supplied to cut stems of rootless tobacco seedlings. The retardant (at least at one concentration) also inhibited sterol biosynthesis and mevalonate production in cell-free tobacco seedling preparations. These observations, coupled with the known ability of Amo 1618 to slow the growth rate of tobacco (Marth *et al.*, 1953) raised the question of whether this and other plant growth retardants could possibly exert their growth retarding effects via an inhibition of sterol biosynthesis.

To test this hypothesis three retardants (viz. Amo 1618, CCC and Phosfon D) were examined for their effects on sterol biosynthesis in, and growth of tobacco seedlings. Some of this work has recently been accepted for publication (see Preface).

1. Effect of the Retardants on Sterol Biosynthesis

a. Experiments with rootless seedlings

The three retardants were tested for their effects on the incorporation of DL-2-^{14}C-mevalonate into sterols and sterol precursors in 21-day-old rootless tobacco seedlings (Methods 5). Non-saponifiable lipids were extracted from the seedlings [Methods 7.a.(i)] and separated into the five main sterol and hydrocarbon fractions by T.L.C. (Methods 8.a.).

Amo 1618 inhibition of mevalonate incorporation into sterol showed a concentration dependence in much the same manner as shown previously (Table 5). As the concentration of Amo 1618 increased, there was an increased inhibition of mevalonate incorporation into all of the sterol fractions (in particular the 4-desmethylsterols), accompanied by an increased accumulation of squalene-2,3-epoxide (figure 15).

Identification of the accumulation product was confirmed by co-chromatography on thin-layer silica gel G plates using 5% (v/v) ethyl acetate in n-hexane (R_f = 0.38) and 25% (v/v) ethyl acetate in n-hexane (R_f = 0.87) as developing solvents (figure 16) followed by gas-liquid chromatography with authentic squalene-2,3-epoxide and ^{14}C-squalene-2,3-epoxide standards (figure 17). The results confirmed those previously published (Douglas and Paleg, 1972) and demonstrated, once again that Amo 1618 is apparently an effective inhibitor of squalene-2,3-epoxide cyclase activity in rootless tobacco seedlings.

Figure 15. The effect of several concentrations of Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol intermediates of rootless tobacco seedlings.

Percentage incorporation of L-2-^{14}C-mevalonate into ● 4-desmethylsterols, □ 4-methylsterols, ▽ 4,4'-dimethylsterols, ○ squalene-2,3-epoxide, and △ squalene is shown.

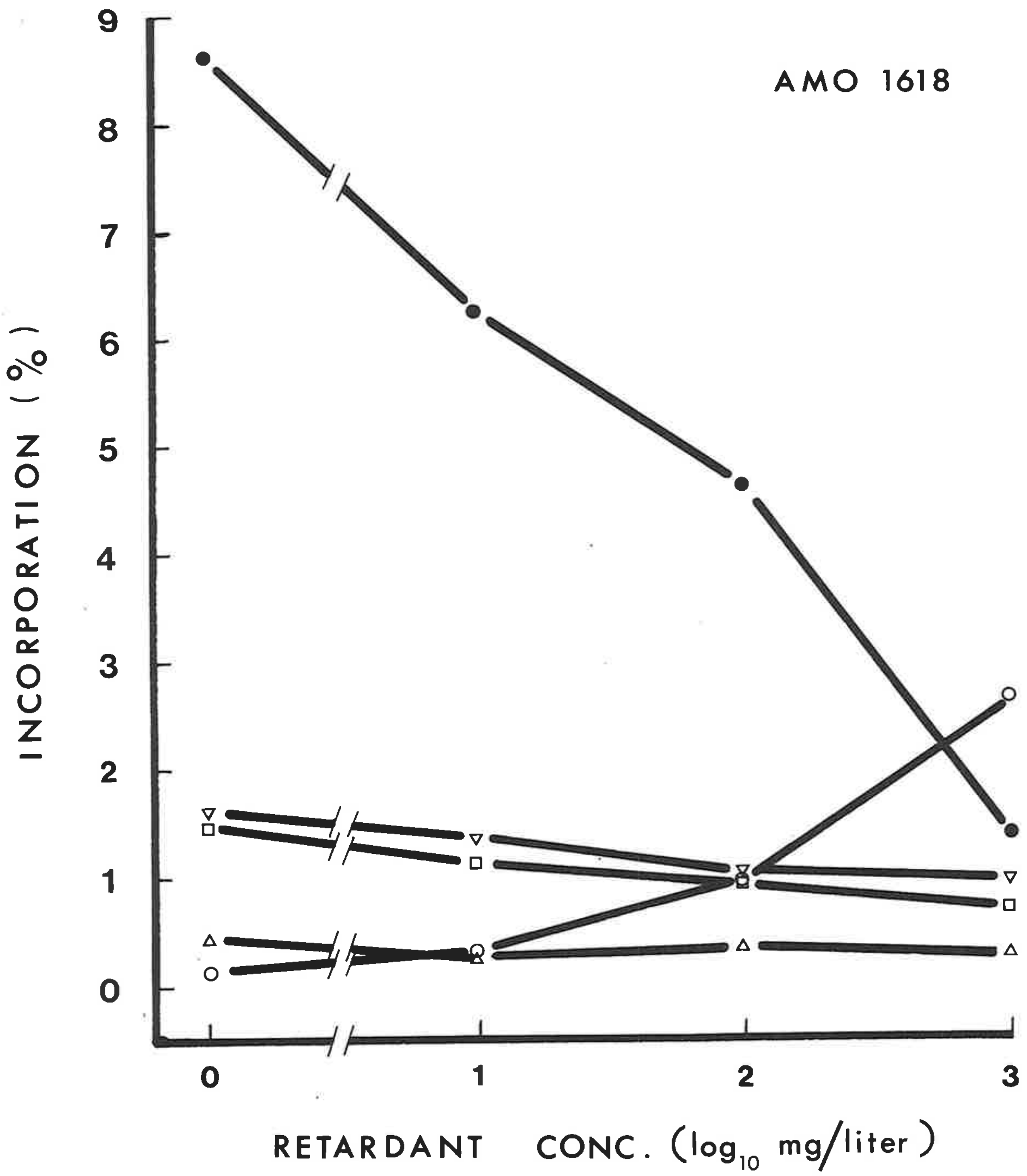
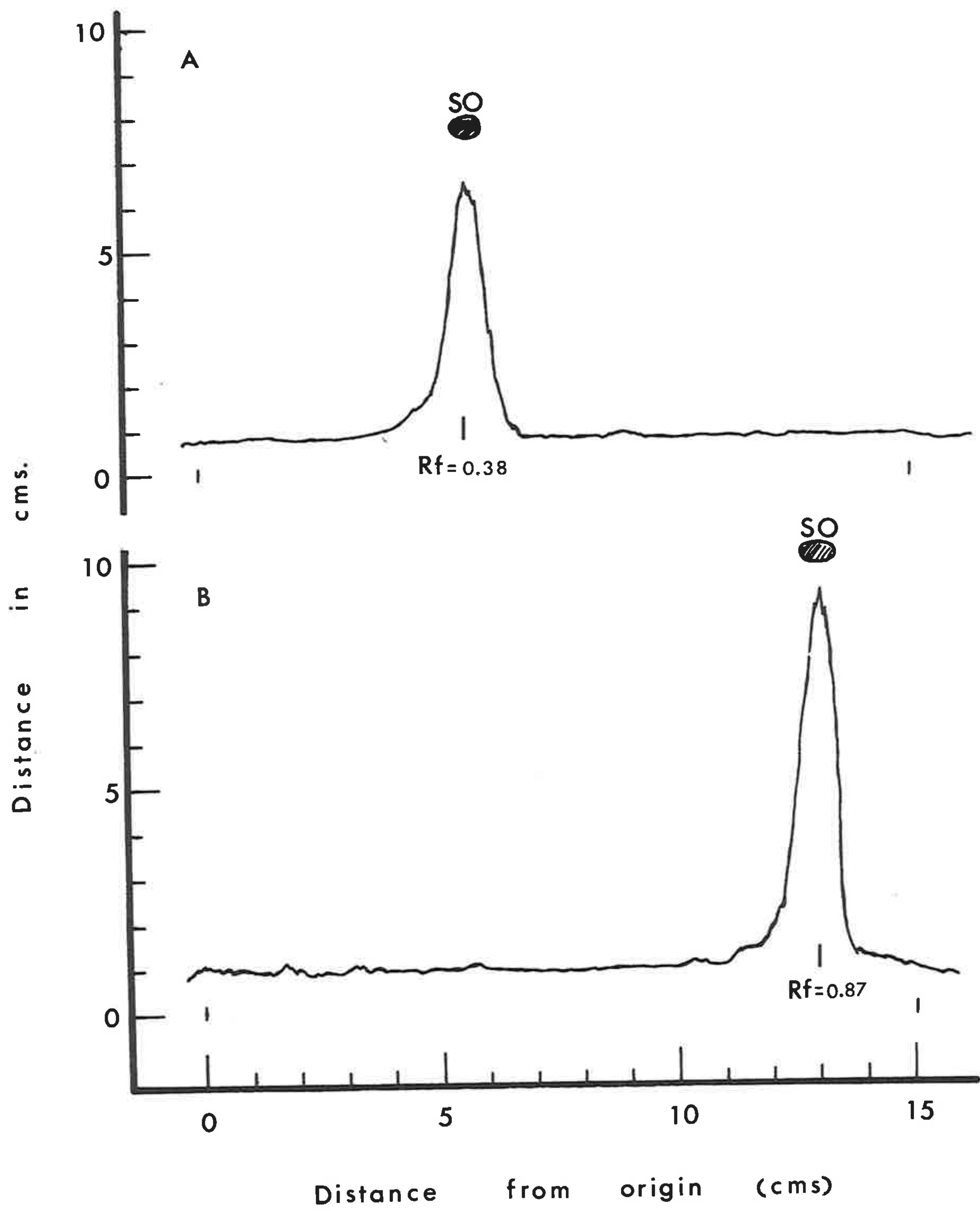

AMO 1618
INCORPORATION (%)
RETARDANT CONC. (log10 mg/liter)
0
1
2
3
4
5
6
7
8
9

Figure 16. Scans of radioactivity from squalene-2,3-epoxide region eluted from thin-layer chromatograms of non-saponifiable lipid fraction of Amo 1618-treated rootless tobacco seedlings.

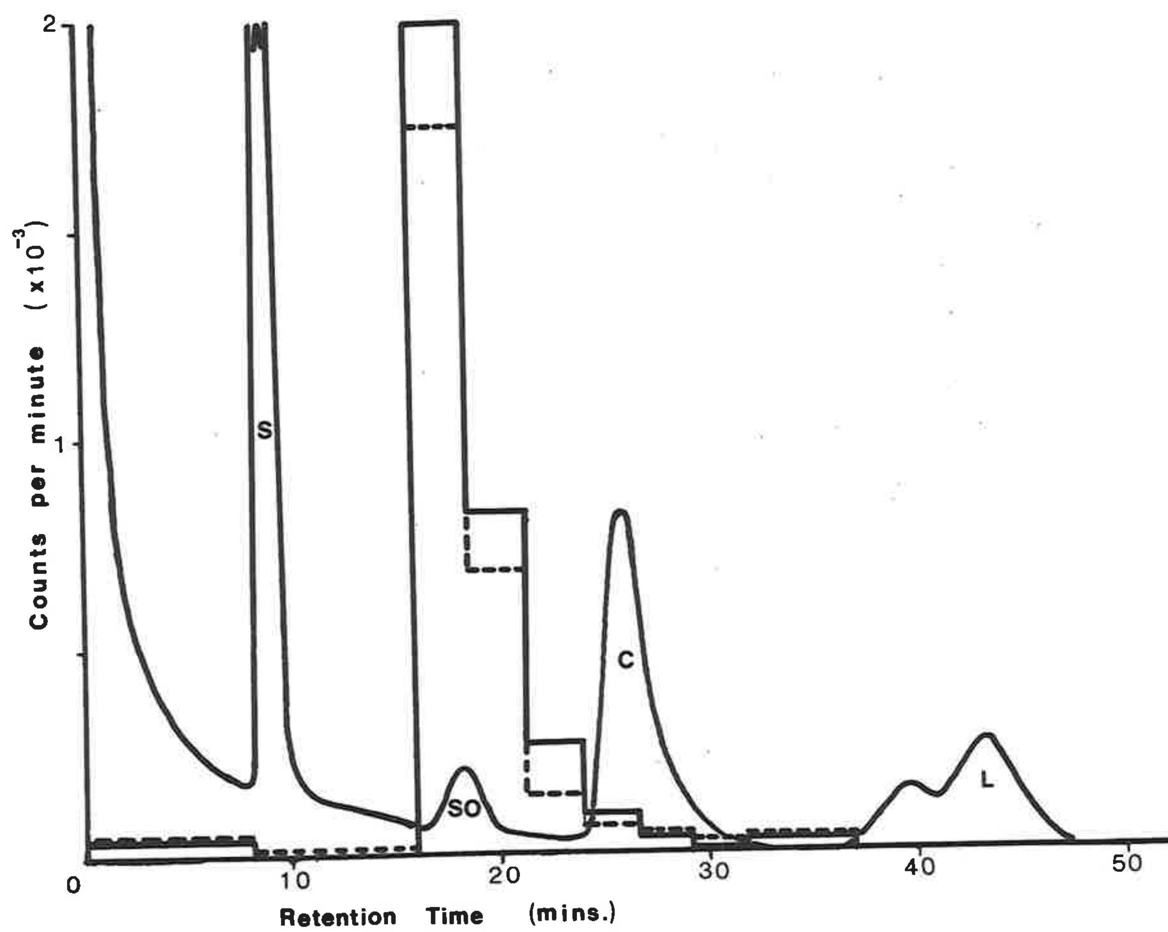
The solvent systems used are: A, 5% (v/v) ethyl acetate in n-hexane, and B, 25% (v/v) ethyl acetate in n-hexane. SO = squalene-2,3-epoxide.



A
B
SO
SO
Rf=0.38
Rf=0.87
10
5
0
10
5
0
Distance in cms.
0
5
10
15
Distance from origin (cms)

Figure 17. Distribution of radioactivity in fractions collected from G.L.C. eluate of the accumulation product resulting from treatment of rootless tobacco seedlings with 1,000 μg/ml Amo 1618.

Radioactivity from accumulation product (solid lines) and authentic ^{14}C-squalene-2,3-epoxide (broken lines) is shown. The curve shows the mass peaks of squalane (S), squalene-2,3-epoxide (SO), cholesterol (C), and lanosterol (L) standards.



Counts per minute (x10^{-3})
2
1
0
10
20
30
40
50
S
SO
C
L
Retention Time (mins.)

CCC also inhibits sterol biosynthesis in rootless tobacco seedlings both on a per gram fresh weight (Table 9) and an absolute incorporation (figure 18) basis when supplied at concentrations greater than 5 μg/ml. At the lowest concentration, however, an apparent stimulation in the incorporation of mevalonate into all sterol and squalene fractions was observed. These findings parallel the effects of very low concentrations of Amo 1618 on sterol biosynthesis reported earlier (Results A.2.). Concentrations of the retardant which inhibited sterol biosynthesis also produced an accumulation of squalene-2,3-epoxide in a similar fashion to Amo 1618. The accumulation product from the 500 μg/ml CCC treatment was identified by co-chromatography on T.L.C. and G.L.C. (figure 19) by the same methods employed for squalene-2,3-epoxide from Amo 1618-treated tobacco seedlings.

The two retardants, Amo 1618 and CCC, appear to have a common site of action (viz. the squalene-2,3-epoxide cyclase step) although much greater levels of CCC are required to produce an inhibition of sterol biosynthesis. For example, on an absolute basis, 10 μg/ml Amo 1618 produced about 29% inhibition of mevalonate incorporation into 4-desmethylsterols whereas 50 μg/ml CCC was required to cause a 22% reduction in 4-desmethylsterol production. At 1,000 μg/ml the inhibitions of incorporation into 4-desmethylsterols were about 83% for Amo 1618 and 62% for CCC.

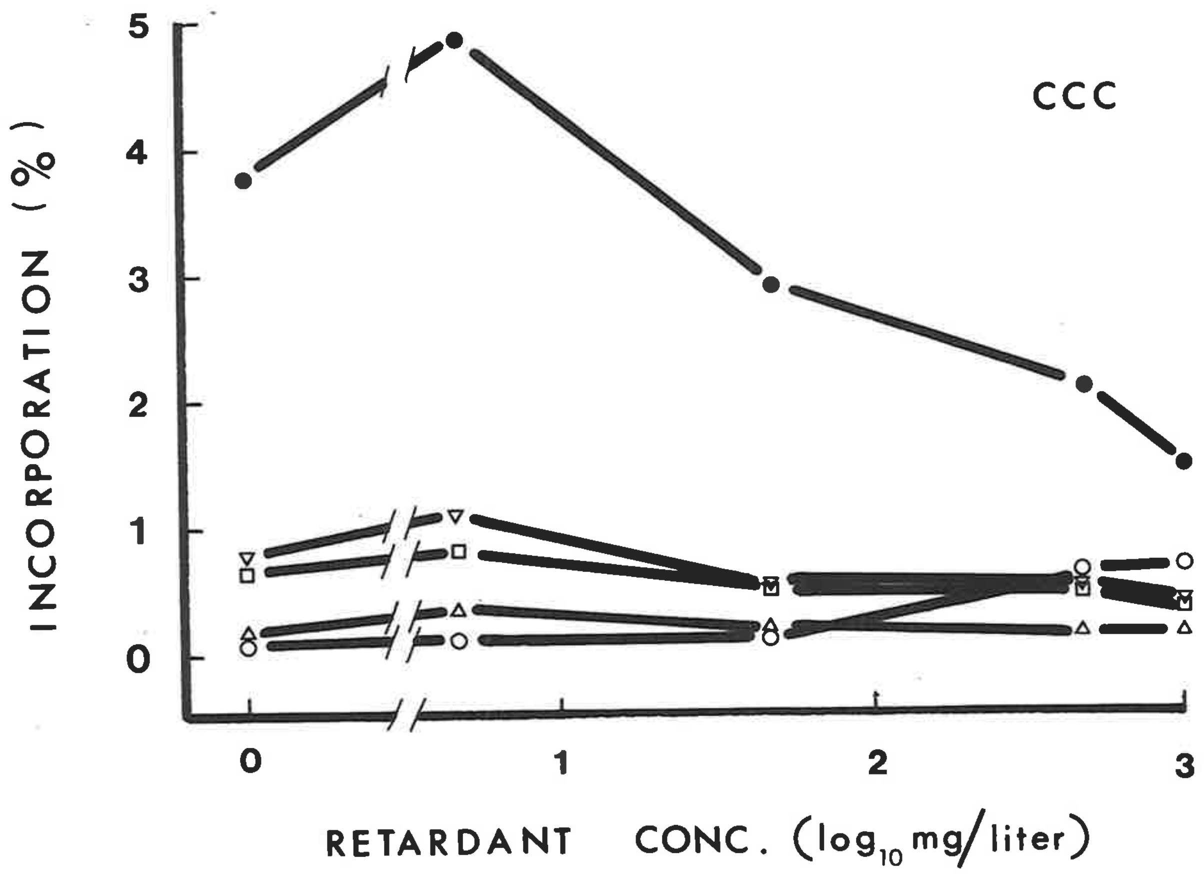
Table 9. Effect of CCC on incorporation of DL-2-^{14}C-mevalonic acid into tobacco seedling sterols and sterol precursors.

(Sterols and sterol intermediates separated by T.L.C. of total non-saponifiable lipid fraction).

Retardant concen- tration	Incorporation of L-2-^{14}C-mevalonate into				
	4-desmethyl- sterols	4-methyl- sterols	4,4'-dimethyl- sterols	squalene- 2,3-epoxide	squalene
μg/ml	c.p.m./gm F.W.				
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
1,000	65,786	15,008	17,799	29,958	6,268

Figure 18. The effect of several concentrations of CCC on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol intermediates of rootless tobacco seedlings.

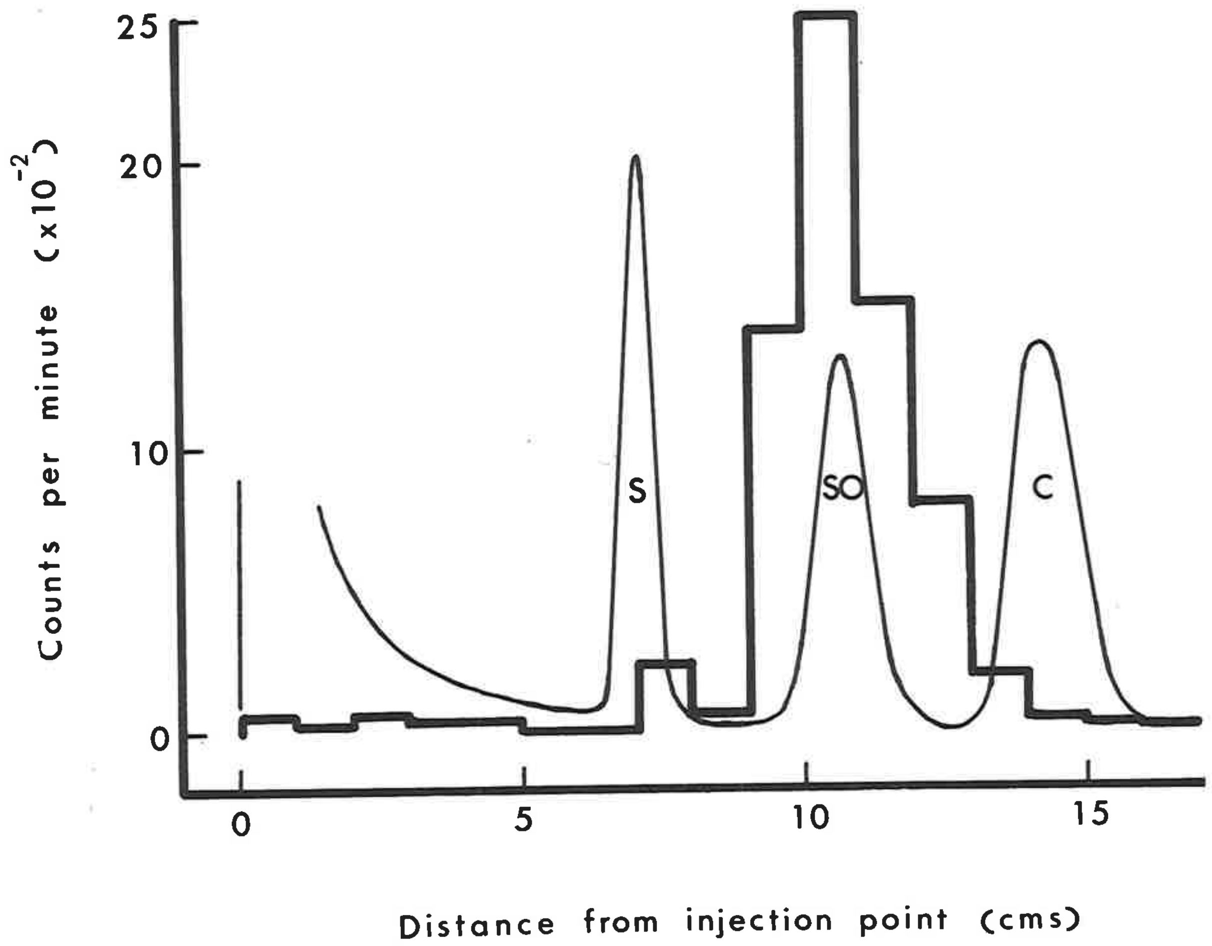
Percentage incorporation of L-2-^{14}C-mevalonate into ● 4-desmethylsterols, □ 4-methylsterols, ▽ 4,4'-dimethylsterols, ○ squalene-2,3-epoxide, and △ squalene is shown.



CCC
INCORPORATION (%)
5
4
3
2
1
0
0
1
2
3
RETARDANT CONC. (log10 mg/liter)

Figure 19. Distribution of radioactivity in fractions collected from G.L.C. eluate of the accumulation product resulting from treatment of rootless tobacco seedlings with 1,000 μg/ml CCC.

Radioactivity associated with fractions (solid lines) and mass peaks of standards (curve) are shown. S = squalene; SO = squalene-2,3-epoxide, and C = cholesterol.



25
20
10
0
Counts per minute (x10^{-2})
S
SO
C
0
5
10
15
Distance from injection point (cms)

Of the three retardants tested, the most powerful inhibitor of mevalonate incorporation into 4-desmethylsterols proved to be Phosfon D. A concentration of 50 μg/ml Phosfon D produced 52% inhibition of mevalonate incorporation compared with 46% inhibition by 100 μg/ml Amo 1618.

Pronounced differences existed between the effects of Phosfon D on sterol biosynthesis and the effects of Amo 1618 and CCC. As the concentration of Phosfon D increased, incorporation of the precursor into 4-desmethylsterols showed a similar concentration dependence as was observed with Amo 1618 but the 4-methylsterol and 4,4'-dimethylsterol fractions demonstrated large increases in incorporation up to about 50 μg/ml of the retardant followed by decreases back to almost control values (figure 20). On a per gram fresh weight basis (Table 10) the lowest concentration of Phosfon D tested (5 μg/ml) caused an increase of incorporation into all sterol and hydrocarbon fractions. When calculated on an absolute incorporation basis, however, only the 4,4'-dimethylsterols showed a significant increase in radioactivity (<u>ca</u>. 2.8-fold) while the 4-desmethylsterols actually showed about a 14% reduction in radioactivity. It seems likely therefore, that the major sites of sterol inhibition by Phosfon D are the demethylation steps in the biosynthetic sequence.

In this experiment it was noted that the two highest concentrations of the retardant caused visible wilting of the experimental plant material. In fact, 1,000 μg/ml of Phosfon D caused marked wilting in

Figure 20. The effect of several concentrations of Phosfon D on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol intermediates of rootless tobacco seedlings.

Percentage incorporation of L-2-^{14}C-mevalonate into ● 4-desmethylsterols, □ 4-methylsterols, ▽ 4,4'-dimethylsterols, ○ squalene-2,3-epoxide, and △ squalene is shown.

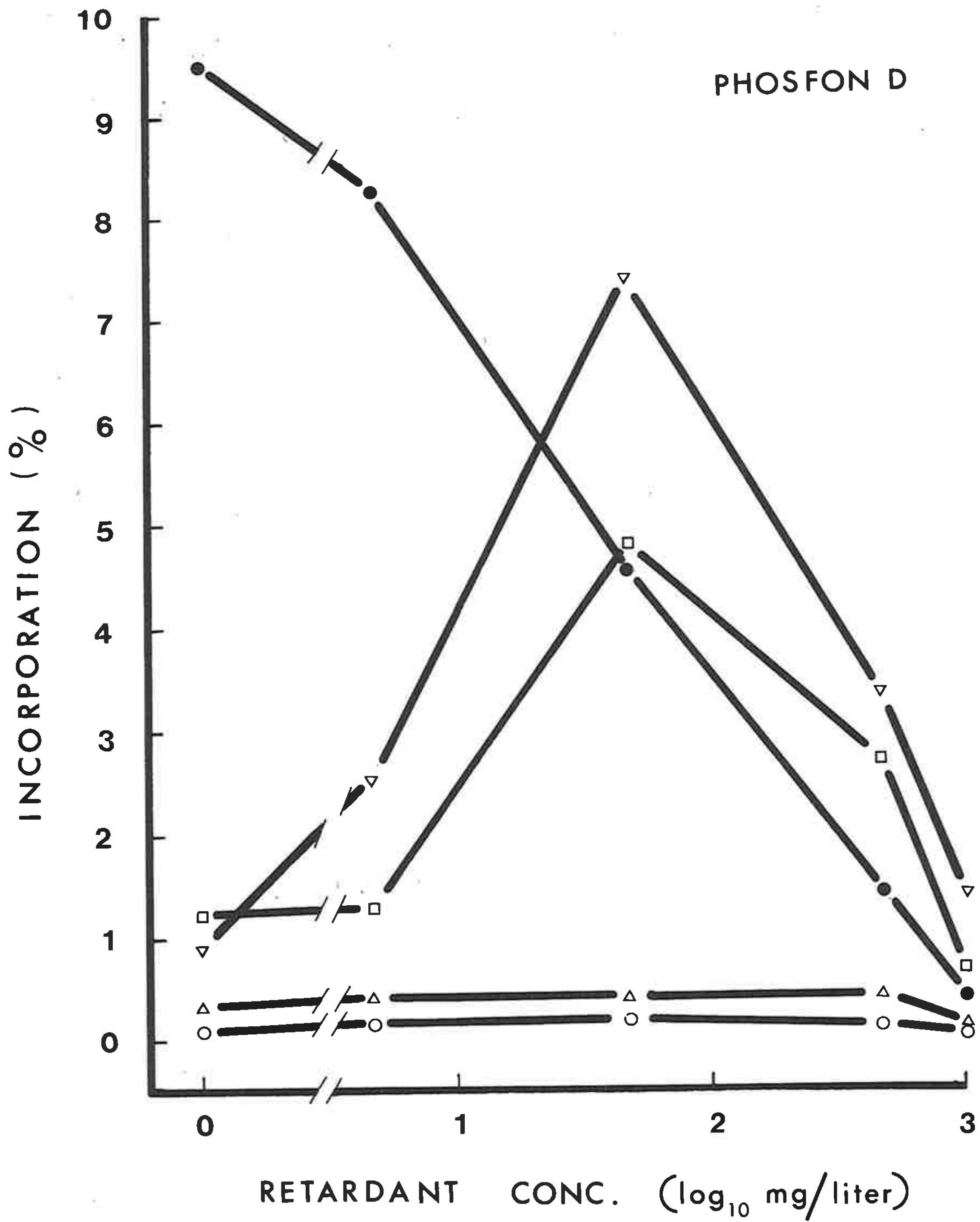

PHOSFON D
INCORPORATION (%)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
RETARDANT CONC. (log10 mg/liter)

Table 10. Effect of Phosfon D on incorporation of DL-2-^{14}C-mevalonic acid into tobacco sterols and sterol precursors.

(Sterols and sterol intermediates separated by T.L.C. of total non-saponifiable lipid fraction).

Retardant concen-tration	Incorporation of L-2-^{14}C-mevalonate into				
	4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
µg/ml	c.p.m./gm F.W.				
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
1,000	22,766	37,687	77,385	2,877	7,643

all of the seedlings in the test solution. The severe stressing of plant material could lead to an overall reduction in metabolic activity and might account for the decline in accumulation of the intermediate sterols observed at these high levels of the retardant.

Other possible explanations for this reduction in the accumulation of radioactivity into the 4-methylsterols and 4,4'-dimethylsterols at high Phosfon D concentration will be discussed later in this chapter.

A similar site of action of Phosfon D was demonstrated in cholesterol biosynthesis in cell-free rat liver homogenates. Rat liver preparations (Methods 13.b.) were incubated in the presence of DL-2-^{14}C-mevalonate under aerobic conditions in the presence or absence of 1,000 μg/ml Phosfon D. After saponification of the incubation mixture, the non-saponifiable lipids were extracted into petroleum spirit (b.p. 60-80 C) and examined by co-chromatography with authentic standards by T.L.C. (Methods 13.b.).

The retardant inhibited the incorporation of precursor into cholesterol by about 83% and increased the incorporation into the lanosterol fraction by 3 to 4-fold. Incorporation into the hydrocarbon fractions was relatively unchanged (Table 11). These results are consistent with an inhibition of cholesterol biosynthesis by Phosfon D at a site (or sites) between lanosterol and cholesterol in the biosynthetic pathway and support the findings in tobacco seedlings.

Table 11. Effect of Phosfon D on the incorporation of DL-2-^{14}C-mevalonic acid into cholesterol and intermediates on the cholesterol pathway in cell-free rat liver homogenates.

(Sterols and sterol precursors separated by T.L.C. of the total non-saponifiable lipid fraction).

Sterol fraction	Incorporation of L-2-^{14}C-mevalonate		% of control
	Control	Phosfon D-treated	
	c.p.m.		
cholesterol	127,257	21,151	16.6
lanosterol	7,023	24,072	342.8
squalene-2,3-epoxide	10,867	12,910	118.8
squalene	14,116	15,001	106.3

b. Effect of Amo 1618 on sterol biosynthesis in intact tobacco seedlings

In order to relate effects of the retardants on sterol biosynthesis with growth, the effects of one retardant (Amo 1618) were tested on sterol biosynthesis in intact tobacco seedlings.

A single application of Amo 1618 (300 μg) was made to the stem apex of individually potted tobacco seedlings along with a total of 2 μCi of DL-2-^{14}C-mevalonic acid. Control plants were administered only the mevalonate. The treated and control plants were then split into two equal groups and one was maintained in the growth cabinet under conditions identical to those used for all growth studies while the other group was placed in the isotope laboratory under the low light intensity conditions used for all rootless seedling experiments. After the usual 24 hr illumination period the sterols were extracted from the whole, washed seedlings [Methods 7.a.(i)] and examined by T.L.C. (Methods 8.a.).

The effect of Amo 1618 (Table 12) on the incorporation of mevalonate into sterols and squalene-2,3-epoxide was less pronounced under conditions of high light intensity (ca. 3-fold increase in squalene-2,3-epoxide) compared with low light intensity (ca. 7-fold increase in squalene-2,3-epoxide). Incorporation of mevalonate into 4-desmethylsterols was also less (on both a % incorporation and per gram fresh weight basis) under conditions of high light intensity. In spite of these observed quantitative differences, however, it may be

Table 12. Effect of 300 μg Amo 1618 and light intensity on incorporation of DL-2-^{14}C-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
	c.p.m./gm F.W.			
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

concluded that, at least with Amo 1618, the absence of roots caused no marked quantitative effect on the incorporation of mevalonate into sterols and sterol precursors in the above ground parts of the plant. This conclusion is supported by work presented later in this thesis in which sterol production in various plant tissues of tobacco is examined more closely. Similarly it can also be assumed that Amo 1618, and probably the other retardants, will affect sterol biosynthesis in growth experiments with intact seedlings in essentially the same way they do in experiments with rootless seedlings.

c. Amo 1618 effects on sterol biosynthesis in tobacco tissues

Twenty one-day-old seedlings were treated with DL-2-^{14}C-mevalonate in the presence or absence of 100 μg Amo 1618 per plant (Methods 3.). The plants were left in the growth cabinet under conditions identical to those used in all growth experiments (Methods 1.) for 24 hr, after which they were removed and the roots washed exhaustively. The plants were then divided up into leaf, stem and root tissues. Incorporation products were extracted from each of the individually weighed tissues [Methods 7.a.(i)] and assayed by T.L.C. (Methods 8.a.) and G.L.C. on 2.5% OV-101 columns (Methods 12).

The retardant inhibited mevalonate incorporation into 4-desmethylsterols and caused some accumulation of squalene-2,3-epoxide in each of the tissues of the plant (figures 21,22 and 23). The stems, however, accounted not only for the greatest uptake of mevalonate into 4-desmethylsterols but also the greatest accumulation of radioactivity

Figure 21. Effect of Amo 1618 on mevalonate incorporation into leaves of intact tobacco seedlings.

The distribution of radioactivity on T.L.C. silica gel G plates is shown for total non-saponifiable lipid extracts from leaves of control (A), and Amo 1618-treated (B) tobacco seedlings. Plates were developed twice in 4% (v/v) diethylether in methylene chloride. C = cholesterol, L = lanosterol, SO = squalene-2,3-epoxide, and S = squalene standard markers.

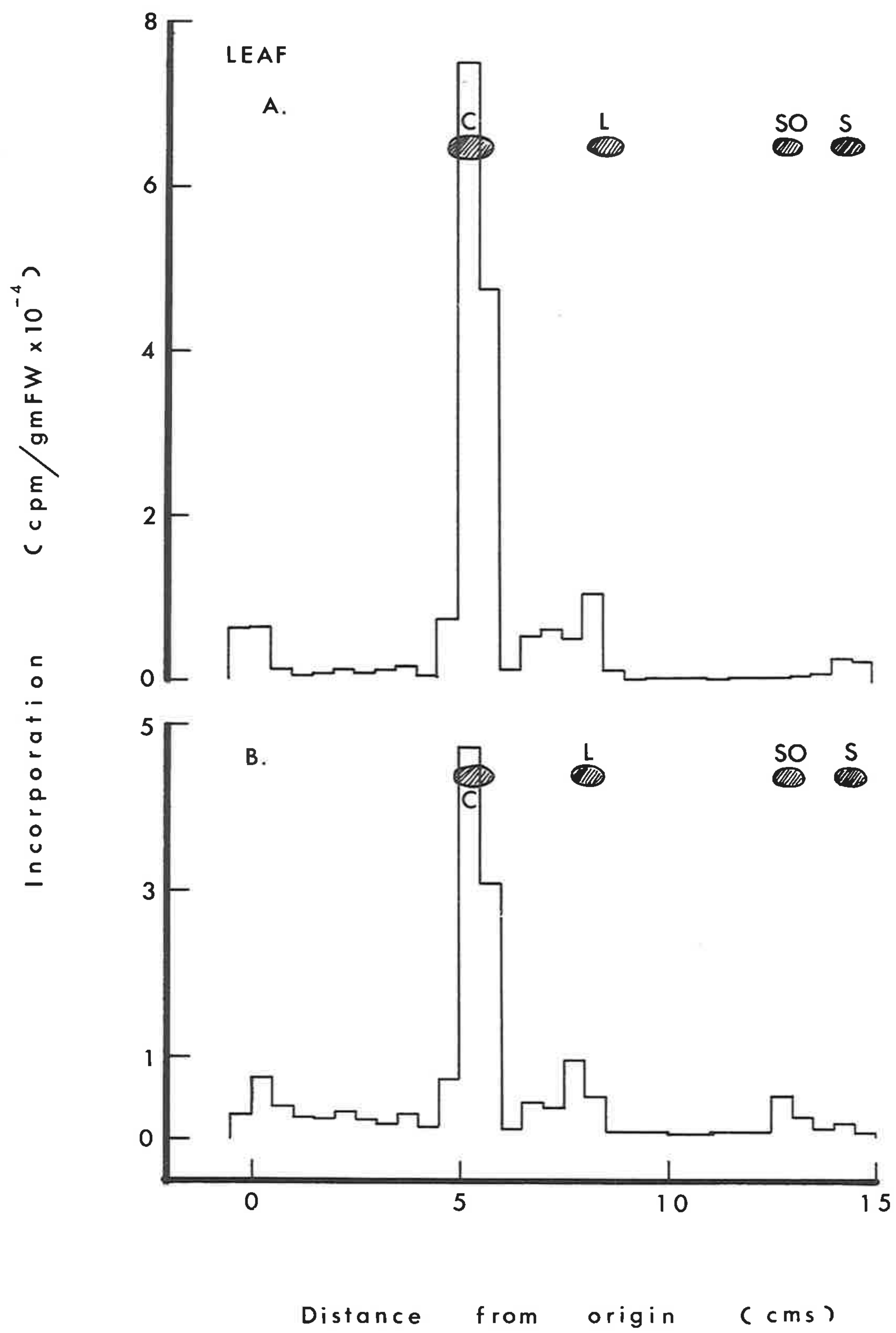

LEAF
A.
B.
C
L
SO
S
Incorporation (cpm/gmFW x 10⁻⁴)
8
6
4
2
0
5
3
1
0
0
5
10
15
Distance from origin (cms)

Figure 22. Effect of Amo 1618 on mevalonate incorporation into stems of intact tobacco seedlings.

The distribution of radioactivity on T.L.C. silica gel G plates is shown for total non-saponifiable lipid extracts from stems of control (A), and Amo 1618-treated (B) tobacco seedlings. Plates were developed twice in 4% (v/v) diethylether in methylene chloride. C = cholesterol, L = lanosterol, SO = squalene-2,3-epoxide, and S = squalene standard markers.

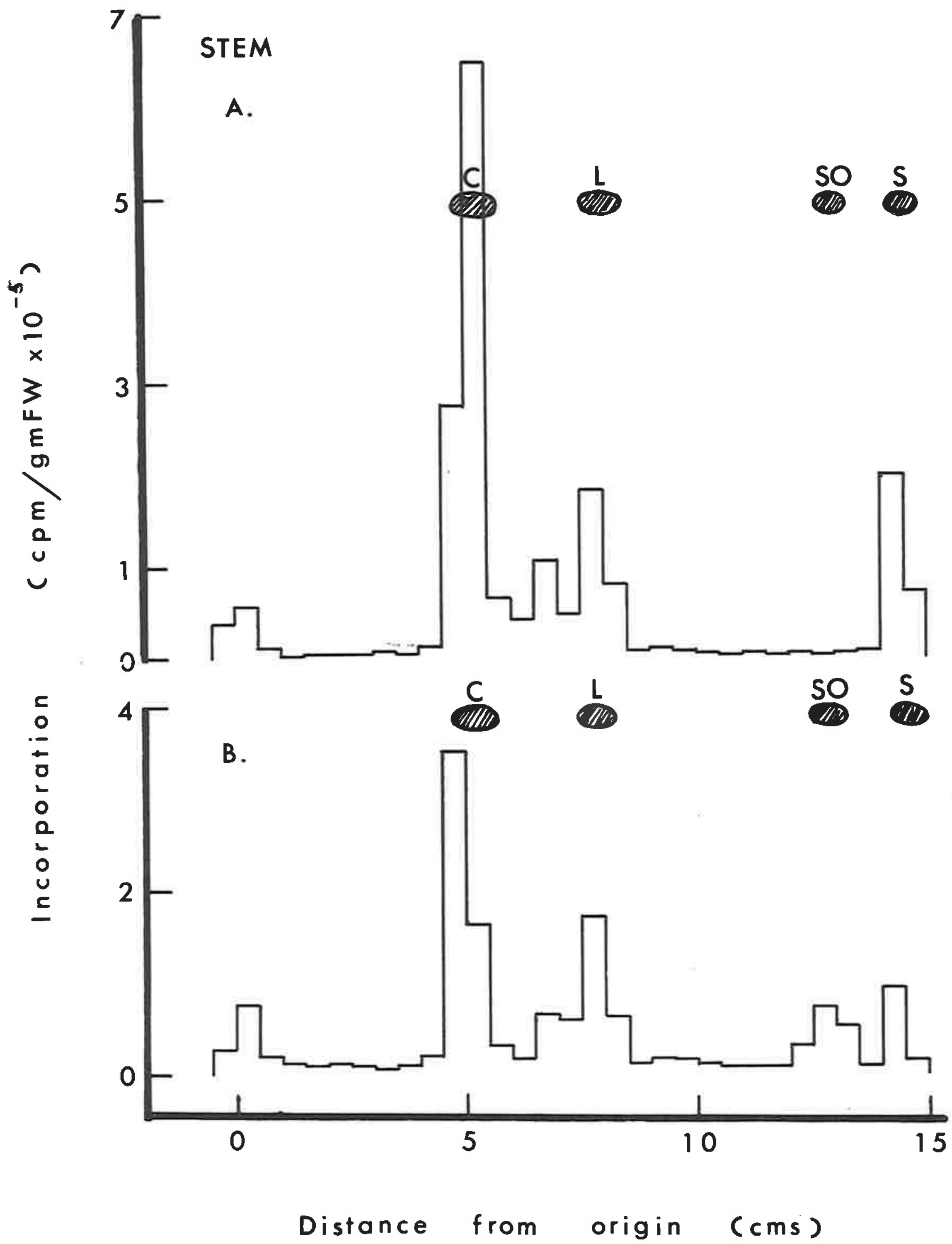
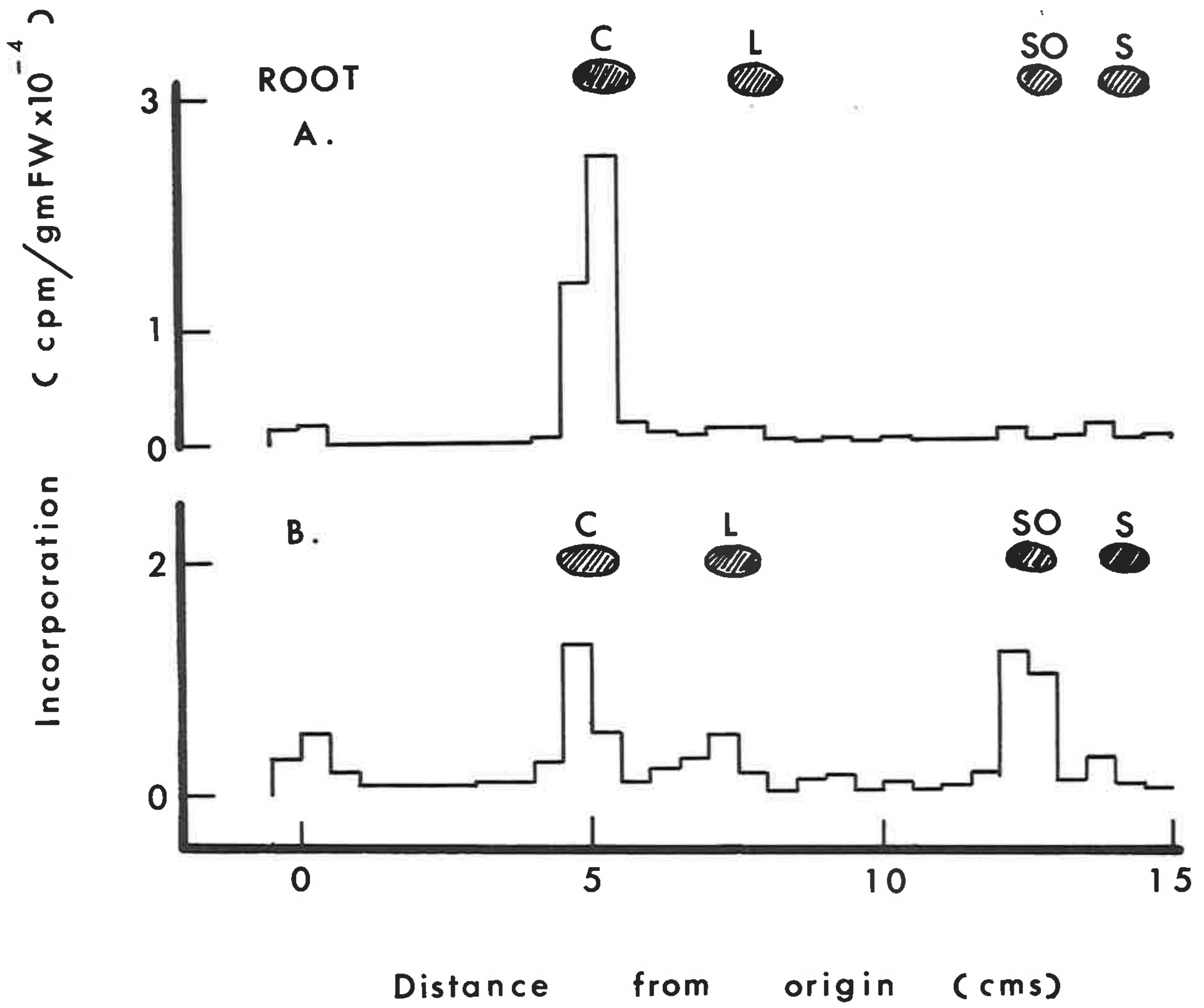

STEM
A.
B.
C
L
SO
S
Incorporation (cpm/gmFW x10^{-5})
Distance from origin (cms)
0
1
3
5
7
2
4
10
15

Figure 23. Effect of Amo 1618 on mevalonate incorporation into roots of intact tobacco seedlings.

The distribution of radioactivity on T.L.C. silica gel G plates is shown for total non-saponifiable lipid extracts from roots of control (A), and Amo 1618-treated (B) tobacco seedlings. Plates were developed twice in 4% (v/v) diethylether in methylene chloride. C = cholesterol, L = lanosterol, SO = squalene-2,3-epoxide, and S = squalene standard markers.


ROOT
A.
B.
C
L
SO
S
Incorporation (cpm/gmFW x 10^{-4})
3
1
0
2
0
0
5
10
15
Distance from origin (cms)

into squalene-2,3-epoxide when calculated on a per gram fresh weight basis. The leaves accounted for _ca_. 79% of the total seedling fresh weight and 59% of labelled desmethylsterols, the stems for _ca_. 7% and 38% respectively, and the roots _ca_. 14% and 3% respectively (Table 13). Further, Amo 1618 treatment inhibited 4-desmethylsterol biosynthesis more strongly in stems (_ca_. 45%) than in leaves (22%) and roots (28%).

Assuming even distribution of ^{14}C-mevalonate and Amo 1618 throughout the various plant tissues this data might suggest that sterol biosynthesis in stems is more susceptible to inhibition (presumably at the squalene-2,3-epoxide cyclase step) by the retardant than in other tissues. The roots contribute very little, quantitatively, to the actual incorporation of ^{14}C-mevalonate into sterols and sterol precursors.

It is interesting to note that Amo 1618 treatment of plants maintained at high light intensity results in a decreased incorporation of mevalonate into the squalene fraction, separated by T.L.C., of the total non-saponifiable lipid fraction. This decrease was observed for whole seedlings (_ca_. 31% reduction) treated with 300 μg of Amo 1618 (Table 12) and also for above ground parts of the plant (e.g. stems _ca_. 44% reduction, and leaves _ca_. 52% reduction) in seedlings treated with 100 μg Amo 1618 (Table 13). No similar reduction in incorporation into squalene was observed in experiments conducted under conditions of low light intensity (e.g. Table 12). This data may imply that Amo 1618 was able to inhibit a pre-squalene step in the biosynthetic

Table 13. Effect of 100 μg Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol precursors in tissues of intact tobacco seedlings.

(Sterols and sterol intermediates separated by T.L.C. of the total non-saponifiable lipid extract).

Tissue	Treatment	Incorporation of L-2-^{14}C-mevalonate into				
		4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene 2,3-epoxide	squalene
		c.p.m.				
Leaves	Control	66,524	5,219	10,533	977	3,289
	Amo 1618-treated	51,902	4,537	8,254	2,549	1,583
Stems	Control	43,449	7,681	11,648	919	12,246
	Amo 1618-treated	24,102	6,570	9,598	5,422	6,902
Roots	Control	3,320	320	795	153	131
	Amo 1618-treated	2,409	247	222	1,728	235

sequence only at high light intensity. The smaller build-up of squalene-2,3-epoxide observed at high light intensity might support such a site of inhibition by the retardant.

Another differential effect between sterol biosynthesis in each of the various plant tissues was observed by G.L.C. analysis of the 4-desmethylsterol fractions of each tissue. Similar (though not identical) patterns were obtained for the mass peaks of the four major sterols in each of the tissues (figure 24) but marked differences in the distribution of radioactivity existed between the above ground tissues and the roots. In both stems and leaves the major radioactive sterol was β-sitosterol whereas campesterol and stigmasterol shared the greatest portion of radioactive label in roots. Amo 1618 inhibited mainly β-sitosterol synthesis in leaves, stigmasterol and β-sitosterol synthesis in stems, and incorporation into all sterols in roots (Table 14).

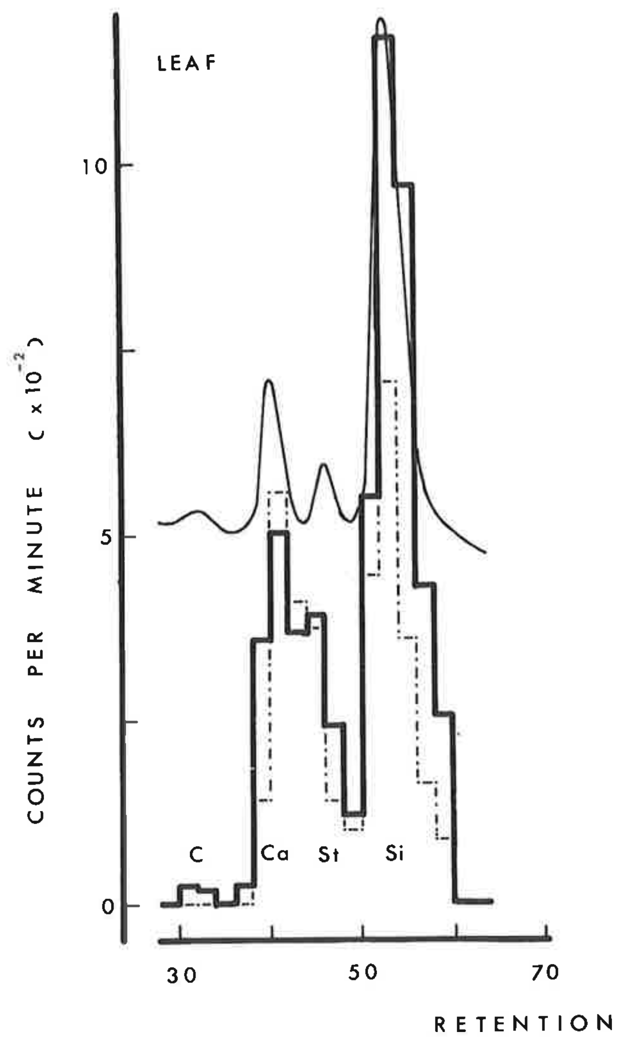
Table 14. Effect of 100 µg Amo 1618 on the incorporation of DL-2-^{14}C-mevalonic acid into 4-desmethylsterols in tissues of intact tobacco seedlings.

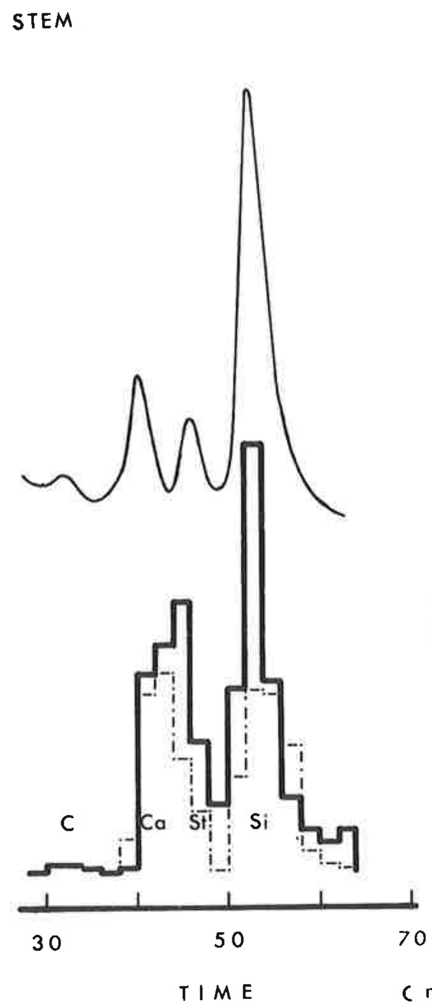
(Individual sterols separated by G.L.C. of the 4-desmethylsterol fraction from T.L.C. plates)

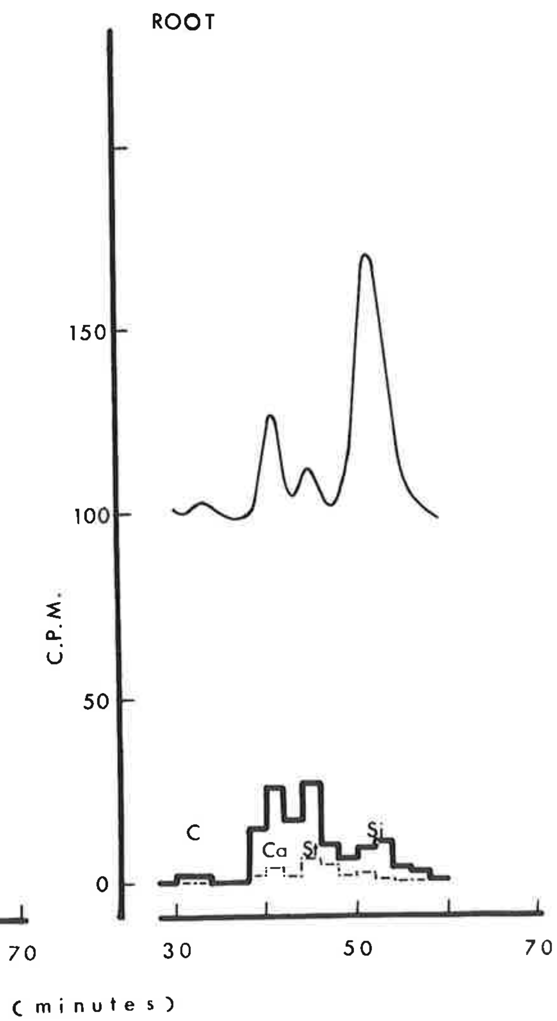
Tissue	Treatment	Incorporation of L-2-^{14}C-mevalonate into			
		Cholesterol	Campesterol	Stigmasterol	β-Sitosterol
		c.p.m.			
Leaves	Control	44	1,068	936	2,871
	Amo 1618-treated	0	900	815	1,357
Stems	Control	22	765	451	1,342
	Amo 1618-treated	0	639	164	742
Roots	Control	10	32	47	27
	Amo 1618-treated	0	14	17	8

Figure 24. G.L.C. analysis of 4-desmethylsterols of tobacco tissues from control and Amo 1618-treated intact tobacco seedlings.

The 4-desmethylsterol fractions from T.L.C. of the non-saponifiable lipid extracts of leaf, stem, and root tissues of intact tobacco seedlings were injected onto a G.L.C. column (2.5% OV-101) and fractions of the eluate collected to determine radioactivity associated with the individual sterols for control (solid lines), and Amo 1618-treated (broken lines) seedlings. The solid curve shows the mass peaks for sterol from the control extracts. C = cholesterol, Ca = campesterol, St = stigmasterol and Si = β-sitosterol.







RETENTION TIME (minutes)

2. Effects of Retardants on Growth of Intact Tobacco Seedlings

It was pointed out earlier (Introduction 2.) that there is not only an apparent lack of correlation between the effects of retardants on growth and taxonomic classification of the plants on which they act, but cultivars of the same species can also vary greatly in their responsiveness to the same retardant. For these reasons it was decided that despite existing reports on the effects of retardants on tobacco (e.g. Marth et al., 1953; Cathey and Stuart, 1961) it was essential to establish an effect of the retardants on the growth of N. tabacum (cv. Turkish Samson) seedlings used in these studies before the effects on sterol biosynthesis could be correlated with effects on growth.

Growth of seedlings was examined following either continuous applications of small doses of the retardant (Methods 3.a.) or single doses of higher levels of the retardant. Three parameters of growth (viz. stem height, leaf length, and leaf width) were measured immediately prior to treatment of the seedlings and thereafter on each alternate day (Methods 2.).

a. Effect of continuous application of retardants

Four concentrations (viz. 1, 3, 10 and 30 μg/plant) of each of the retardants Amo 1618, CCC, and Phosfon D were tested for their effects on 17 day-old tobacco seedlings. The retardants were applied as single 5 μl drops of a solution in 0.05% (w/v) tween-20 and controls were treated with the tween solution only. All of the measurements

for each plant during the experimental period were converted to logarithms and statistical analysis of the log values was carried out by computer.

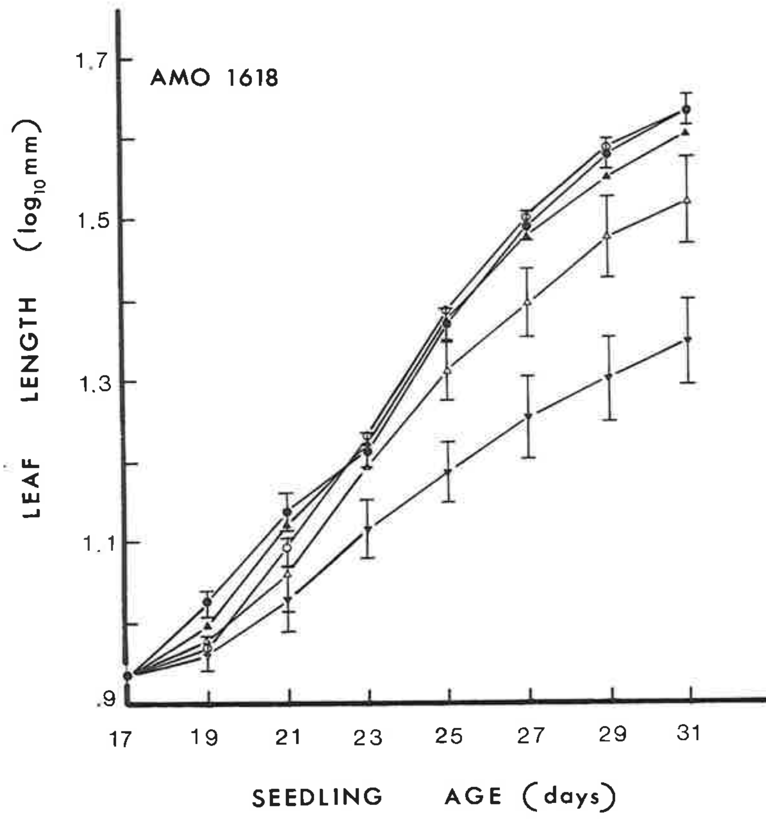
Neither stem nor leaf growth was retarded by continuous application of 1 or 3 μg of Amo 1618 but both parameters were significantly inhibited by 30 μg Amo 1618 treatments (figures 25, 26). Treatment of seedlings with 10 μg quantities of Amo 1618 produced some retardation of leaf growth (in particular leaf length) and a more pronounced inhibition of stem elongation. It would therefore appear that continuous application of levels of Amo 1618 greater than 10 μg/plant are required to produce significant retardation of growth of tobacco seedlings at this age (17 days) and under the conditions (constant temperature, continuous light) employed.

CCC appeared to be less effective than Amo 1618 in retarding tobacco seedling growth as 1, 3 and 10 μg treatments were all relatively ineffective in slowing either stem or leaf growth and only 30 μg/plant was able to cause an inhibition of both parameters (figures 27, 28). Further, 30 μg CCC treatments were only about as effective in retarding stem elongation as 10 μg Amo 1618 treatments.

By far the most potent growth retardant was Phosfon D. As little as 1 μg of this retardant/plant was able to cause marked reductions in both leaf and stem growth (figures 29, 30). The 30 μg Phosfon D treatments caused severe tissue damage after only a few applications and finally proved fatal to the seedlings (data not shown).

Figure 25. Continuous application of several levels of Amo 1618 to 17 day-old tobacco seedlings.

The effect of the retardant on leaf length and leaf width is shown for control (●) seedlings and seedlings treated with 1 μg (○), 3 μg (▲), 10 μg (△), or 30 μg (▼) of Amo 1618.


AMO 1618
LEAF LENGTH (log10 mm)
SEEDLING AGE (days)
1.7
1.5
1.3
1.1
.9
17
19
21
23
25
27
29
31

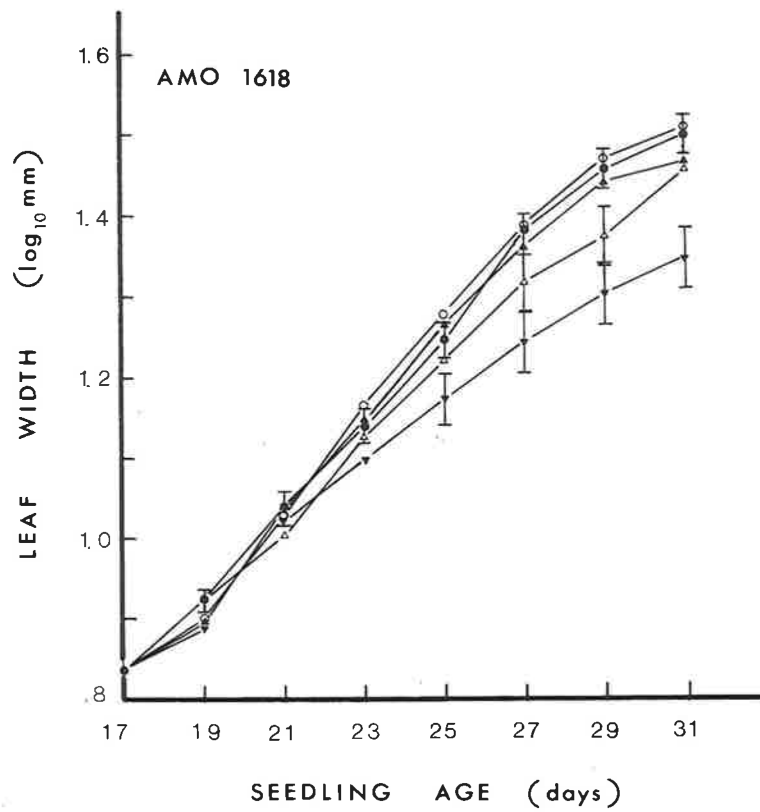

AMO 1618
LEAF WIDTH (log10 mm)
SEEDLING AGE (days)
1.6
1.4
1.2
1.0
.8
17
19
21
23
25
27
29
31

Figure 26. Effect of continuous application of Amo 1618 on stem elongation of tobacco seedlings.

The stem growth of control (●) seedlings is compared with that of seedlings treated with 1 μg (○), 3 μg (▲), 10 μg (△), or 30 μg (▼) of retardant.

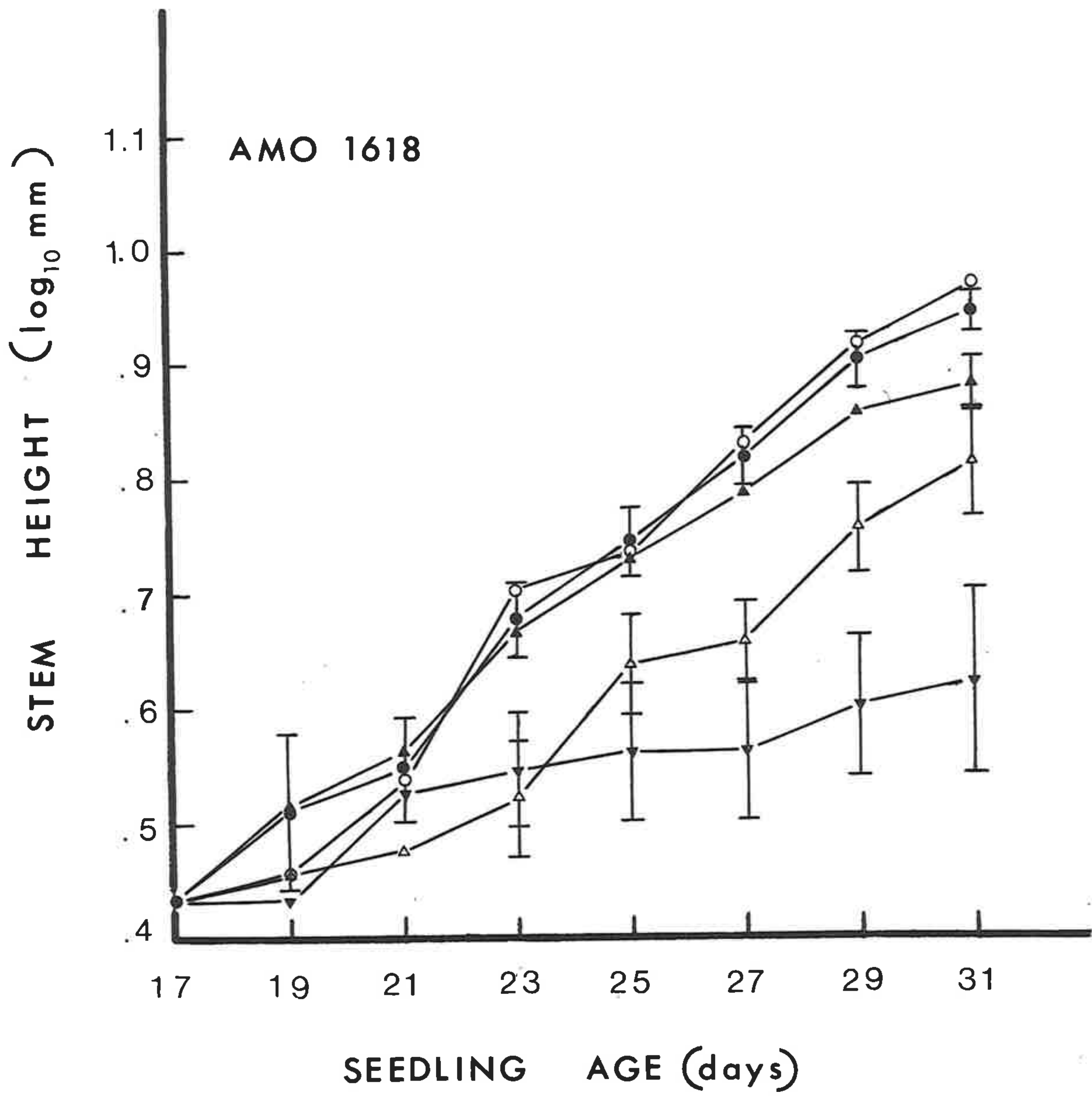
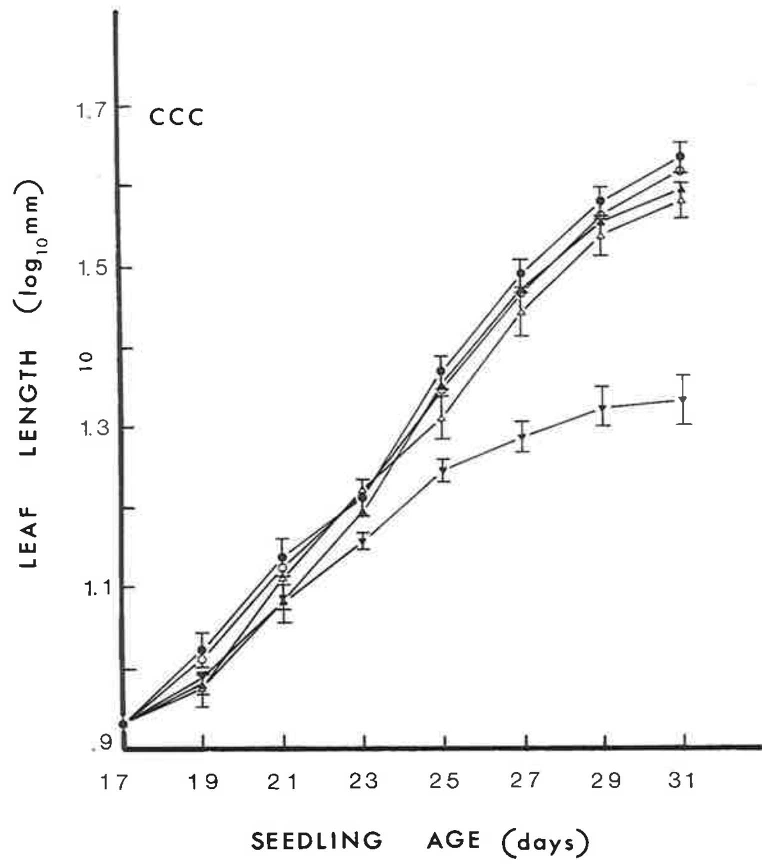

AMO 1618
STEM HEIGHT (log10 mm)
1.1
1.0
.9
.8
.7
.6
.5
.4
17
19
21
23
25
27
29
31
SEEDLING AGE (days)

Figure 27. Continuous application of several levels of CCC to 17 day-old tobacco seedlings.

The effect of the retardant on leaf length and leaf width is shown for control (●) seedlings and seedlings treated with 1 μg (○), 3 μg (▲), 10 μg (△), or 30 μg (▼) of CCC.


CCC
LEAF LENGTH ($\log_{10}$ mm)
1.7
1.5
1.3
1.1
.9
17
19
21
23
25
27
29
31
SEEDLING AGE (days)

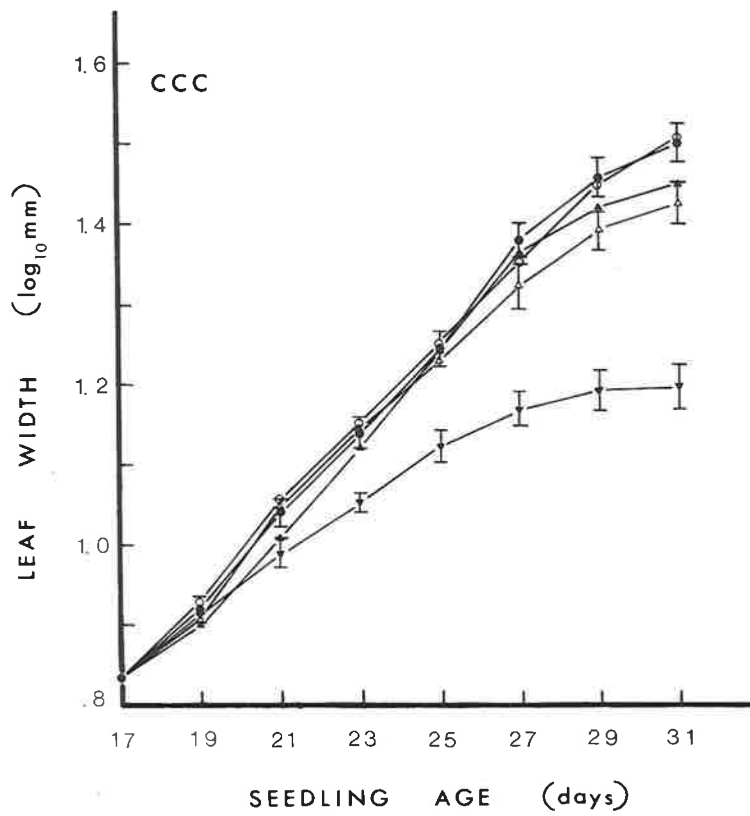
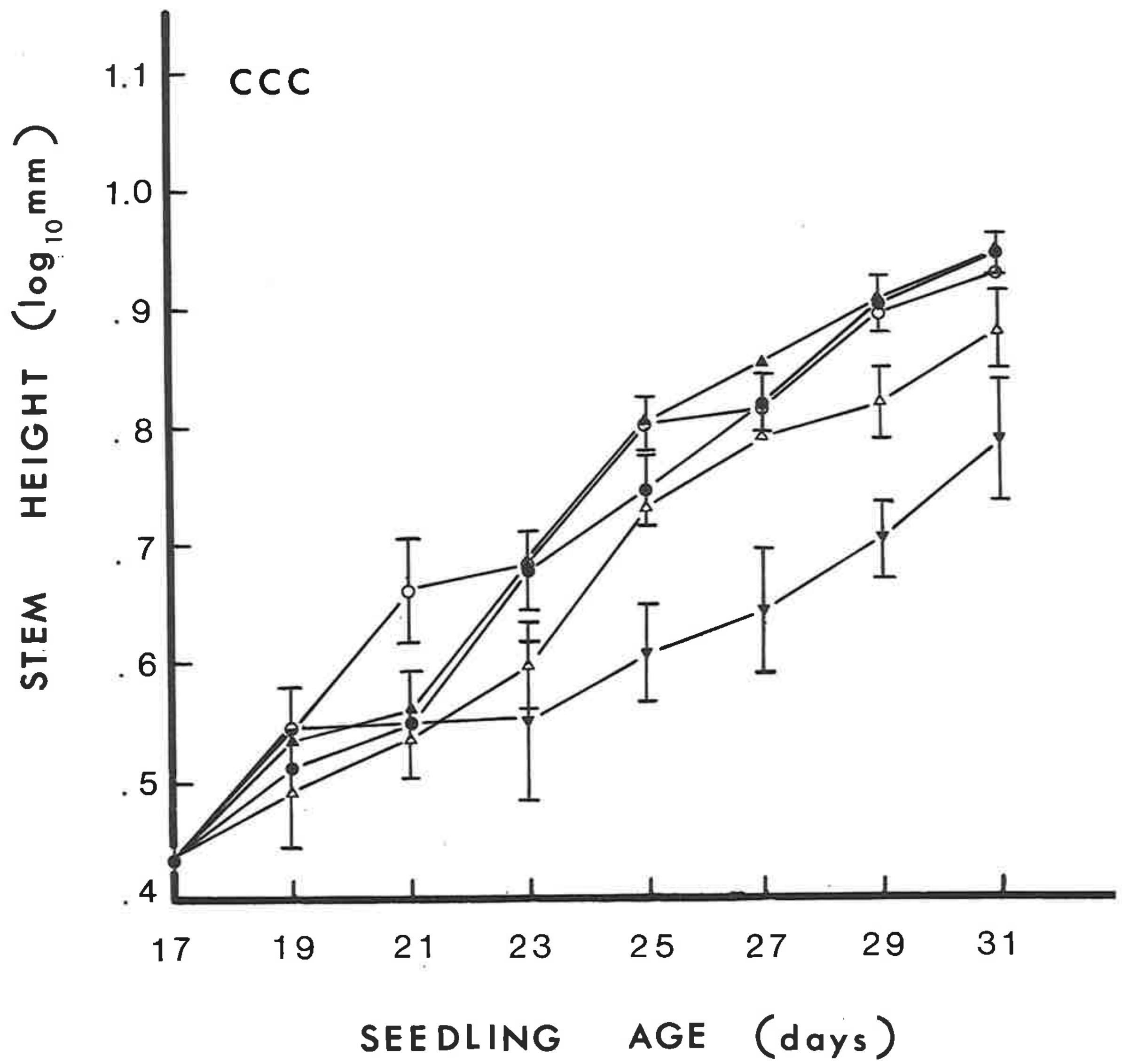

CCC
LEAF WIDTH ($\log_{10}$ mm)
1.6
1.4
1.2
1.0
.8
17
19
21
23
25
27
29
31
SEEDLING AGE (days)

Figure 28. Effect of continuous application of CCC on stem elongation of tobacco seedlings.

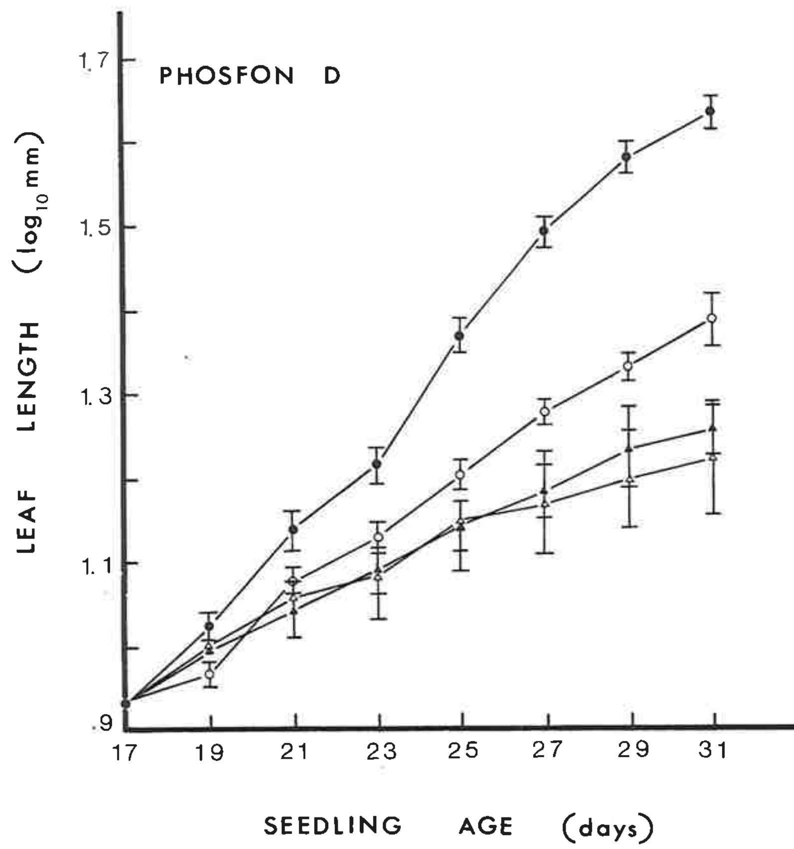
The stem growth of control (●) seedlings is compared with that of seedlings treated with 1 μg (○), 3 μg (▲), 10 μg (△), or 30 μg (▼) of retardant.



CCC
STEM HEIGHT (log10 mm)
1.1
1.0
.9
.8
.7
.6
.5
.4
17
19
21
23
25
27
29
31
SEEDLING AGE (days)

Figure 29. Continuous application of several levels of Phosfon D to 17 day-old tobacco seedlings.

The effect of the retardant on leaf length and leaf width is shown for control (●) seedlings and seedlings treated with 1 μg (○), 3 μg (▲), or 10 μg (△) of Phosfon D.


PHOSFON D
LEAF LENGTH (log_{10} mm)
SEEDLING AGE (days)
1.7
1.5
1.3
1.1
.9
17
19
21
23
25
27
29
31

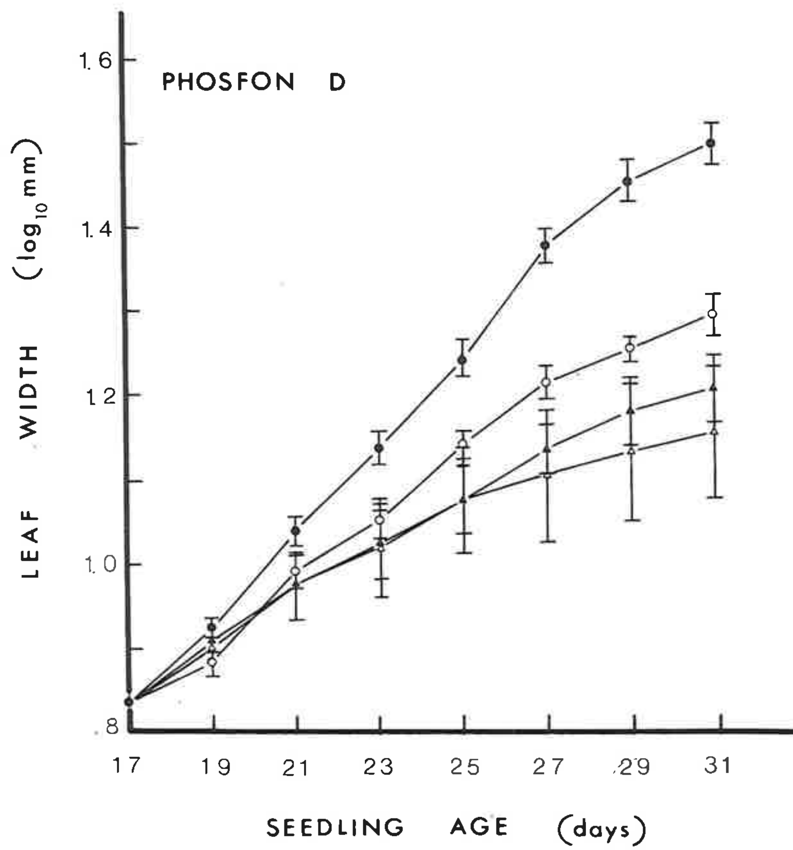
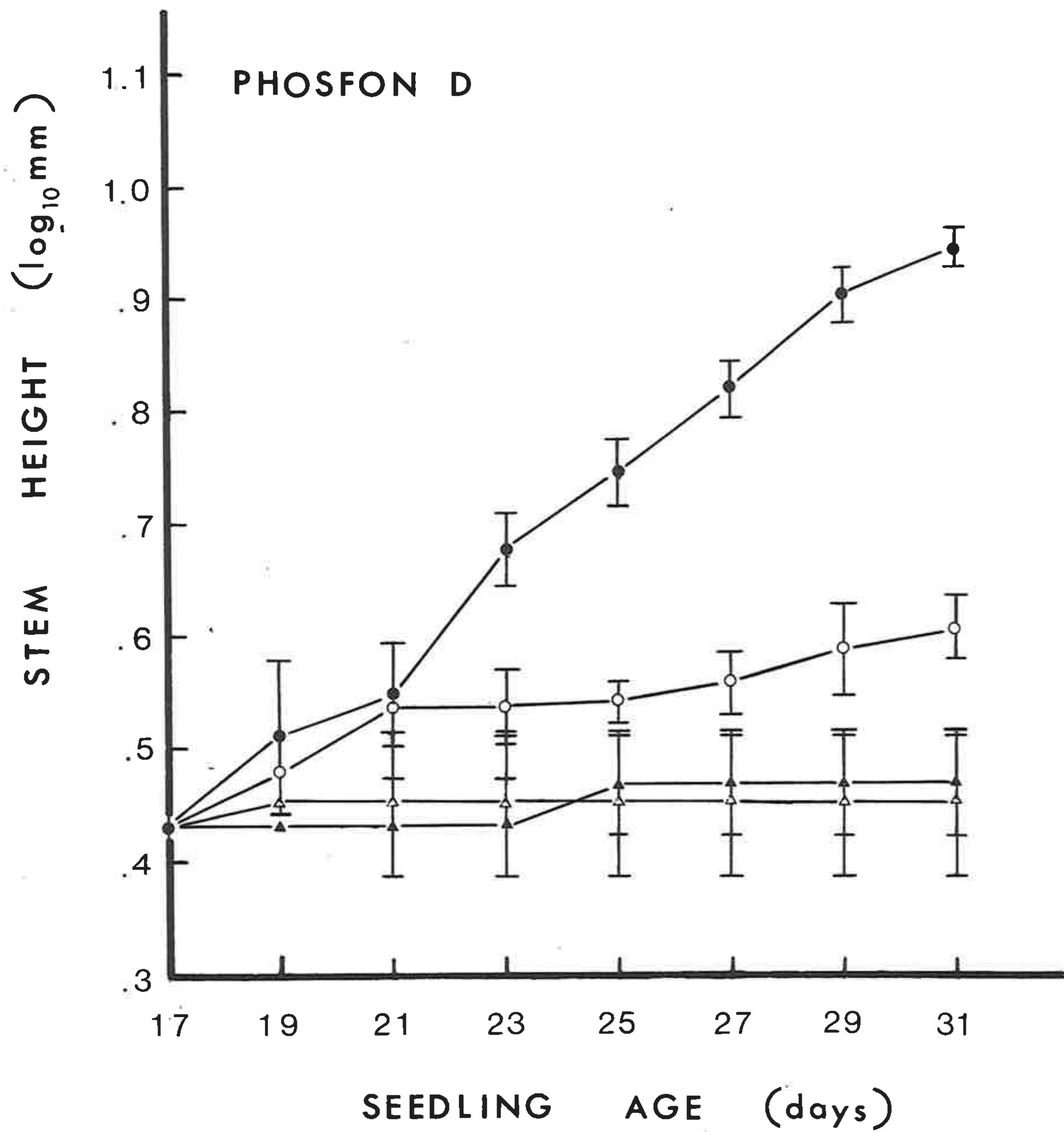

PHOSFON D
LEAF WIDTH (log_{10} mm)
SEEDLING AGE (days)
1.6
1.4
1.2
1.0
.8
17
19
21
23
25
27
29
31

Figure 30. Effect of continuous application of Phosfon D on stem elongation of tobacco seedlings.

The stem growth of control (●) seedlings is compared with that of seedlings treated with 1 μg (○), 3 μg (▲), or 10 μg (△) of retardant.


PHOSFON D
STEM HEIGHT (log_{10} mm)
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
17
19
21
23
25
27
29
31
SEEDLING AGE (days)

The higher levels of the retardants often lead to the production of visible morphological changes in the seedlings. Some of the observed changes included thicker, darker green leaves (in particular with Amo 1618 treatments) and chlorosis or bleaching of leaves of CCC-treated plants. All of these observed changes have been well documented for many different plant species.

Marked similarities between the effects of the retardants on sterol biosynthesis and growth are evident from this data. Each of the retardants inhibit sterol biosynthesis and each has been shown to retard growth of tobacco seedlings. The effects of the retardants on both sterol biosynthesis and growth are concentration-dependant and higher levels of the retardants produce greater inhibitions of both processes. Stems exhibited a greater sensitivity to retardation of growth and inhibition of sterol biosynthesis by the retardants than leaves. The order of effectiveness of the three retardants (viz. Phosfon D > Amo 1618 > CCC) was apparent for both sterol biosynthesis inhibition and retardation of seedling growth. Taken as a whole these data demonstrate a correlation between the effects of the retardants on sterol biosynthesis in, and growth of tobacco seedlings.

b. Effect of a single application of the retardants on tobacco stem growth and sterol biosynthesis.

Thus far, a correlation between the effects of the retardants on sterol biosynthesis in 21 day-old rootless tobacco seedlings and growth of intact tobacco seedlings has been demonstrated. To

better correlate these two effects of the retardants stem elongation and mevalonate incorporation in 4-desmethylsterols were examined for the same plants 24 hr after treatment with a single dose of 300 μg Amo 1618. A set of 10 seedlings (21 days old) was measured for stem height and then treated with 300 μg of Amo 1618 and 0.5 μCi of DL-2-^{14}C-mevalonic acid each. A control set of 10 seedlings was treated only with mevalonate. 24 hr after the addition of the mevalonate precursor stem heights were again measured and then the excised stems of plants from each treatment were weighed and the incorporation of precursor into 4-desmethylsterols was determined (Methods 7.a.). Raw data obtained for stem heights was examined statistically and incorporation into 4-desmethylsterols was expressed on both an absolute incorporation basis and a per gram fresh weight basis (Table 15).

From the data presented it may be seen that a close correlation between the effects of the retardant on stem elongation and sterol biosynthesis was evident. The retardant was able to reduce stem elongation by _ca._ 80% and inhibit mevalonate incorporation into 4-desmethylsterols by _ca._ 85% during the 24 hr test period.

c. Effect of very low levels of Amo 1618 on stem growth and sterol biosynthesis in tobacco seedlings.

Earlier in this chapter (Results A.2.a.) it was shown that very low levels of Amo 1618 produced an apparent stimulation of mevalonate incorporation into sterols in rootless tobacco seedlings. The effects of similar levels of the retardant were therefore tested

Table 15. Simultaneous effect of 300 μg 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		
	%	c.p.m. per g fresh wt	Initial	After 24 hr	Δ
				mm	
Control	1.40	139,735	2.2	4.2	2.0
Amo 1618	0.20	21,059	2.2	2.6*	0.4
Percent inhibition due to Amo 1618	85.7	84.9			80

* Significantly different from control at $p = < 0.01$

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on intact tobacco seedlings to observe whether the correlation between the effects of the retardant on sterol biosynthesis and seedling growth operated at low levels of the retardant as well.

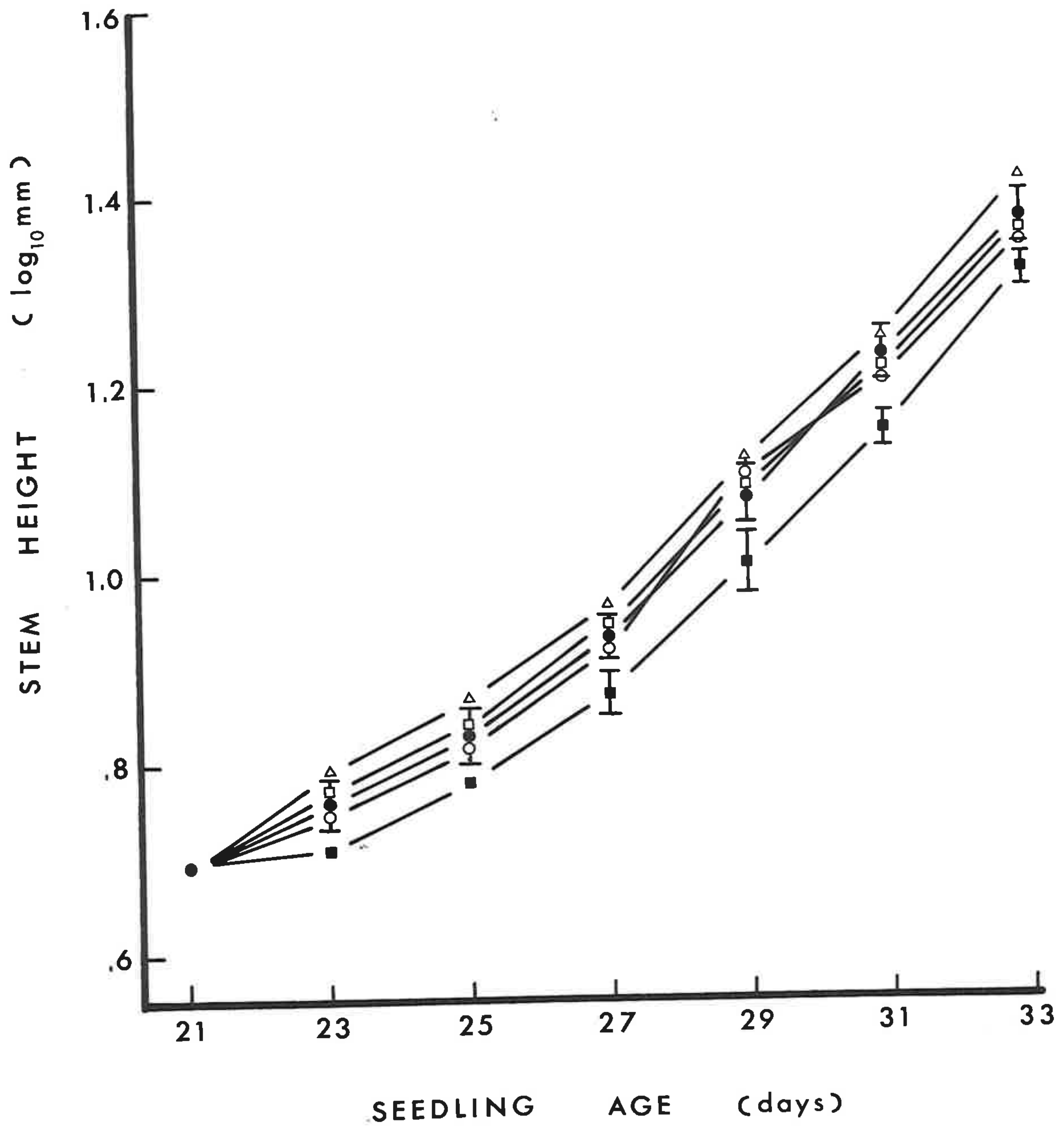
Twenty one-day-old tobacco seedlings were treated with 0, 0.1, 0.5, 1.0 or 3.0 μg retardant/plant in the presence or absence of 2-^{14}C-mevalonic acid. To study the effects of the retardant on tobacco seedling growth, the same levels of Amo 1618 were applied continuously (on alternate days) and stem and leaf growth was measured throughout the test period (Methods 3.a.). For incorporation experiments one set of test plants was kept under conditions identical to those used for growth experiments (Methods 1.) and another set of identically treated plants was placed under the same conditions used for rootless tobacco seedlings [constant temperature (23°) and low light intensity].

Seedling growth appeared to be relatively unaffected by the levels of Amo 1618 used in these experiments, the only level which produced any significant effect was 3 μg which caused a very small retardation of stem elongation after several applications (figure 31). None of the retardant treatments caused a significant effect either stimulatory or inhibitory on leaf growth (data not shown).

Incorporation of mevalonate into sterols and sterol precursors followed a similar pattern to that observed in rootless tobacco seedlings, i.e. an apparent initial stimulation of incorporation up to 1 μg Amo 1618 followed by a slight decline in incorporation at 3 μg

Figure 31. The effect of low levels of Amo 1618 on stem growth of tobacco seedlings.

The stem growth of 21 day-old tobacco seedlings is shown for control (●) plants and plants treated continuously with 0.1 μg (○), 0.5 μg (□), 1 μg (△), or 3 μg (■) of retardant.



STEM HEIGHT (log10 mm)
1.6
1.4
1.2
1.0
.8
.6
21
23
25
27
29
31
33
SEEDLING AGE (days)

Amo 1618. Light intensity had no effect on the stimulation of mevalonate incorporation induced by the low levels of the retardant although some quantitative differences did exist between the two light treatments (Table 16 A and B). Under high light intensity the increased incorporation into 4-desmethylsterols was accompanied by a decreased incorporation into other sterol and hydrocarbon fractions (Table 16A). Incorporation into all sterol and hydrocarbon fractions was stimulated at low light intensity (Table 16B). Apparently light intensity plays some part in either the metabolism of sterol intermediates (e.g. for triterpenoid production) or the channelling of precursor into isoprenoid pathways. Other possibilities will be discussed later in the thesis.

G.L.C. analysis of the 4-desmethylsterols from each treatment showed a general 'across the board' increase in radioactivity associated with each of the four major sterols (except cholesterol and stigmasterol at 3 μg of Amo 1618/plant) at high light intensity (Table 17A). A similar, though not identical pattern was observed for mevalonate incorporation into the 4-desmethylsterols under low light intensity (Table 17B).

Using α-cholestane as internal standard the absolute levels of each sterol, calculated by measuring peak area, remained unchanged 24 hr after retardant treatment (data not presented). It is therefore probable that the apparent increases in sterol biosynthesis observed at low levels of Amo 1618 reflect an increase in specific activity rather than increased sterol production. Because 24 hr is such a

Table 16. The effect of very low levels of Amo 1618 on mevalonate incorporation into sterols and sterol precursors in intact tobacco seedlings.

The incorporation of mevalonate into sterol and hydrocarbon fractions (separated by T.L.C. of the non-saponifiable lipid fraction) is shown for plants maintained under high light intensity (growth conditions) (A), and plants placed under low light intensity conditions (B) for 24 hr.

Retardant concentration	Incorporation of L-2-^{14}C-mevalonate into				
	4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
μg/plant	c.p.m./gm F.W.				
A					
0	233,846	58,426	66,124	3,176	7,360
0.1	453,688	35,794	62,382	1,126	1,858
0.5	443,497	25,884	51,259	1,722	5,710
1.0	402,831	36,478	49,071	2,171	5,670
3.0	348,831	30,365	47,665	3,989	4,207
B					
0	304,059	20,026	37,955	997	1,824
0.1	407,264	21,256	45,664	1,112	2,609
0.5	469,281	25,133	46,636	1,136	2,883
1.0	480,028	43,471	94,231	2,930	5,178
3.0	354,651	18,874	44,751	1,121	3,379

Table 17. The effect of very low levels of Amo 1618 on the incorporation of mevalonate into 4-desmethylsterols in intact tobacco seedlings.

Mevalonate incorporation into 4-desmethylsterols (as determined by G.L.C.) is shown for 21 day-old tobacco seedlings maintained under high light intensity (A), and low light intensity (B) conditions.

Retardant concentration	Incorporation of L-^{14}C-mevalonate into				
	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol	Total
μg/plant			c.p.m.		
A					
0	169	773	371	1,163	2,476
0.1	224	1,151	670	2,086	5,131
0.5	233	892	697	1,644	3,466
1.0	1,231	801	513	3,031	5,576
3.0	128	937	264	1,014	2,343
B					
0	320	1,467	703	2,207	4,697
0.1	371	1,906	1,109	3,455	6,841
0.5	402	1,538	1,201	2,835	5,976
1.0	1,312	1,854	547	3,231	6,944
3.0	204	1,489	420	1,612	3,725

short time in which to detect changes in sterol levels the latter possibility is not over-ruled, however.

No correlation was apparent between the effects of the very low levels of Amo 1618 on the growth of tobacco seedlings and incorporation of mevalonate into the 4-desmethylsterols.

3. The Effects of Animal Steroid Biosynthesis Inhibitors on Growth of Tobacco Seedlings

The animal steroid biosynthesis inhibitors which are structurally similar to the plant growth retardants, exhibit many similar effects to the retardants. Both types of compounds have been shown to act similarly in inhibiting sterol biosynthesis in rat liver preparations and some plant species (see Introduction 4.) and both also possess the ability to retard the growth of certain higher plants. It was therefore argued that if sterol biosynthesis were a requirement for plant growth then inhibitors of sterol biosynthesis should also retard the growth of tobacco seedlings in much the same manner observed for the retardants. As one of the SK and F compounds has already demonstrated the ability to inhibit sterol biosynthesis in *Nicotiana tabacum* (Reid, 1968a) it was assumed that the animal steroid biosynthesis inhibitors would also act on the tobacco seedlings used in this study. Only the effects of these compounds on the growth of tobacco seedlings was therefore examined.

Three steroid inhibitors (viz. SK and F 7997-A_3, SK and F 7732-A_3, and AY-9944) were each tested for their effects on stem and leaf growth of 21 day-old tobacco seedlings. One concentration of Amo 1618 was also tested in the same experiment for comparison with the inhibitors. A single application of either 0, 100 or 300 μg of each inhibitor [in 0.05% (v/v) tween-20 solution] was made to the stem apex of 21 day-old tobacco seedlings and measurements of leaf growth and

stem elongation were recorded each alternate day (Methods 2.).

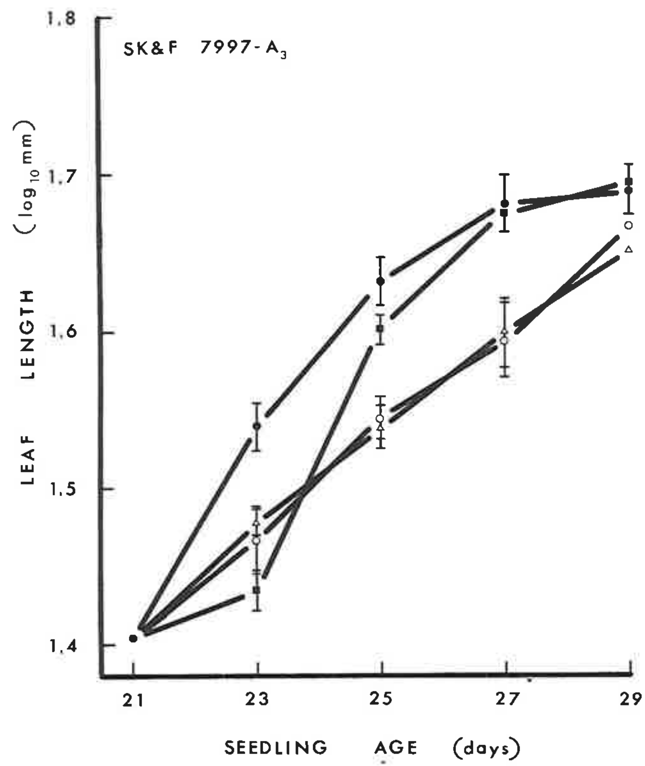
SK and F 7997 caused an initial retardation of leaf growth (in particular leaf length) which was eventually reversed by 8 days after application of the inhibitor. The observed inhibition of leaf growth was not concentration dependant since 100 μg of the inhibitor was as effective in retarding leaf length as 300 μg (figure 32). However, stem elongation was retarded by both levels of the inhibitor and 300 μg proved to be far more effective than the lower level of the inhibitor (figure 33). By comparison with Amo 1618, the inhibitor was equally effective in inhibiting stem elongation and perhaps slightly more effective in retarding leaf growth, i.e. a close similarity between the effects of Amo 1618 and SK and F 7997 on tobacco seedling growth was observed.

The other SK and F compound tested (SK and F 7732-A_3) was not as effective as SK and F 7997 in slowing the rate of leaf growth. Neither level of the inhibitor caused a significant decrease in leaf growth (figure 34). Stem elongation, however, was retarded by both 100 and 300 μg of the inhibitor although SK and F 7732 appeared to be less effective than Amo 1618 in this regard (figure 35).

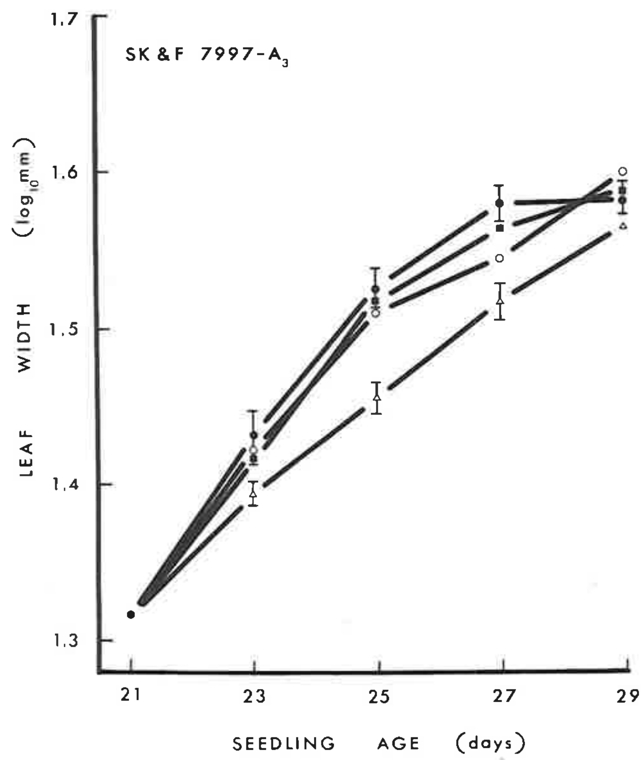
AY-9944 was the least effective growth inhibitor tested although solubility problems were encountered in endeavouring to make up a sufficiently concentrated solution for administering the drug in as small a volume as possible. Further, when 300 μg of the inhibitor was placed onto stem apices, some of the compound crystallized from

Figure 32. Effect of SK and F 7997-A_3 on leaf growth of intact tobacco seedlings.

The effects of single applications of 100 µg (△) or 300 µg (○) of SK and F 7997 on leaf length and leaf width are compared with the effects of 100 µg Amo 1618 (■) and control (●).



SK&F 7997-A3
LEAF LENGTH (log10 mm)
1.8
1.7
1.6
1.5
1.4
21
23
25
27
29
SEEDLING AGE (days)



SK&F 7997-A3
LEAF WIDTH (log10 mm)
1.7
1.6
1.5
1.4
1.3
21
23
25
27
29
SEEDLING AGE (days)

Figure 33. The effects of SK and F 7997-A_3 on stem elongation of intact tobacco seedlings.

Stem elongation of control (●) plants is compared with seedlings treated with either 100 μg (△) or 300 μg (○) of the inhibitor, or 100 μg of Amo 1618 (■).

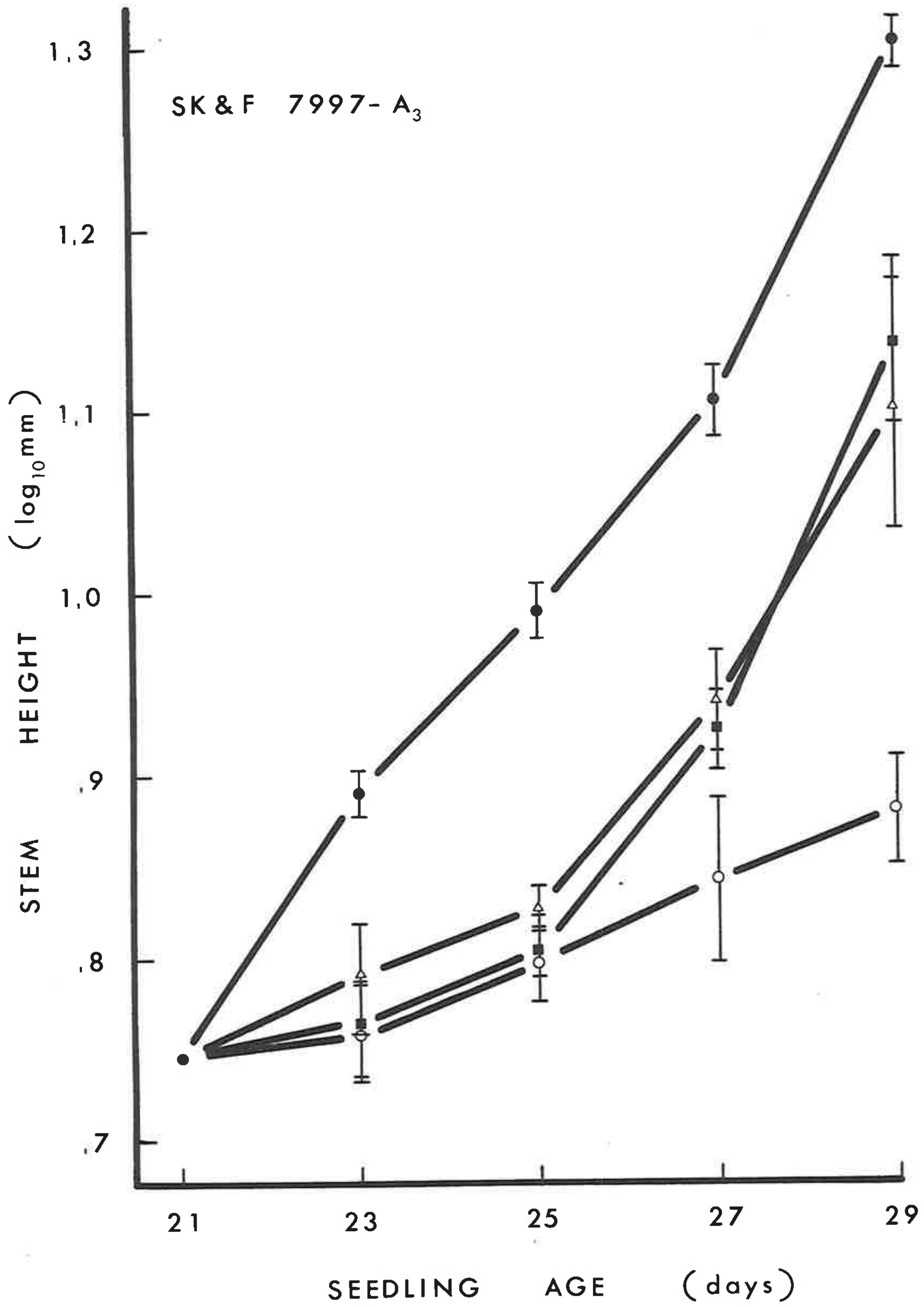
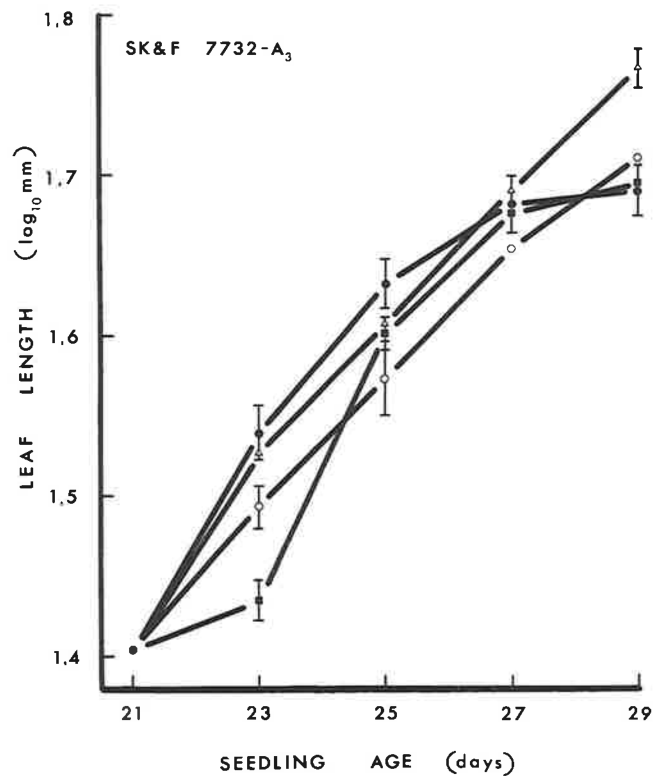

SK&F 7997- A3
1,3
1,2
1,1
1,0
.9
.8
.7
STEM HEIGHT (log10 mm)
21
23
25
27
29
SEEDLING AGE (days)

Figure 34. Effect of SK and F 7732-A_3 on leaf growth of intact tobacco seedlings.

The effects of single applications of 100 μg (△) or 300 μg (○) of SK and F 7732 on leaf length and leaf width are compared with the effects of 100 μg Amo 1618 (■) and control (●).


SK&F 7732-A3
LEAF LENGTH (log10 mm)
1,8
1,7
1,6
1,5
1,4
21
23
25
27
29
SEEDLING AGE (days)

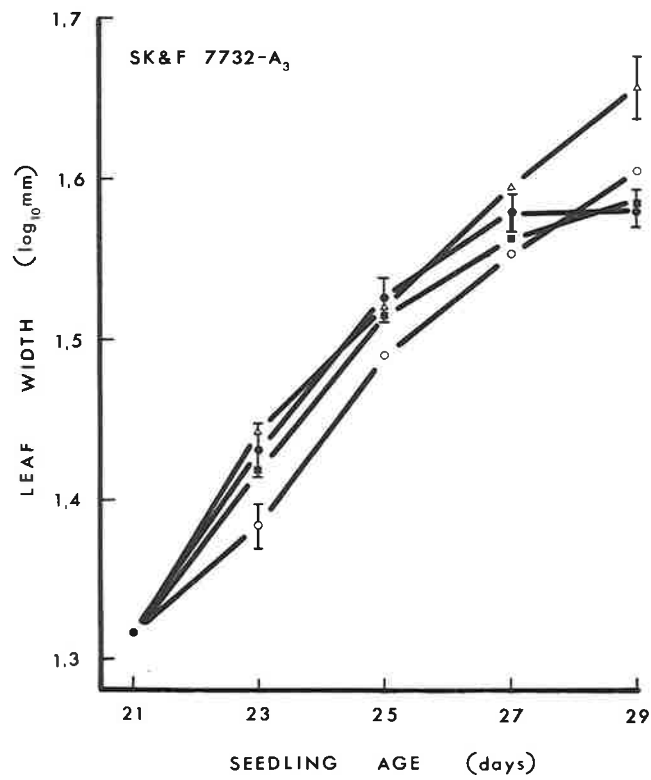
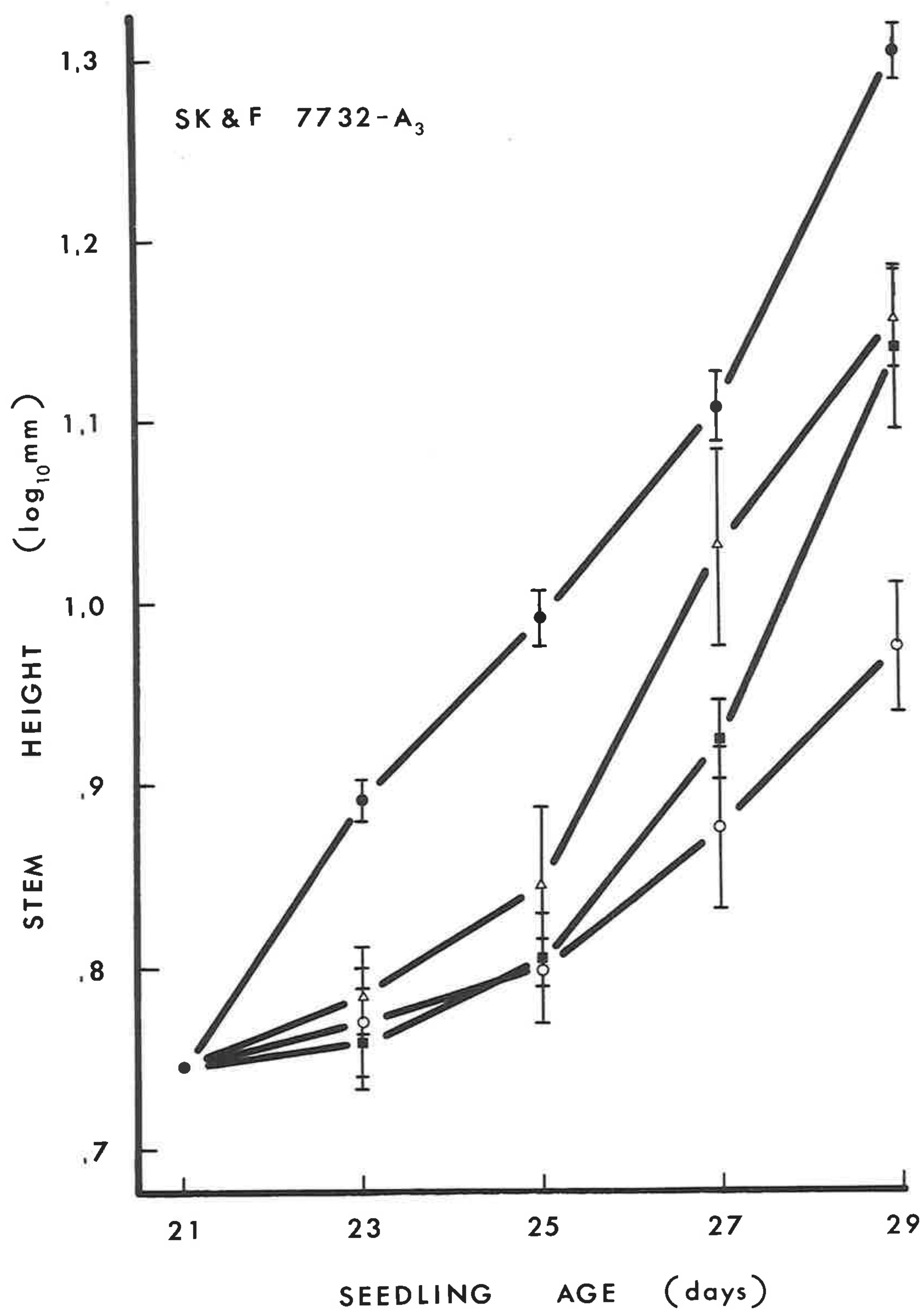

SK&F 7732-A3
LEAF WIDTH (log10mm)
1,7
1,6
1,5
1,4
1,3
21
23
25
27
29
SEEDLING AGE (days)

Figure 35. The effects of SK and F 7732-A_3 on stem elongation of intact tobacco seedlings.

Stem elongation of control (●) plants is compared with seedlings treated with either 100 μg (△) or 300 μg (○) of the inhibitor, or 100 μg of Amo 1618 (■).



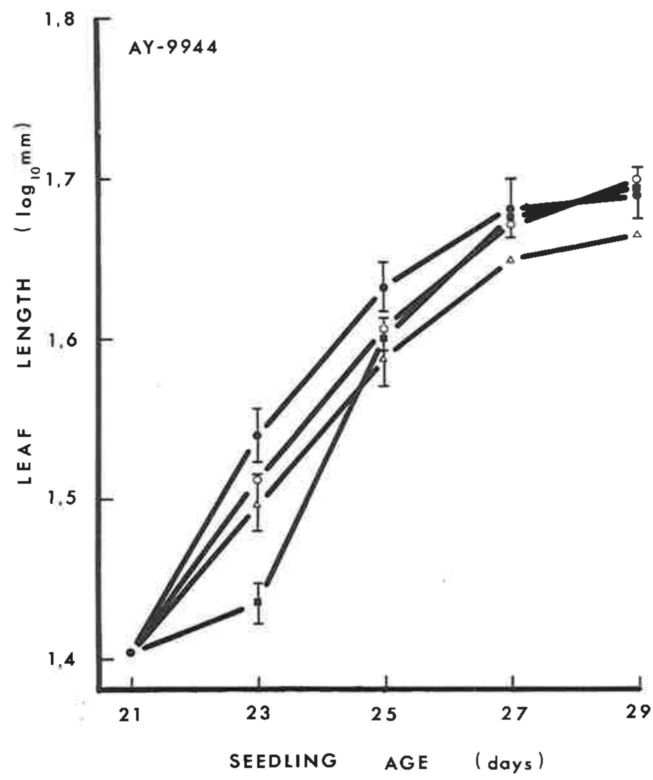
SK & F 7732-A3
1,3
1,2
1,1
1,0
.9
.8
.7
STEM HEIGHT (log10mm)
21
23
25
27
29
SEEDLING AGE (days)

solution and was therefore not taken up by the plants. Neither level of AY-9944 was effective in retarding leaf growth (figure 36) and, although both levels caused a decrease rate of stem growth, the inhibitor was less potent than Amo 1618 in reducing stem growth (figure 37). The failure of seedlings to take up all of the applied inhibitor (above) could explain why 100 μg was equally effective as 300 μg of AY-9944 in retarding stem growth. It should be pointed out that AY-9944 has not been shown to inhibit sterol biosynthesis.

Despite differences between the quantitative effects of each of the inhibitors on growth, each steroid biosynthesis inhibitor demonstrated the ability to retard stem growth without formative effects. With each of the SK and F compounds the retardation of stem growth was concentration dependant, a finding which is consistent with the effects of the retardants already presented (Results B.2.). The animal steroid biosynthesis inhibitors are able, therefore, to cause growth inhibition in intact tobacco seedlings (in particular stem growth) in a similar manner to that observed for at least one retardant and the results, at least with SK and F 7997-A_3, would support the contention that sterol biosynthesis is a requirement for plant growth.

Figure 36. Effect of AY-9944 on leaf growth of intact tobacco seedlings.

The effects of single applications of 100 μg (△) or 300 μg (○) of AY-9944 on leaf length or leaf width are compared with the effects of 100 μg Amo 1618 (■) and control (●).


AY-9944
1,8
1,7
1,6
1,5
1,4
LEAF LENGTH ($\log_{10}$ mm)
21
23
25
27
29
SEEDLING AGE (days)

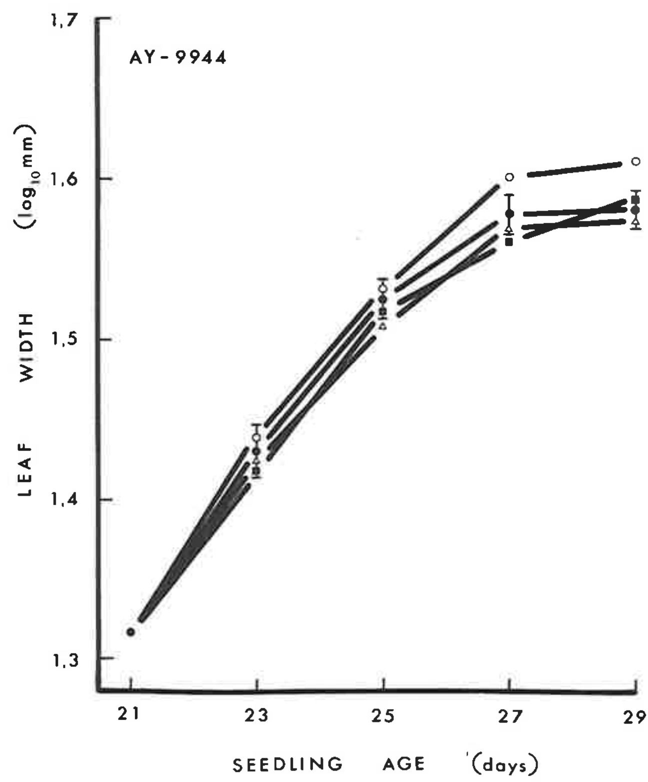
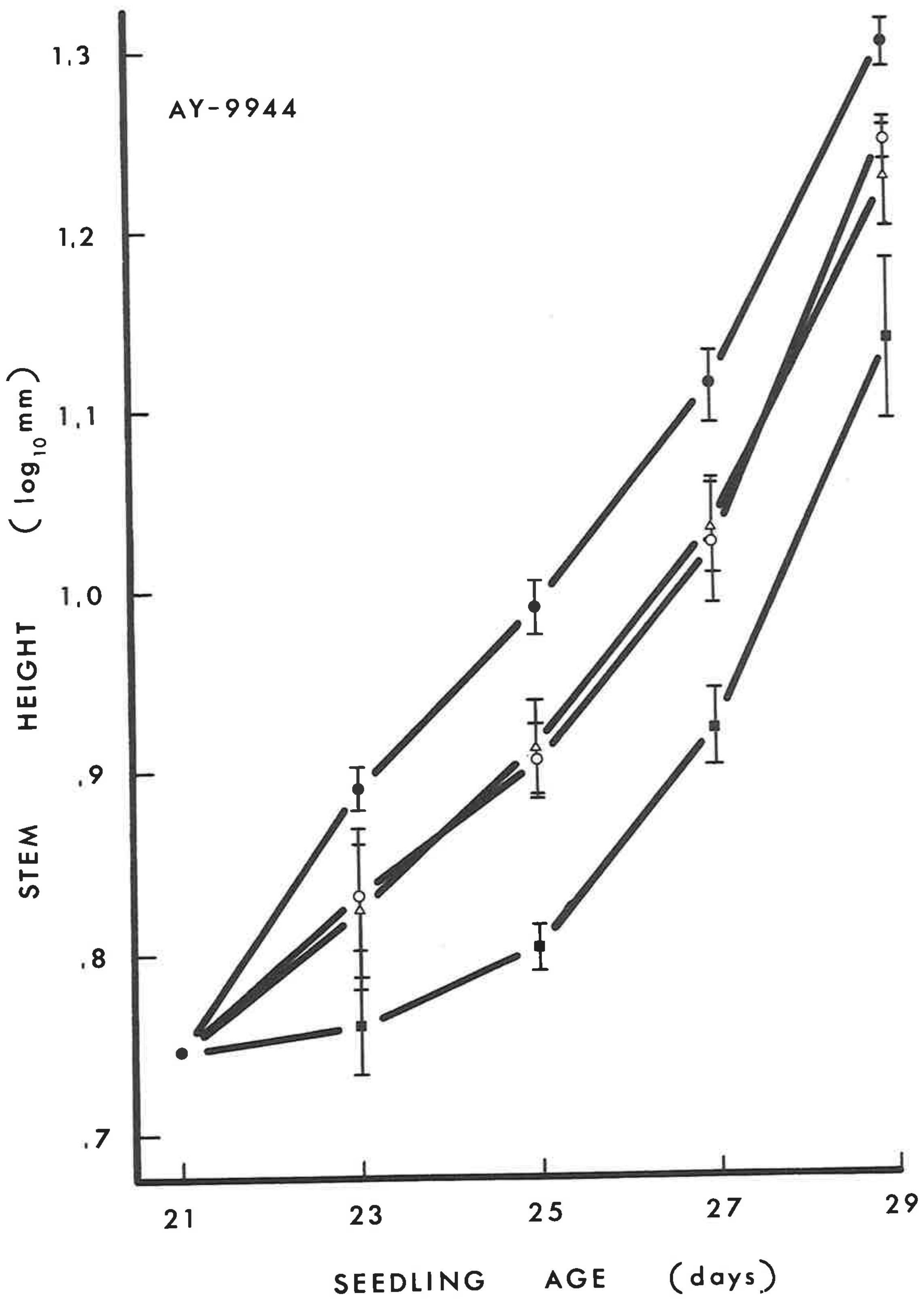

AY-9944
1,7
1,6
1,5
1,4
1,3
LEAF WIDTH ($\log_{10}$ mm)
21
23
25
27
29
SEEDLING AGE (days)

Figure 37. The effects of AY-9944 on stem elongation of intact tobacco seedlings.

Stem elongation of control (●) plants is compared with seedlings treated with either 100 μg (△) or 300 μg (○) of the inhibitor, or 100 μg of Amo 1618 (■).



AY-9944
1.3
1.2
1.1
1.0
.9
.8
.7
STEM HEIGHT ($\log_{10}$ mm)
21
23
25
27
29
SEEDLING AGE (days)

4. The Effect of Sterol Addition to Retardant-Treated Plants

All of the data presented so far implies that sterol biosynthesis is a requirement for tobacco seedling growth. To test this hypothesis in the simplest possible way, sterols were added (as aqueous emulsions; Methods 3.c.) to 21 day-old seedlings which had previously been treated with retardants, to observe whether sterol application was able to reverse, either partially or wholly, the growth retardation induced by retardants.

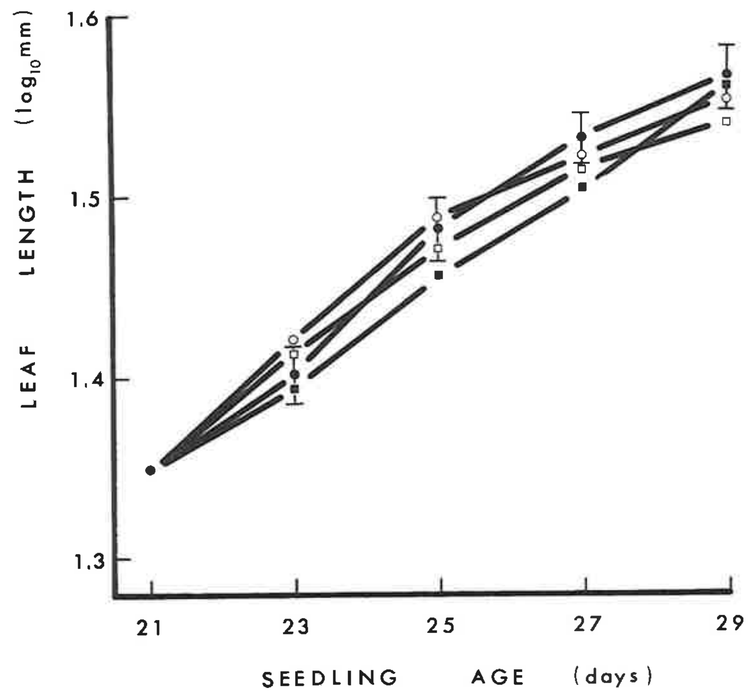
a. β-Sitosterol addition to Amo 1618-treated tobacco seedlings

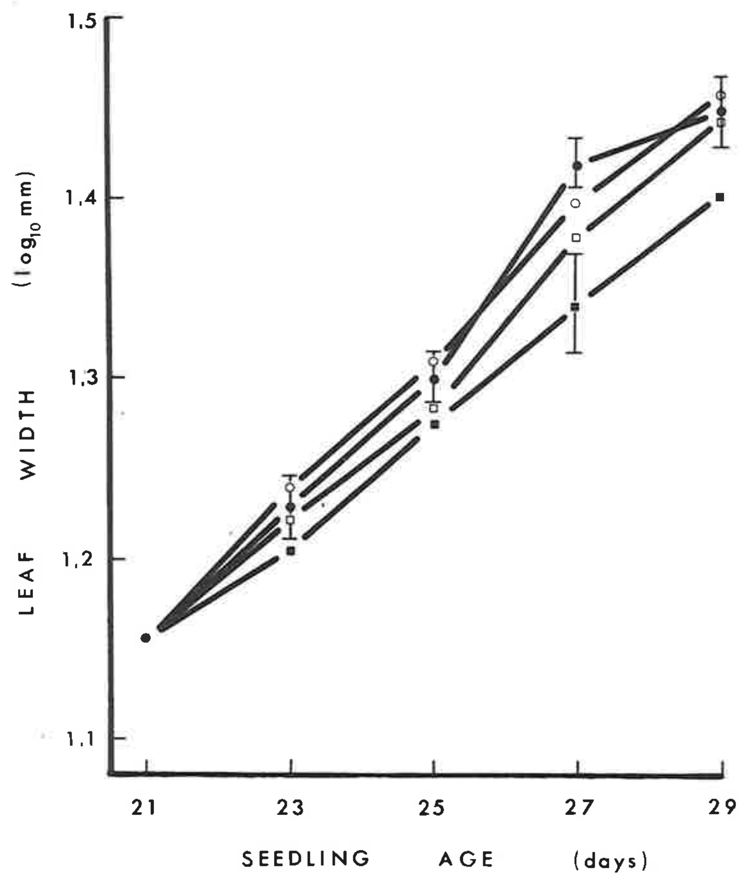
A single application of Amo 1618 (100 μg/plant) was employed in this experiment. Sets of 10 seedlings were prepared and two sets (20 seedlings) were treated with Amo 1618 and another two sets (20 seedlings) were used as controls. After all of the retardant solution (or just tween-20 solution in the case of controls) had been absorbed by the seedlings, one set of Amo 1618-treated and one set of control seedlings were sprayed with an aqueous emulsion of 0.003% (w/v) β-sitosterol in 0.004% (w/v) Pluronic F68. The other control and Amo 1618-treated seedlings were sprayed with an equal volume of only 0.004% (w/v) Pluronic F68 solution (Methods 3.c.). Measurements of leaf and stem growth were both made on alternate days and analysed as previously described (Methods 4.).

Neither the retardant nor the sterol (alone or in combination) had any significant effect on leaf growth (figure 38). Stem height,

Figure 38. Effect of β-sitosterol on leaf growth of control and Amo 1618-treated tobacco seedlings.

The effects of β-sitosterol addition to control (○) plants and plants treated with 100 μg of Amo 1618 (□) were compared with control (●) and Amo 1618-treated (■) plants to which no sterol was applied.


LEAF LENGTH (log10 mm)
1,6
1,5
1,4
1,3
21
23
25
27
29
SEEDLING AGE (days)


LEAF WIDTH (log10 mm)
1,5
1,4
1,3
1,2
1,1
21
23
25
27
29
SEEDLING AGE (days)

however, was considerably reduced as a result of retardant treatment but β-sitosterol had no effect of its own on stem elongation (figure 39). Amo 1618-induced retardation of stem elongation was significantly and almost completely reversed by β-sitosterol application.

b. β-sitosterol addition to tobacco seedlings treated with Amo 1618, CCC, or Phosfon D

The levels of each retardant used in this experiment were 100 μg Amo 1618, 250 μg CCC, and 30 μg Phosfon D. All methodology was exactly as in the preceding experiment.

Once again, the retardants exerted no significant effect on leaf growth (data not presented) so only the effects on stem elongation will be discussed here. Sterol application was effective in completely reversing the effects of both Amo 1618 and CCC on stem elongation but was ineffective on Phosfon D-treated plants (figure 40).

c. Addition of other sterols to Amo 1618-treated tobacco seedlings

Applications of stigmasterol, cholesterol and β-sitosterol emulsions to tobacco seedlings treated with 100 μg of Amo 1618 (exactly as previously described; Results B.4.a.) showed that each of the sterols was equally effective in completely reversing the reduced rate of stem growth induced by the retardant (figure 41). As was previously shown for β-sitosterol (Results B.4.a.), none of the sterols exerted any effects of their own (either stimulatory or

Figure 39. The effects of β-sitosterol on stem elongation of control and Amo 1618-treated tobacco seedlings.

Stem growth of control seedlings in the presence (○) or absence (●) of β-sitosterol and Amo 1618-treated plants in the presence (□) or absence (■) of β-sitosterol is shown.

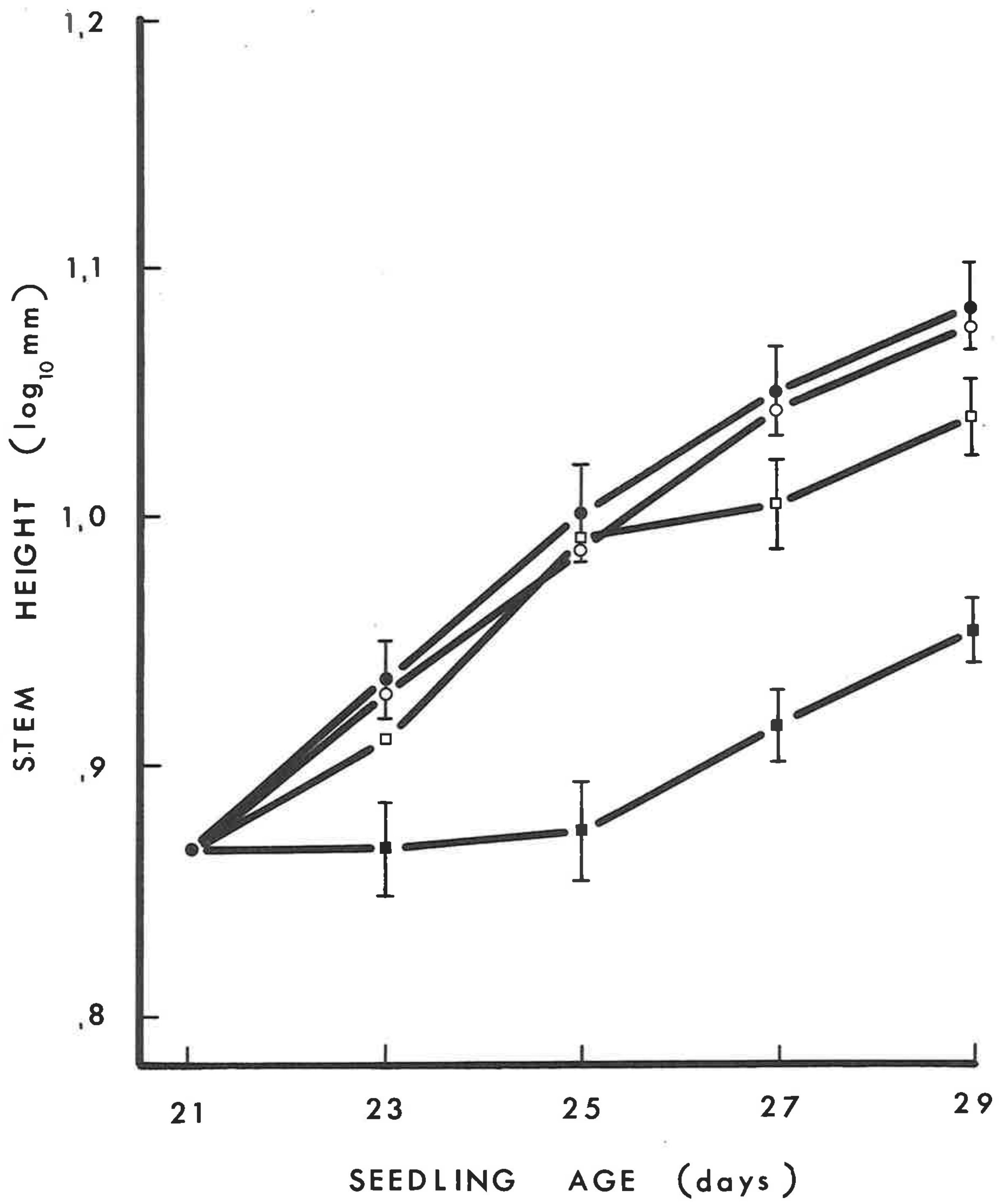
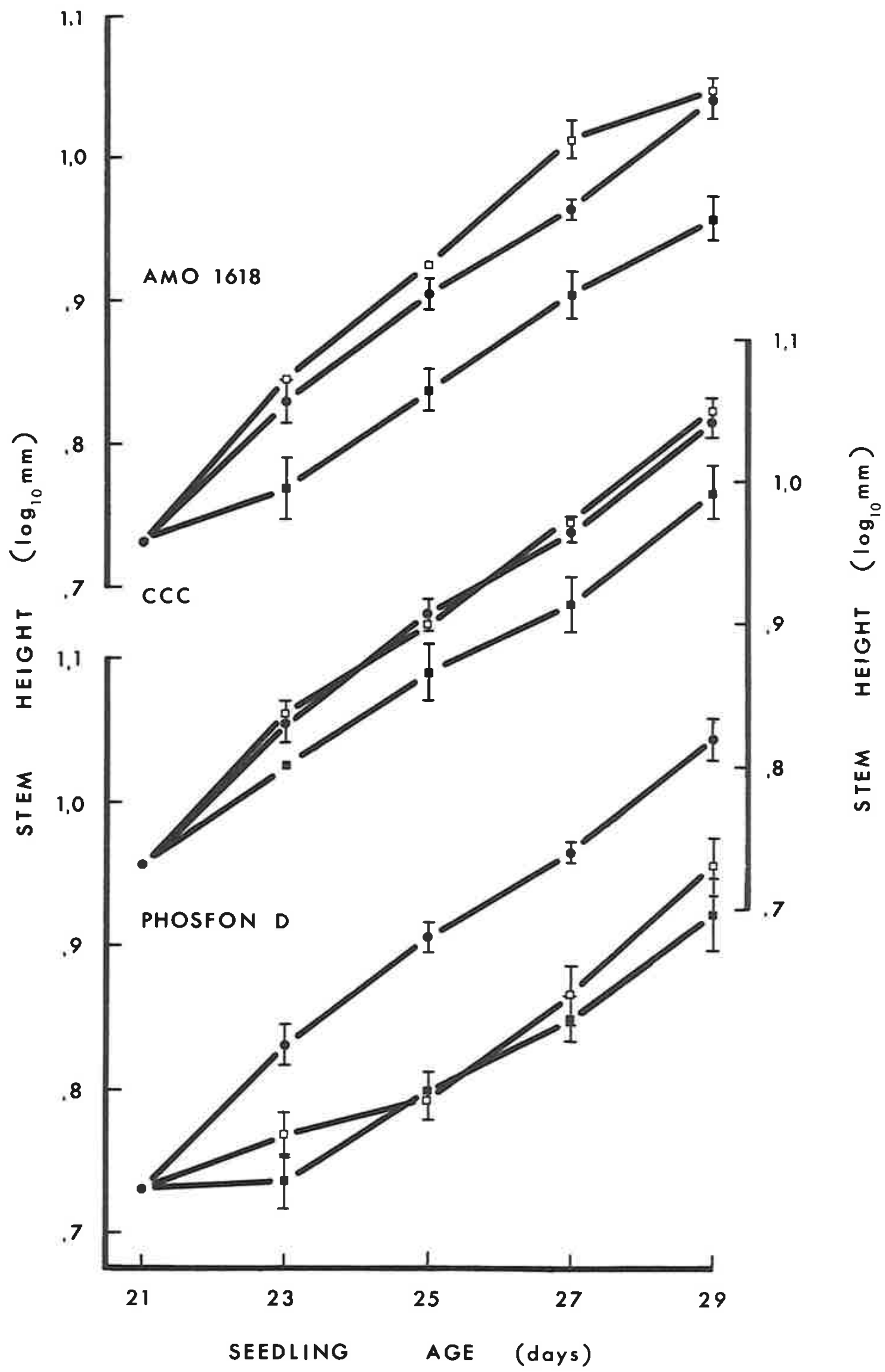

1,2
1,1
1,0
,9
,8
STEM HEIGHT (log10 mm)
21
23
25
27
29
SEEDLING AGE (days)

Figure 40. The effect of β-sitosterol addition to Amo 1618, CCC, and Phosfon D-treated intact tobacco seedlings.

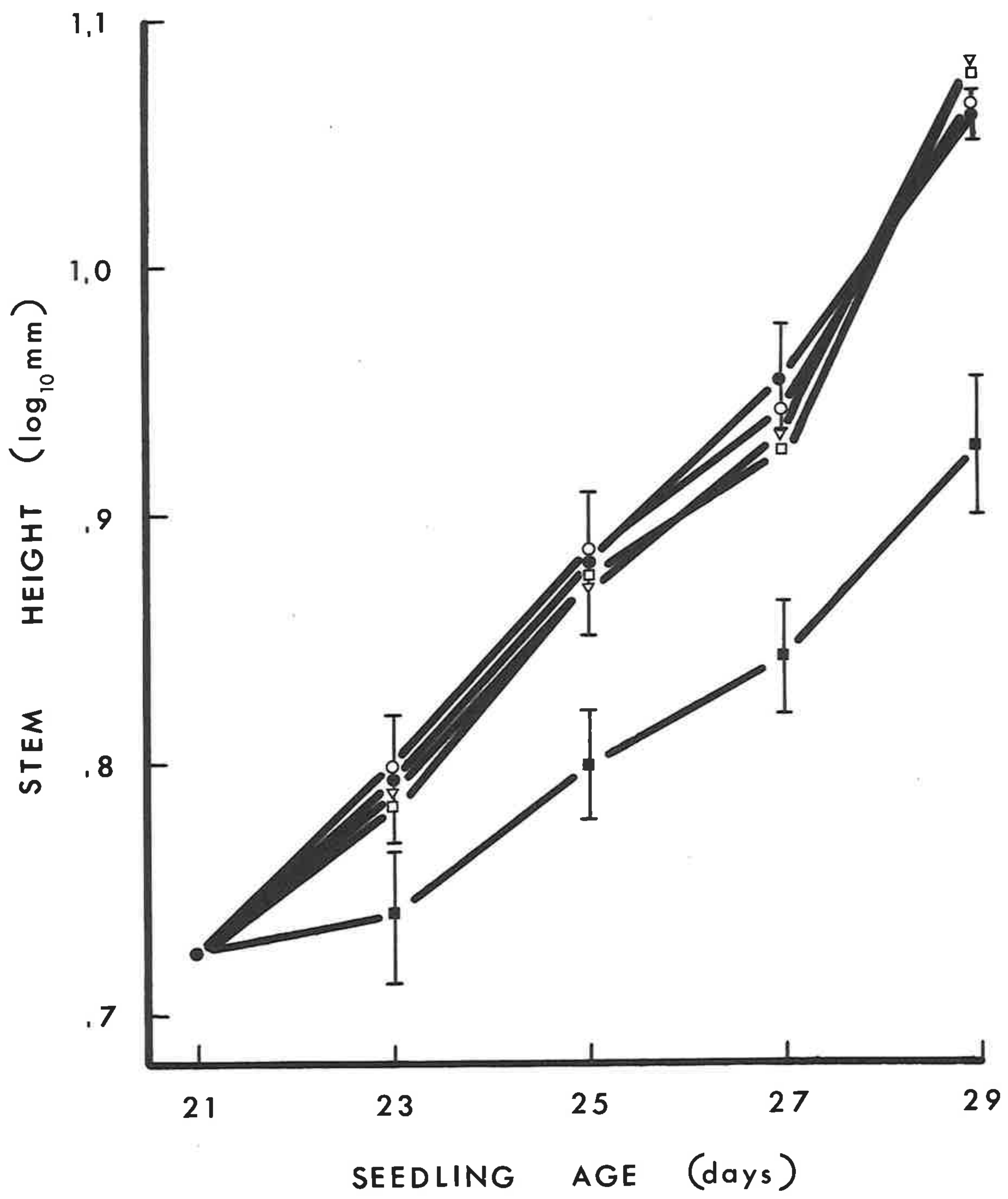
Stem elongation of control (●) seedlings is compared with seedlings treated with retardant in the presence (□) or absence (■) of β-sitosterol.



AMO 1618
CCC
PHOSFON D
STEM HEIGHT ($\log_{10}$ mm)
1,1
1,0
,9
,8
,7
21
23
25
27
29
SEEDLING AGE (days)

Figure 41. The effects of several sterols on reduced stem growth of intact tobacco seedlings treated with Amo 1618.

Stem growth of control (●) seedlings is compared with seedlings treated with 100 μg Amo 1618 either alone (■) or in combination with β-sitosterol (□), cholesterol (○), or stigmasterol (▽).


1,1
1,0
.9
.8
.7
STEM HEIGHT (log_{10}mm)
21
23
25
27
29
SEEDLING AGE (days)

inhibitory) on stem growth (data not presented).

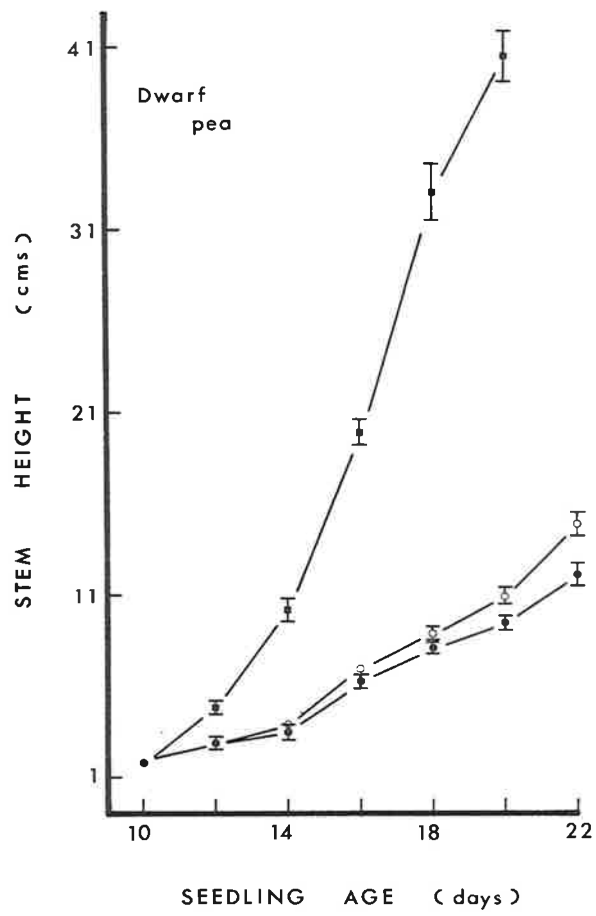
All of the above data suggests that sterol biosynthesis is a requirement for plant growth (in particular stem growth) and that the effects of retardants on sterol biosynthesis and plant growth might even be causally related.

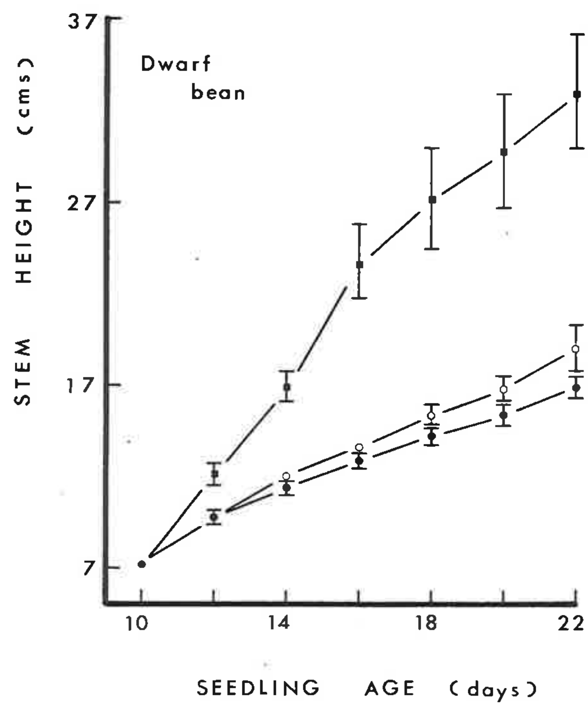
d. Effect of sterol application on stem elongation in dwarf varieties of pea and bean

As a corollary to the effects of sterol application on retardant induced inhibition of stem growth, the effects of exogeneous sterol on stem elongation in dwarf pea and dwarf bean was tested. Using gibberellin-treated (30 μg/plant) dwarf plants as "tall" controls and untreated plants as "dwarf" controls it was observed that a mixture of plant sterols (viz. cholesterol, campesterol, stigmasterol, and β-sitosterol) was able to cause a slight but significant increase in stem height but unable to induce "tall" growth in either pea or bean plants (figure 42). The data suggests that dwarfism in the varieties of plant tested is not a result of reduced rates of sterol production.

Figure 42. Effect of sterol application on stem elongation of dwarf pea and dwarf bean seedlings.

Stem growth of control (●) seedlings is compared with that of seedlings sprayed with an aqueous emulsion of sterols (O) or treated with a single application of 30 μg GA_3 (■).


Dwarf pea
STEM HEIGHT (cms)
41
31
21
11
1
10
14
18
22
SEEDLING AGE (days)


Dwarf bean
STEM HEIGHT (cms)
37
27
17
7
10
14
18
22
SEEDLING AGE (days)

5. Effect of Amo 1618 on Sterol Biosynthesis in Sub-cellular Fractions of Tissues from Intact Tobacco Seedlings

Thus far the plant growth retardants have been demonstrated to act as inhibitors of sterol biosynthesis in tobacco seedlings and a strong correlation has been shown to exist between the effects of the retardants on sterol biosynthesis and growth. The sterols are structural and/or functional components of membranes and therefore it may be reasoned that by inhibiting sterol biosynthesis the retardants might deplete membranes of essential sterols and thus impare membrane function leading ultimately to reduced cellular activity and hence growth. To investigate this possibility more closely, sterols and sterol intermediates were examined from sub-cellular fractions of Amo 1618-treated and control intact tobacco seedlings which had been supplied with DL-2-^{14}C-mevalonic acid.

Ten 21 day-old seedlings were treated with 300 μg each of Amo 1618 (Methods 3.a.) and 1 μCi of DL-2-^{14}C-mevalonic acid in 0.05% (w/v) tween-20 solution. Control plants were treated with only tween-20 solution. Similar sets of plants were treated as above with the omission of DL-2-^{14}C-mevalonate and were used for growth studies (Methods 2.). 24 hr after application of the ^{14}C-precursor, seedlings were removed from the growth cabinet, the roots washed exhaustively with water to remove all soil etc. and, after drying by pressing light-ly between filter papers, they were divided into leaf, stem and root tissues. Each of the tissues thus obtained were weighed and then

immediately transferred into twice their own weight of ice-cold solution A and homogenized (Methods 6.b.). The homogenate was then subjected to three centrifugations (after first filtering through gauze) to obtain three pellet fractions (P_1, P_{20} and P_{200}) and a supernatant fraction (S_{200}) (Methods 6.b.). The 1,000 x g pellet (P_1) was discarded as it would have contained some unbroken cells and cell debris and the purpose of this study was to examine only intra-cellular fractions. The 20,000 x g pellet (P_{20}), 200,000 x g pellet (P_{200}) and 200,000 x g supernatant (S_{200}) were examined for incorporation products by extracting sterols and sterol intermediates as outlined [Methods 7. a.(ii)] and separating the different compounds thus obtained by subjecting the total lipid extract to T.L.C. (Methods 8.a.). By this method sterol esters were obtained as well as free sterols and the esters were treated separately to the free sterols. On T.L.C. (above) the sterol esters co-chromatographed with squalene-2,3-epoxide and were separated from the hydrocarbon intermediate by saponifying the silica gel eluent in methanolic KOH [Methods 7.a.(i)] followed by T.L.C. of the non-saponifiable lipids.

The incorporation of mevalonate into free sterols and sterol intermediates is inhibited by Amo 1618 in much the same manner as previously described (Results B.1.) and, once again the retardant appeared to act at the cyclase step in the biosynthetic pathway (Table 18). A summation of total incorporation of mevalonate into each sterol fraction for all of the intracellular fractions of all of the tissues examined revealed that the retardant reduced incorporation into

Table 18. The effect of 300 μg of Amo 1618 per plant on DL-2-^{14}C-mevalonate incorporation into free sterols and sterol precursors of sub-cellular organelles of intact tobacco seedlings.

(Sterols and sterol intermediates separated by T.L.C. of the un-saponified lipid extract from the sub-cellular fractions; squalene-2,3-epoxide values obtained after T.L.C. of the non-saponifiable lipid extract). P_{20} = 20,000 x g pellet; P_{200} = 200,000 x g pellet; S_{200} = 200,000 x g supernatant.

Table 18 A = P_{20} fraction

Table 18 B = P_{200} fraction

Table 18 C = S_{200} fraction

Table 18A. P_{20} fraction

Tissue	Treatment	Incorporation of L-2-^{14}C-mevalonate				
		4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
				c.p.m.		
Leaf	Control	689,579	53,160	41,625	16,011	80,651
	Amo 1618-treated	105,223	18,792	12,717	19,734	19,788
Stem	Control	182,556	6,798	4,610	1,606	7,356
	Amo 1618-treated	19,674	4,759	3,023	3,245	9,994
Root	Control	516	201	178	96	292
	Amo 1618-treated	249	103	62	137	83
Total	Control	872,651	60,159	46,413	17,713	88,299
	Amo 1618-treated	125,146	23,654	15,802	23,116	29,865
% inhibition or stimulation		85.7	60.7	65.9	130.5	66.2

Table 18B. P_{200} fraction

Tissue	Treatment	Incorporation of L-2-^{14}C-mevalonate: 4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
				c.p.m.		
Leaf	Control	340,206	27,482	30,512	11,102	40,739
	Amo 1618-treated	115,441	23,558	29,280	14,567	24,751
Stem	Control	48,007	1,447	1,372	458	2,011
	Amo 1618-treated	13,809	1,505	1,333	4,779	3,074
Root	Control	453	161	221	117	241
	Amo 1618-treated	101	2	44	81	57
Total	Control	388,666	29,090	32,105	11,677	42,991
	Amo 1618-treated	129,351	25,065	30,657	19,427	27,882
% inhibition or stimulation		66.7	13.8	4.5	166.4	35.1

Table 18C. S_{200} fraction

Tissue	Treatment	Incorporation of L-2-^{14}C-mevalonate				
		4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
				c.p.m.		
Leaf	Control	248	1,832	577	560	2,283
	Amo 1618-treated	223	862	729	4,448	2,704
Stem	Control	45	169	82	47	59
	Amo 1618-treated	110	169	150	91	199
Root	Control	28	13	1	39	34
	Amo 1618-treated	17	65	26	72	45
Total	Control	321	2,014	660	645	2,376
	Amo 1618-treated	350	1,096	905	4,611	2,948
% inhibition or stimulation		109.0	45.6	137.1	705.0	124.1

4-desmethylsterols by 79.8%, 4-methylsterols by 45.9%, 4,4'-dimethylsterols by 40.2%, squalene by 54.6% and increased incorporation into squalene-2,3-epoxide by ca. 1.6-fold (Table 18).

The P_{20} fraction accounted for 69.2% of total radioactivity associated with 4-desmethylsterols (Table 18A), the P_{200} fraction for 30.8% (Table 18B), and the S_{200} fraction only 0.03% (Table 18C). Although the P_{20} fraction contained in excess of double the amount of radioactive incorporation products that the P_{200} fraction contained, it would also have accounted for a much greater weight of material and therefore, if calculated on a per gram of tissue basis, the P_{20} fraction would probably account for less incorporation than the P_{200} fraction. Unfortunately no weight measurements were taken for these intracellular fractions and the above reasoning is based only on the observation that the P_{20} fraction contained considerably more material than the P_{200} fraction. The S_{200} fraction accounted for very little radioactivity and most of that which was present in this fraction was associated principally with the hydrocarbon (squalene and squalene-2,3-epoxide) fractions.

Amo 1618 inhibited the incorporation of mevalonate into sterols far more effectively in the P_{20} fraction than the P_{200} fraction and had little to no effect on the incorporation into sterols associated with the S_{200} fraction (Table 18). A greater accumulation of squalene-2,3-epoxide was observed in the S_{200} and P_{200} fractions of retardant-treated plants than in the P_{20} fraction. This observation could

reflect a greater participation of the 200,000 x g pellet and supernatant fractions in sterol biosynthesis, particularly the mevalonate to squalene-2,3-epoxide steps of the sequence.

The greatest inhibitions of mevalonate incorporation into 4-desmethylsterols and the greatest accumulations of squalene-2,3-epoxide into each of the pellet fractions were observed in stem tissues. In both pellet fractions from stem tissue the incorporation into squalene did not decrease as a result of retardant treatment as was observed in both leaf and root tissues (Table 18). Once again, this could imply that sterol biosynthesis proceeds at a greater rate in stems than in leaves.

When the data from this experiment was calculated as incorporation into total leaf, stem, and root tissue (Table 19) similar results were obtained to those presented earlier (Table 13). On an absolute incorporation basis leaf tissue accounted for the vast majority of radioactivity associated with 4-desmethylsterols (81.6%), stem tissue accounted for most of the remainder (18.3%) and roots only accounted for 0.08% (Table 19A). On a per gram fresh weight basis, stems accounted for the major portion of radioactivity associated with 4-desmethylsterols (75.6%) followed by leaves (24.1%) and roots (0.24%). Biosynthesis of 4-desmethylsterols was inhibited more strongly in stems on both an absolute (85.4%) and per gram fresh weight (84.9%) basis than in leaves (78.6% and 80.7%, respectively) or roots (63.2% and 66.7%, respectively). Further, accumulation of squalene-2,3-epoxide

Table 19. The effect of 300 μg of Amo 1618 per plant on the incorporation of DL-2-^{14}C-mevalonate into free sterols and sterol precursors from tissues of intact tobacco seedlings.

Values presented in Table 18 are presented as total mevalonate incorporation in each tissue as expressed on an absolute (A) and a per gram fresh weight (B) basis.

Tissue	Treatment	Incorporation of L-2-^{14}C-mevalonate				
		4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
A				c.p.m.		
Leaves	Control	1,030,033	82,474	72,714	27,673	123,673
	Amo 1618-treated	220,887	43,212	42,726	38,749	47,243
% inhibition or stimulation		78.6	47.6	41.2	140.0	61.8
Stems	Control	230,608	8,414	6,064	2,111	9,426
	Amo 1618-treated	33,593	6,433	4,506	8,115	13,267
% inhibition or stimulation		85.4	23.5	25.7	384.4	140.7
Roots	Control	997	375	400	252	567
	Amo 1618-treated	367	170	132	290	185
% inhibition or stimulation		63.2	54.7	67.0	115.1	67.4
B				c.p.m./gm F.W.		
Leaves	Control	334,426	26,777	23,608	8,984	40,153
	Amo 1618-treated	64,738	12,664	12,522	11,356	13,846
% inhibition or stimulation		80.7	52.7	46.9	126.4	65.5
Stems	Control	1,048,218	38,245	27,563	9,595	42,845
	Amo 1618-treated	158,457	30,344	21,254	28,278	62,580
% inhibition or stimulation		84.9	20.6	22.7	398.9	146.1
Roots	Control	3,379	1,271	1,355	854	1,922
	Amo 1618-treated	1,125	521	404	889	567
% inhibition or stimulation		66.7	58.9	70.1	104.2	70.5

was considerably higher in stems (*ca.* 4-fold) than in leaves (*ca.* 1.5-fold) or roots (no change).

The inhibition of incorporation into 4-methylsterols and 4,4'-dimethylsterols was greatest in roots and least in stems (Table 20) implying that the effects of the retardant on sterol biosynthesis differ from tissue to tissue within the plant. In this instance, for example, it seems that the retardant may inhibit sterol biosynthesis in stems at the demethylation steps in the pathway as well as the cyclization step thus leading to a greater inhibition of 4-desmethyl-sterol biosynthesis than in leaves or roots and a backing-up of 4-methyl- and 4,4'-dimethyl- sterol intermediates. In leaves and roots, on the other hand, an inhibition by the retardant of a pre-squalene step seems likely.

Once again 300 μg of Amo 1618 caused a marked reduction in stem elongation (data not presented) pointing to a close correlation between the effects of the retardant on sterol biosynthesis in, and stem growth of tobacco seedlings.

An examination of sterol esters from the intra-cellular tissue fractions revealed that the vast majority (99.1%) of sterol esters in the plant were associated with leaf tissue and that the P_{20} fraction accounted for a major portion (74.1%) of this total. The stem tissue accounted for the remainder of sterol esters (0.9%) and most of this remainder was located in the P_{20} fraction (Table 20). No sterol esters were detected in either the supernatant fraction or root tissues.

Table 20. Effect of 300 μg Amo 1618 per plant on the incorporation of DL-2-^{14}C-mevalonic acid into sterol esters from sub-cellular organelles of intact tobacco seedlings.

The sterol esters-squalene-2,3-epoxide region from T.L.C. plates on which the total un-saponified lipid extract was developed, was saponified according to the text and the free sterols thus obtained were separated from squalene-2,3-epoxide by T.L.C.

Tissue	Treatment	Incorporation of L-2-^{14}C-mevalonate into sterol esters from		
		P_{20}	P_{200}	S_{200}
			c.p.m.	
Leaves	Control	83,167	28,076	N.D.
	Amo 1618-treated	11,297	2,625	N.D.
% inhibition		86.4	90.6	-
Stems	Control	1,021	20	N.D.
	Amo 1618-treated	21	0	N.D.
% inhibition		97.9	100	-
Roots	Control	N.D.	N.D.	N.D.
	Amo 1618-treated	N.D.	N.D.	N.D.
% inhibition		-	-	-

N.D. = none detected.

Amo 1618 inhibited mevalonate incorporation into sterol esters quite strongly (greater than 86%) suggesting that esterification follows sterol synthesis and that inhibition of sterol ester formation was probably the result of inhibition of sterol biosynthesis.

It is evident from the above data that the sterols, sterol intermediates and sterol esters present in all tobacco seedling tissues as incorporation products from DL-2-^{14}C-mevalonic acid are located almost exclusively in membraneous organelles. Amo 1618 treatment of the seedling leads to a marked reduction in the levels of incorporation products associated with these organelles and could therefore be interfering with the structural integrity, or function, of membraneous organelles. It is postulated that such an action of the retardant could account, at least in part, for its growth retarding ability.

6. Discussion

The three retardants tested were all able to inhibit the incorporation of mevalonate into 4-desmethylsterols in rootless tobacco seedlings and in each case the inhibition was concentration dependant. Phosfon D was the most potent and CCC the least effective of the three retardants. Each retardant, at least at middle of the range concentrations, appears to act at different points in the biosynthetic pathway. Amo 1618 (and CCC at higher concentrations) appeared to be most effective at the squalene-2,3-epoxide cyclase step and Phosfon D was apparently more effective in inhibiting demethylation reactions in the sequence. Two of the retardants (Amo 1618 and Phosfon D) act at the same steps in the sterol pathway of tobacco as they do in the cholesterol pathway in rat liver preparations. Further, both of these retardants are potent inhibitors of sterol biosynthesis in both tobacco and in rat liver homogenates (Paleg, 1970a). Although less effective than Amo 1618 or Phosfon D, CCC inhibited sterol biosynthesis in tobacco; a finding which was not observed in the rat liver system as Paleg (1970a) demonstrated that CCC (at concentrations as high as 1,000 μg/ml) was completely ineffective in inhibiting mevalonate incorporation into cholesterol in rat liver homogenates.

The inhibition of sterol biosynthesis induced by Amo 1618 in rootless tobacco seedlings was also observed in intact seedlings and was largely independent of light intensity. Although small quantitative differences in the incorporation of mevalonate into sterols were

observed under the two different light treatments (high and low intensity) the retardant was equally as effective in inhibiting 4-desmethylsterol biosynthesis under each of the conditions employed. The same apparent site of Amo 1618 action (viz. the squalene-2,3-epoxide cyclase step) was observed in each case but there was also some indication of a possible pre-squalene site of inhibition evident under high light intensity treatments. Despite these quantitative differences, however, it was concluded that the effects of Amo 1618 (and by analogy the other retardants) on sterol biosynthesis in intact tobacco seedlings, will follow fairly closely the observed effects on rootless seedlings of the same age.

The effect of high concentrations of Phosfon D on mevalonate incorporation into sterols and sterol precursors, as mentioned earlier in this section (Results B.1.a.), differ slightly from the effects of lower concentrations of the retardant. The dramatically increased incorporation of precursor into the 4-methylsterols and 4,4'-dimethylsterols is reduced at the high retardant concentrations. At the same time incorporation into squalene and squalene-2,3-epoxide is reduced. The data could suggest that at high concentration, the retardant becomes effective in inhibiting sterol biosynthesis at a pre-squalene site, or sites, in the pathway, as well as at the demethylation reactions. Such a mechanism would account for a reduction in the accumulation of the 4-methyl- and 4,4'-dimethyl- sterols due to a reduced synthesis of hydrocarbon precursors of these sterol intermediates. Thus a scheme involving several points of inhibition by

Phosfon D in the sterol biosynthetic pathway is envisaged. The points of inhibition could be differentially sensitive to the retardant concentration. At low Phosfon D concentration the primary site of sterol inhibition (on an absolute basis) appeared to be the demethylation of 4,4'-dimethylsterols and as the retardant concentration increased the demethylation of 4-methylsterols, and finally, the biosynthesis of squalene, were also inhibited.

Another, perhaps less likely, explanation of the data is that the accumulated 4,4'-dimethylsterols and 4-methylsterols at high retardant concentration eventually result in feed-back inhibition of their own synthesis.

A pre-squalene site of inhibition in the biosynthetic pathway was also observed for Amo 1618 treatment of intact tobacco seedlings grown under high light intensity. This point of inhibition was demonstrated at all levels of Amo 1618 [viz. from 0.1 to 3 μg/plant (Results B.2.c.), 100 μg/plant (Results B.1.b.), and 300 μg/plant (Results B.1.b. and B.5.)] and is therefore a most sensitive site of inhibition of the retardant. It is possible that squalene may be shunted into other triterpenoid pathways as a result of Amo 1618 inhibition of sterol biosynthesis only at high light intensity but why high light intensity should render a particular part of the sterol pathway (in this case squalene production from mevalonate) sensitive to inhibition by a plant growth retardant is unclear.

Treatment of rootless tobacco seedlings with very low levels of the retardants Amo 1618 and CCC resulted in an apparent stimulation of mevalonate incorporation into sterols and sterol intermediates. Further, when intact tobacco seedlings of the same age were treated with low levels of Amo 1618, a similar, though not identical, set of results was obtained, especially under the same low light intensity conditions used for rootless tobacco seedling work. Assuming these apparent stimulations of sterol biosynthesis to reflect an increased sterol production, the results parallel very closely those showing increased gibberellin production as a result of CCC treatment of pea seedlings (Carr and Reid, 1968; Reid and Crozier, 1970) and gladiolus (Halevy and Shilo, 1970).

G.L.C. analysis of the 4-desmethylsterols from control and low concentration Amo 1618-treated intact tobacco seedlings, however, showed that the absolute levels of the individual sterols remained unchanged even though radioactivity associated with them increased significantly. Due to the rather slow metabolic turn-over of sterols in plants one might expect to observe an increase in sterol levels during a 24 hr period if the sterol biosynthetic rate were increased up to almost double that of control. As no such increase was observed it may be concluded that the apparent stimulation of sterol biosynthesis at low levels of retardant reflected an increased specific activity of sterols rather than an increased sterol production by the tobacco seedlings. These findings would suggest, therefore, that the low levels of Amo 1618 are able to somehow lower the pool size of

available mevalonate in the seedlings without inhibiting sterol biosynthesis at any later stage in the pathway, resulting in a greater uptake of the supplied ^{14}C-precursor by the plants. There are perhaps two main ways in which the retardant could lower mevalonate levels; by an inhibition of HMG-CoA reductase activity (as demonstrated in Results A.1.) or an inhibition of acetylcholine esterase activity (Riov and Jaffe, 1973c). The former mode of action would result in a direct reduction in mevalonate production in the plant and thus lead to the synthesis of sterols of higher specific activity than those in control seedlings. An inhibition of acetylcholine esterase activity, on the other hand, could lower the acetate pool size and might lead eventually to a reduction in mevalonate production and thus to sterols of higher specific activity than controls.

Repeated applications of the retardants to intact tobacco seedlings showed that each of the retardants possessed the ability to retard leaf growth and, more effectively, stem elongation. A concentration dependance was exhibited for the effects of each of the retardants on stem growth and the data indicated that the retardants were differentially effective. Thus, a correlation between the effects of the retardants on sterol biosynthesis in, and growth of tobacco seedlings was observed.

Single applications of higher levels of the retardants were also effective in inhibiting stem elongation but were ineffective in retarding leaf growth. An examination of sterol biosynthesis in each of the

seedling tissues revealed that, on a per gram fresh weight basis, sterol biosynthesis in stems proceeded at a greater rate and was inhibited by Amo 1618 to a larger extent than in leaves or roots. A quantitative relationship between the effects of Amo 1618 on stem growth and sterol biosynthesis was demonstrated during a 24 hr test period.

The greater sensitivity of growth of tobacco stems than leaves to retardant application is to some extent paralleled by the greater sensitivity of sterol biosynthesis in stems to Amo 1618 application. Leaves, which exhibit a greater resistance to sterol biosynthesis inhibition by Amo 1618, show a reduced rate of growth only when repeated applications of relatively high levels of retardant are administered.

Treatment of intact tobacco seedlings with Amo 1618 led to marked reductions in the levels of sterols associated with membraneous components of the cells, particularly in stem tissue. As sterols are components of cell membranes the retardant(s) effects on growth of plants may be at least, in part, due to a change in the sterol content and/or composition of membraneous organelles which, in turn, leads to impaired function of the organelles and consequently reduced growth. Inhibition of sterol biosynthesis in yeast has been demonstrated to reduce respiratory capacity of the organism which can be restored by addition of ergosterol (Parks and Starr, 1963). As the P_{20} fraction examined in this work would be rich in mitochondria it

is not hard to imagine a similar process occurring in tobacco seedlings treated with Amo 1618 (and, by analogy, other retardants). Inhibition of sterol biosynthesis by the retardants could lead to reduced respiratory function of mitochondria and the reduced respiration rate could, in turn, lead to reduced metabolic activity and hence growth. The addition of sterols back to the retardant-treated plants could repair mitochondrial membranes, restoring their full respiratory capacity and hence growth.

Specific animal steroid biosynthesis inhibitors have been shown to inhibit the growth of both tall and dwarf varieties of pea (Moore and Anderson, 1966) and inhibit sterol biosynthesis in tobacco seedlings (Reid, 1968a), *Petasites hybridus* (Zabkiewicz *et al*., 1969) and *Xanthium* (Kimura *et al*., 1973). The inhibitors' effects on rat liver cholesterol biosynthesis are also closely paralleled by the plant growth retardants' effects (Paleg, 1970a; Paleg, 1970b). When tested on the growth of intact tobacco seedlings the animal steroid biosynthesis inhibitors each demonstrated growth retarding ability in a manner similar to that observed for the retardants.

The application of sterol to retardant-treated tobacco plants completely reversed the effects of Amo 1618 and CCC (but not Phosfon D) and pointed to a sterol biosynthetic requirement for stem growth.

C. GIBBERELLIC ACID EFFECTS ON GROWTH AND STEROL BIOSYNTHESIS IN TOBACCO SEEDLINGS

Many of the actions of the retardants are virtually the opposite of those of the gibberellins and can be overcome by application of exogeneous gibberellic acid (see Introduction). Further, one site of action of the retardants has been shown to be the gibberellin biosynthetic pathway (Dennis et al., 1965) and specific points in the pathway show different sensitivity to inhibition by the retardants (Schechter and West, 1969).

The work presented in this Thesis so far has demonstrated that the plant growth retardants are powerful inhibitors of sterol biosynthesis in tobacco seedlings and tobacco seedling preparations and that their effects on sterol biosynthesis are correlated with their effects on growth. The implication of this work is that at least a part of the growth retarding activity of the retardants is a result of the inhibition of sterol biosynthesis.

In the light of the reported ability of exogeneous GA_3 to reverse retardant effects, coupled with the known ability of retardants to inhibit GA-biosynthesis, it was of interest to determine whether GA_3 could reverse the effects of the retardants on growth of tobacco seedlings and, if so, whether the hormone mediated its effects on growth via an interaction with sterol biosynthesis. To test these questions an examination of the effects of GA_3 on retardant-induced inhibition of sterol biosynthesis in, and growth of tobacco seedlings was undertaken.

1. Effect of Exogeneous GA_3 on Growth Retardation Induced by Continuous Application of Retardants

For these experiments two levels of each retardant adjudged to be sufficient to retard at least stem elongation were tested for their effects on the growth of 17 day-old tobacco seedlings in the presence or absence of simultaneously applied GA_3. Only a single level of GA_3 (1.5 μg/plant) was employed either alone or in combination with the retardant.

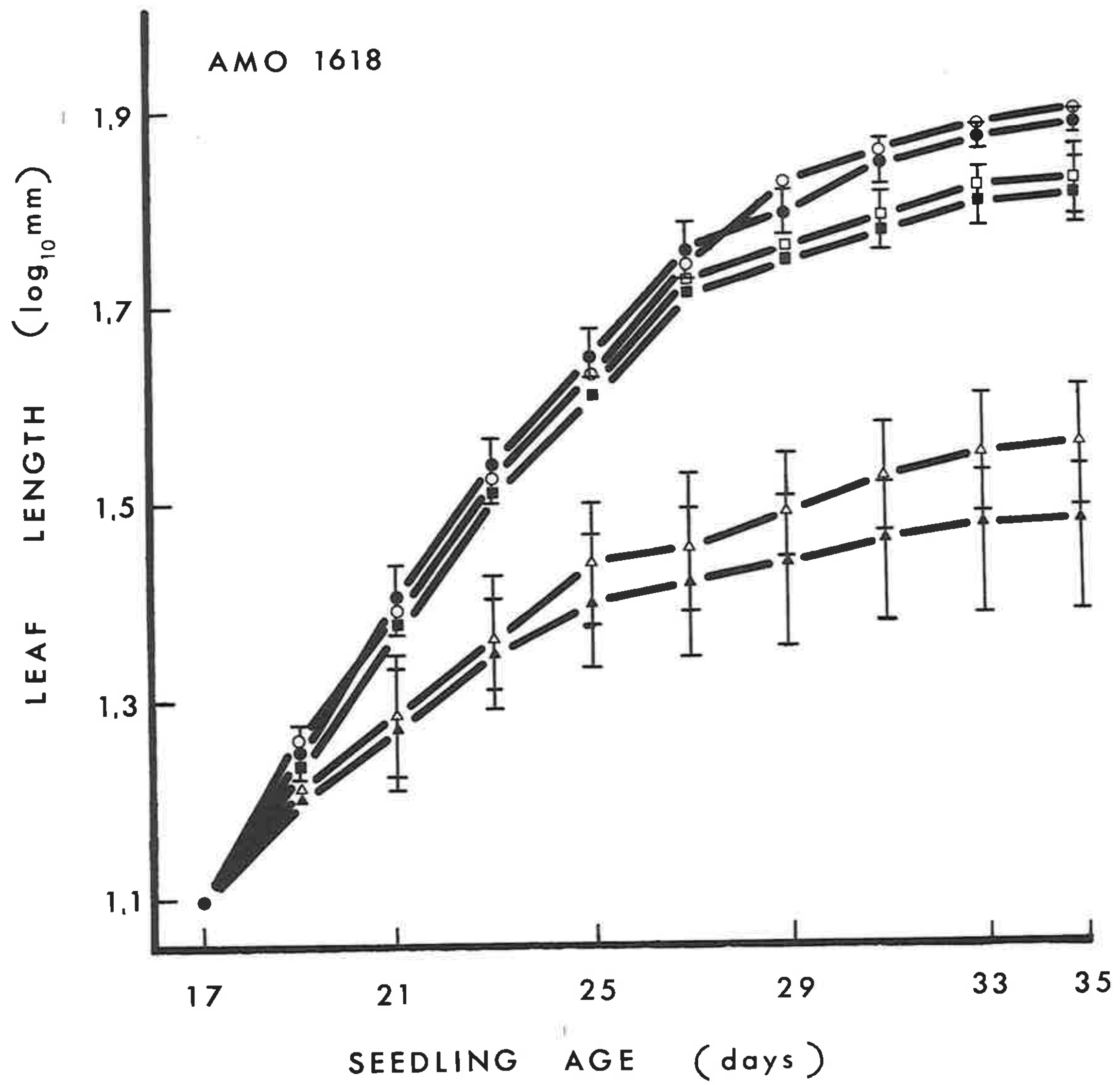
a. Effect on retardation induced by Amo 1618

Seventeen-day-old seedlings were treated with either 10 or 50 μg of Amo 1618/plant, 1.5 μg of GA_3, or a combination of retardant plus GA_3 (above). Applications of the substances were made every second day from the initial date of treatment (Methods 3.a.) and measurements of seedling growth were recorded each second day immediately prior to and following the application of growth substances (Methods 4.).

Exogeneous GA_3 by itself had no effect on leaf growth (figures 43 and 44) and stimulated stem elongation markedly (figure 45). The lower level of the retardant had little effect on leaf growth although some slight inhibition of both leaf length (figure 43) and leaf width (figure 44) was apparent after several applications of the retardant. When applied in combination with this lower level of Amo 1618, GA_3 was able to prevent the eventual retardation of leaf width but not leaf length. Continuous application of 10 μg of Amo 1618 to the seedlings

Figure 43. Effect of GA_3 on leaf length of tobacco seedlings treated with continuous application of Amo 1618.

Leaf length of 17 day-old seedlings was compared for control (●) and GA_3-treated (○) seedlings with seedlings treated with 10 μg of Amo 1618 in the presence (□) or absence (■) of 1.5 μg GA_3 and seedlings treated with 50 μg Amo 1618 in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied every alternate day.



AMO 1618
LEAF LENGTH ($\log_{10}$ mm)
1,1
1,3
1,5
1,7
1,9
17
21
25
29
33
35
SEEDLING AGE (days)

Figure 44. Effect of GA_3 on leaf width of tobacco seedlings treated with continuous application of Amo 1618.

Leaf width of 17 day-old seedlings was compared for control (●) and GA_3-treated (○) seedlings with seedlings treated with 10 μg of Amo 1618 in the presence (□) or absence (■) of 1.5 μg GA_3 and seedlings treated with 50 μg Amo 1618 in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied every alternate day.

AMO 1618

LEAF WIDTH ($\log_{10}$ mm)

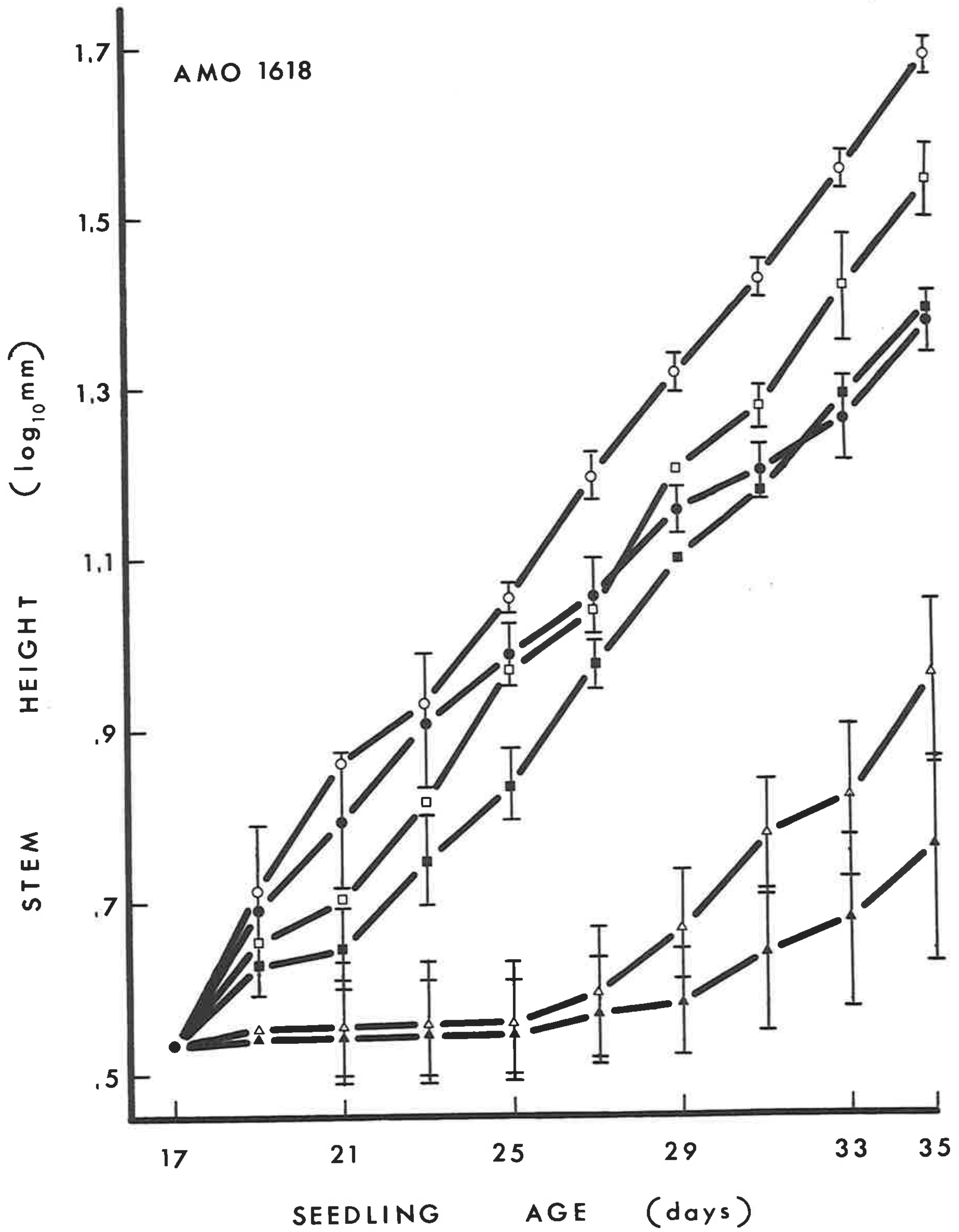
1,8
1,6
1,4
1,2
1,0

17 21 25 29 33 35

SEEDLING AGE (days)

Figure 45. Effect of GA_3 on stem growth of tobacco seedlings treated with continuous application of Amo 1618.

Stem growth of control (●) and GA_3 (○) treated tobacco seedlings are compared with that of seedlings treated with 10 μg Amo 1618/plant in the presence (□) or absence (■) of 1.5 μg GA_3 and seedlings treated with 50 μg Amo 1618/plant in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied on every alternate day.



AMO 1618
STEM HEIGHT ($\log_{10}$mm)
1.7
1.5
1.3
1.1
.9
.7
.5
17
21
25
29
33
35
SEEDLING AGE (days)

also inhibited stem elongation initially, and exogeneous GA_3 reversed this retardation though at a stage considerably later than the effects of the retardant became significant. After several applications of this level of the retardant the retardation of stem elongation observed initially eventually disappeared; at the same time GA_3 caused a slight stimulation of stem elongation when applied in combination with the retardant. The higher level of the retardant induced a marked reduction in stem growth (figure 45) and simultaneous application of GA_3, at the stated level, was unable to cause significant reversal of the retardant effects.

b. Effect on retardation induced by CCC

Both levels of CCC (viz. 25 and 100 μg/plant) retarded leaf length (figure 46) and leaf width (figure 47) but only the higher level was able to induce significant retardation of stem elongation (figure 48). Simultaneous application of GA_3 to the plants not only did not reverse the effect of the retardant (at 25 μg/plant) but actually caused a significant increase in retardation of leaf length and to a lesser extent leaf width and stem elongation. GA_3 was also unable (at this level) to reverse the effects of the higher level of the retardant on either leaf or stem growth.

c. Effect on retardation induced by Phosfon D

Phosfon D caused marked retardation of leaf length (figure 49), leaf width (figure 50), and stem height (figure 51) at both levels

Figure 46. Effect of GA_3 on leaf length of tobacco seedlings treated with continuous application of CCC.

Leaf length of 17 day-old seedlings was compared for control (●) and GA_3-treated (○) seedlings with seedlings treated with 25 μg of CCC in the presence (□) or absence (■) of 1.5 μg GA_3 and 100 μg of CCC in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied each alternate day.

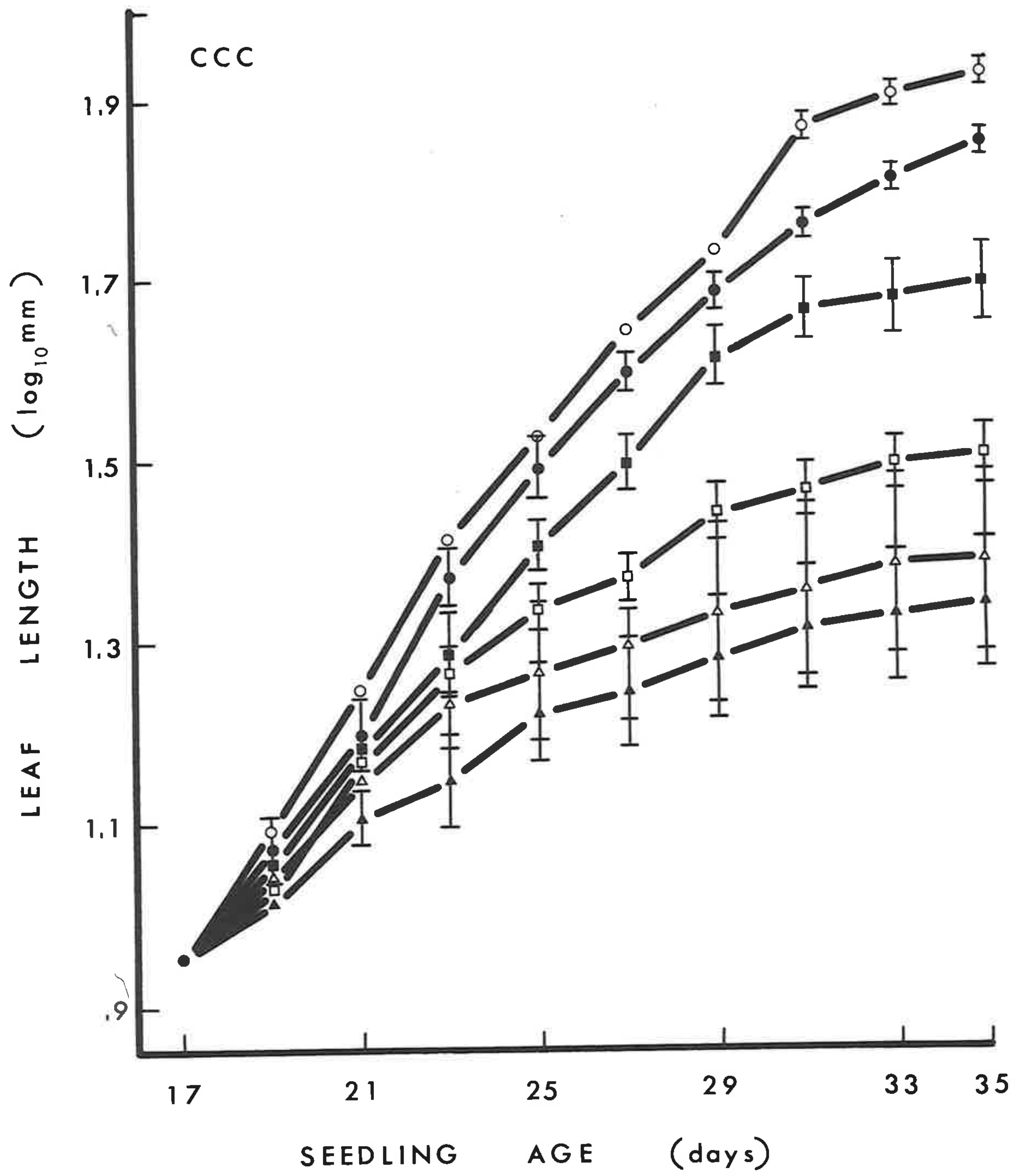

CCC
1,9
1,7
1,5
1,3
1,1
,9
LEAF LENGTH (log10mm)
17
21
25
29
33
35
SEEDLING AGE (days)

Figure 47. Effect of GA_3 on leaf width of tobacco seedlings treated with continuous application of CCC.

Leaf width of 17 day-old seedlings was compared for control (●) and GA_3-treated (○) seedlings with seedlings treated with 25 μg of CCC in the presence (□) or absence (■) of 1.5 μg GA_3 and 100 μg of CCC in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied on each alternate day.

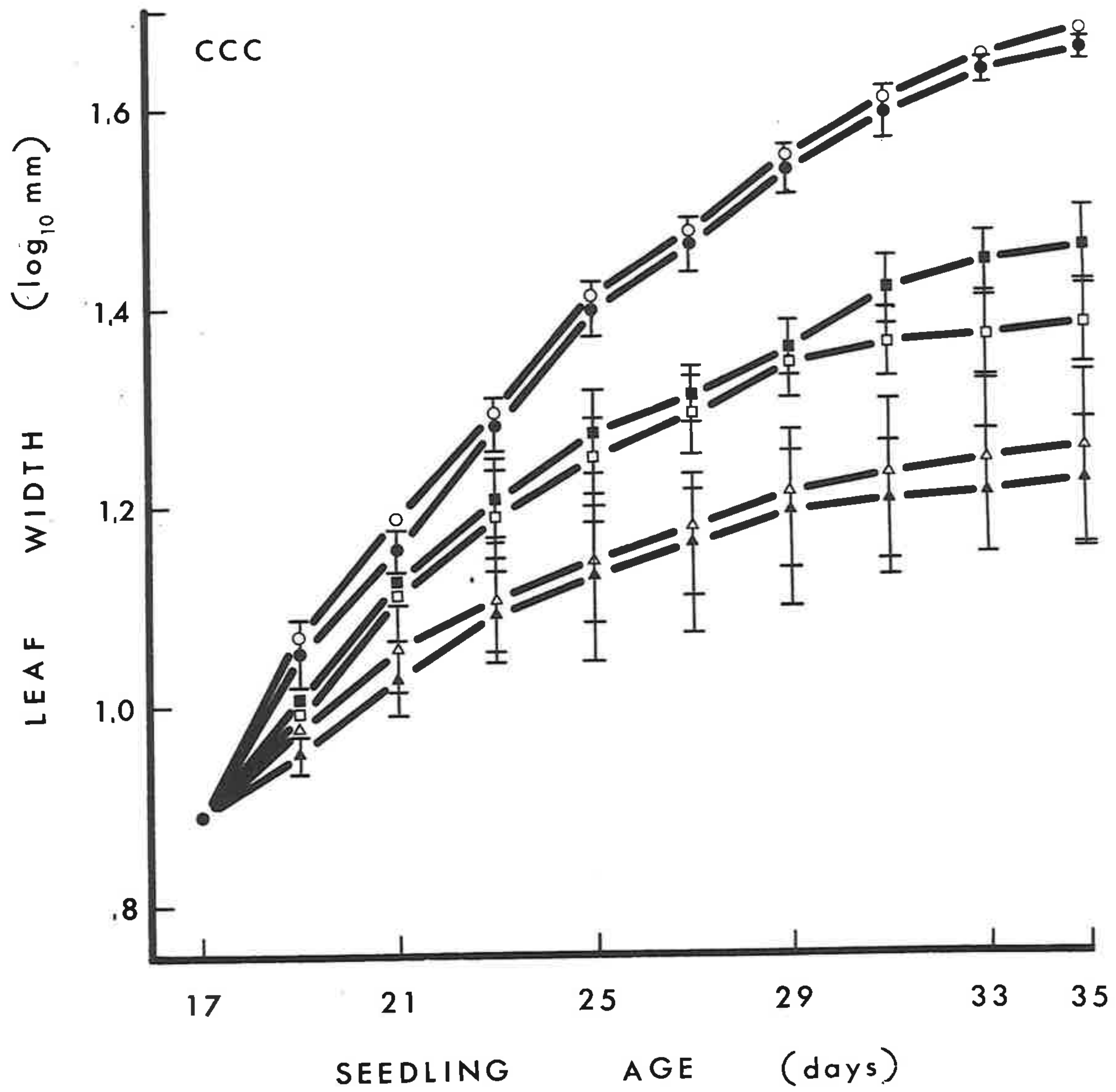

CCC
LEAF WIDTH (log_{10} mm)
1.6
1.4
1.2
1.0
.8
17
21
25
29
33
35
SEEDLING AGE (days)

Figure 48. Effect of GA_3 on stem growth of tobacco seedlings treated with continuous application of CCC.

Stem growth of control (●) and GA_3 (○) treated tobacco seedlings are compared with that of seedlings treated with 25 µg CCC/plant in the presence (□) or absence (■) of 1.5 µg GA_3 and seedlings treated with 100 µg CCC/plant in the presence (△) or absence (▲) of 1.5 µg GA_3. Growth substances were applied on every alternate day.

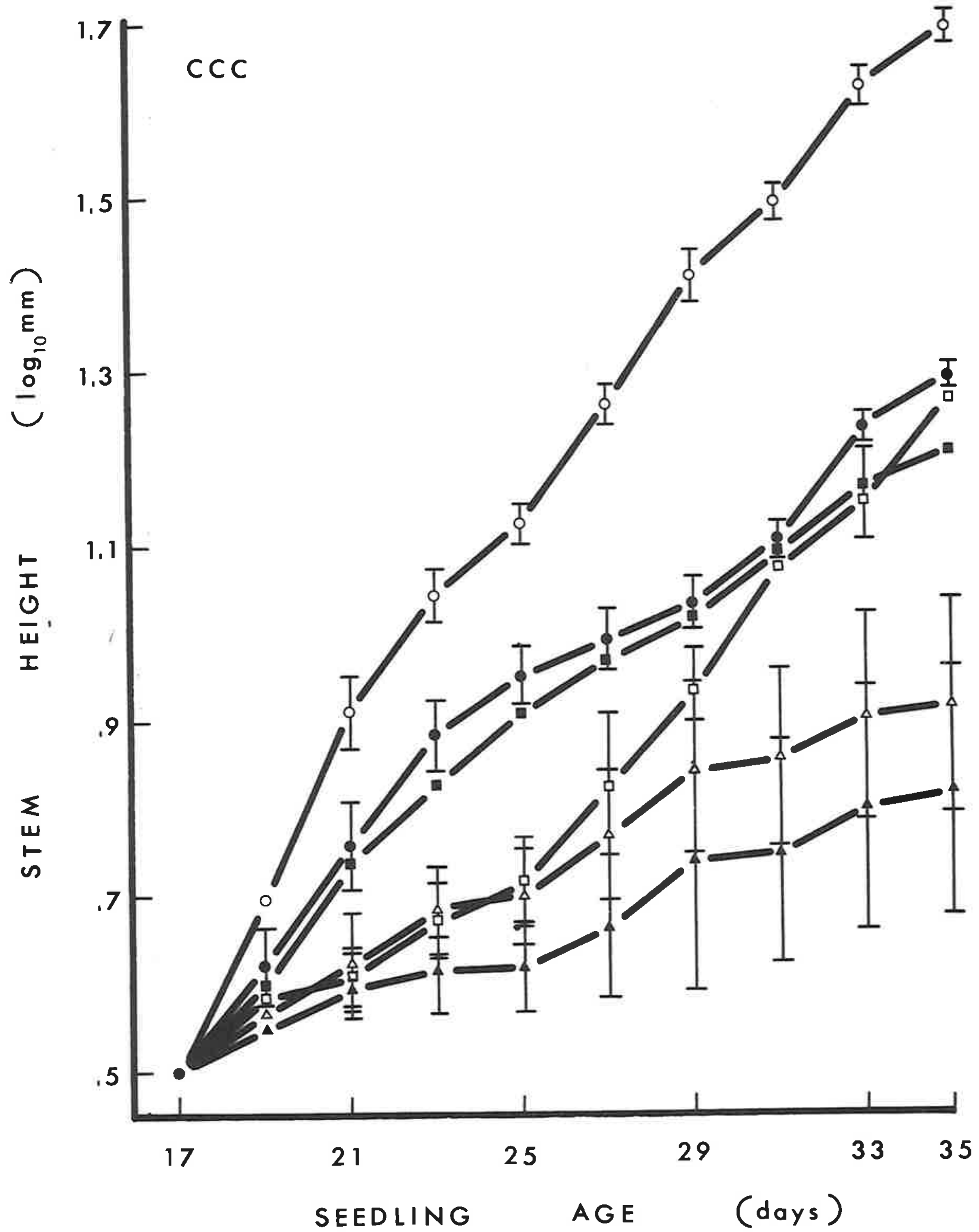
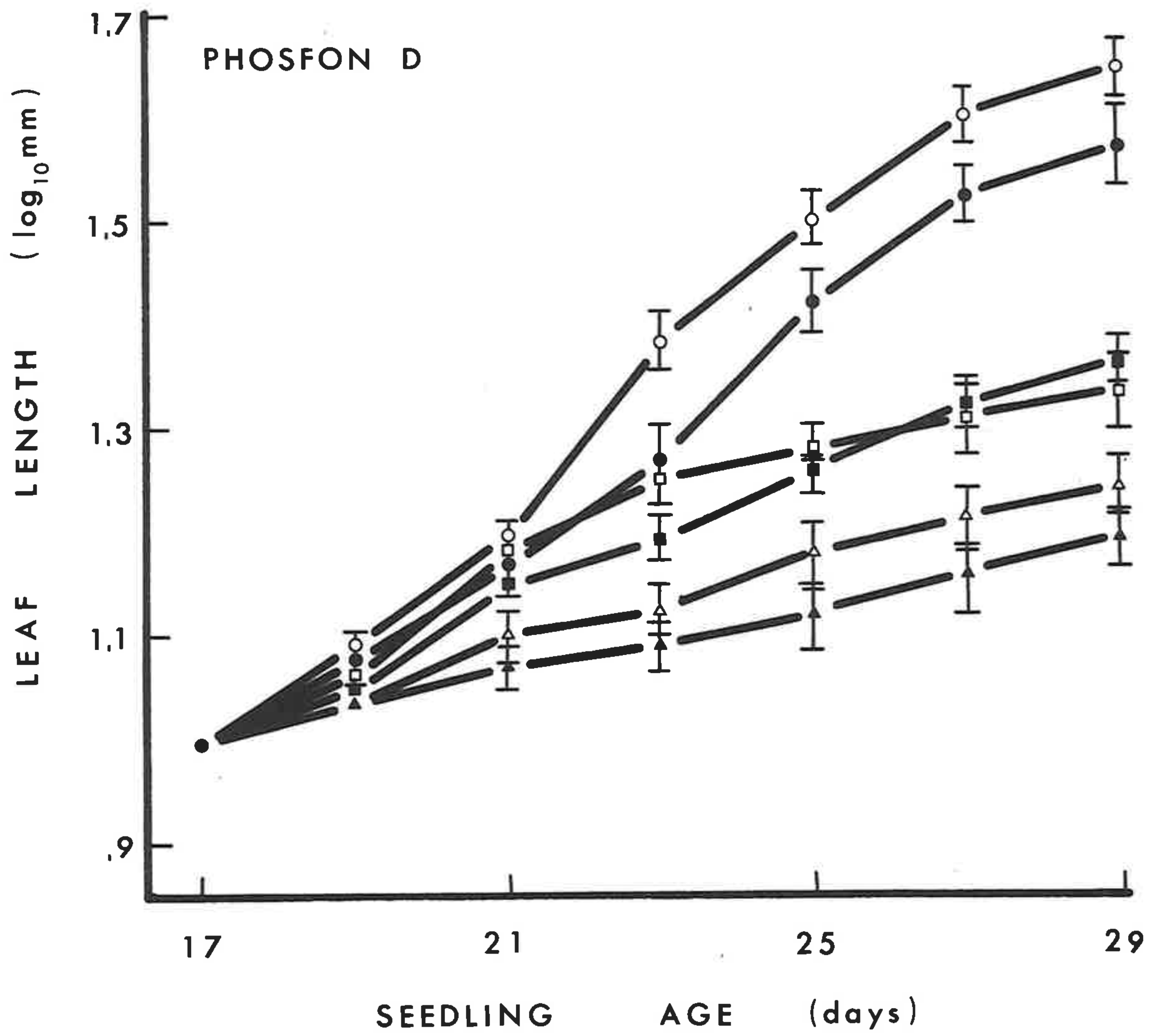

CCC
STEM HEIGHT (log10 mm)
1.7
1.5
1.3
1.1
.9
.7
.5
17
21
25
29
33
35
SEEDLING AGE (days)

Figure 49. Effect of GA_3 on leaf length of tobacco seedlings treated with continuous application of Phosfon D.

Leaf length of control (●) and GA_3-treated (○) tobacco seedlings was compared with that of seedlings treated with 3 μg of Phosfon D in the presence (□) or absence (■) of 1.5 μg GA_3 and seedlings treated with 10 μg of Phosfon D in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied every alternate day.



PHOSFON D
LEAF LENGTH ($\log_{10}$mm)
1,7
1,5
1,3
1,1
,9
17
21
25
29
SEEDLING AGE (days)

Figure 50. Effect of GA_3 on leaf width of tobacco seedlings treated with continuous application of Phosfon D.

Leaf width of 17 day-old seedlings was compared for control (●) and GA_3-treated (○) seedlings with seedlings treated with 3 μg of Phosfon D in the presence (□) or absence (■) of 1.5 μg GA_3 and seedlings treated with 10 μg Phosfon D in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied every alternate day.

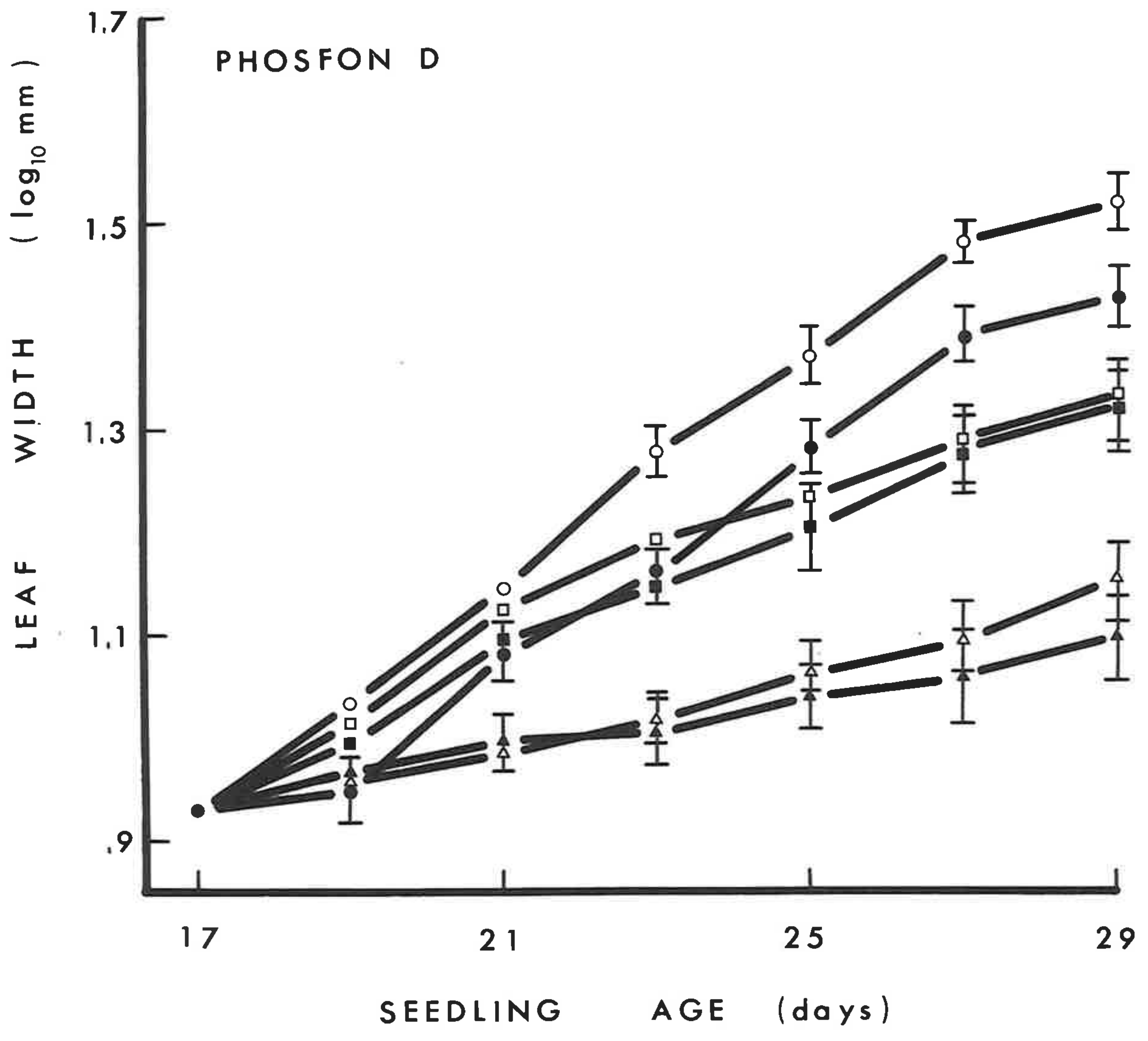
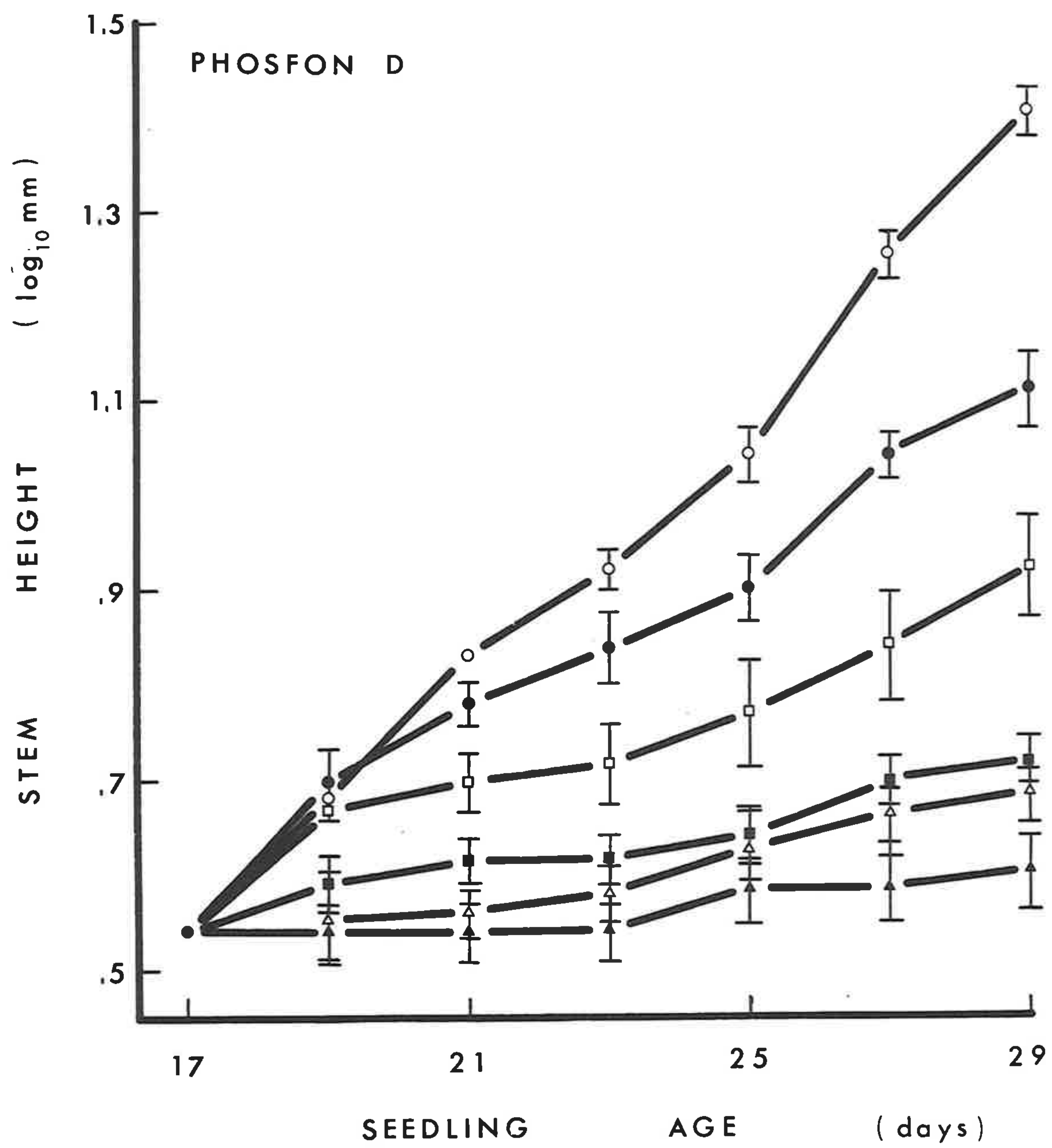

PHOSFON D
LEAF WIDTH (log10 mm)
1,7
1,5
1,3
1,1
,9
17
21
25
29
SEEDLING AGE (days)

Figure 51. Effect of GA_3 on stem growth of tobacco seedlings treated with continuous application of Phosfon D.

Stem growth of control (●) and GA_3 (○) treated tobacco seedlings are compared with that of seedlings treated with 3 μg Phosfon D/plant in the presence (□) or absence (■) of 1.5 μg GA_3 and seedlings treated with 10 μg Phosfon D/plant in the presence (△) or absence (▲) of 1.5 μg GA_3. Growth substances were applied on every alternate day.



PHOSFON D
STEM HEIGHT ($\log_{10}$ mm)
1.5
1.3
1.1
.9
.7
.5
17
21
25
29
SEEDLING AGE (days)

(viz. 3 and 10 µg/plant) tested. Exogeneous GA_3 alone caused a stimulation of stem elongation and to a lesser degree leaf growth but was able to cause only a slight or partial reversal of retardation of stem growth induced by the lower level of the retardant. The hormone had no effect on either leaf or stem growth when applied in combination with the higher level of Phosfon D.

The results presented here indicate that the level of GA_3 tested, although able to induce a marked stimulation of stem elongation by itself, was unable to consistently reverse the retardation of tobacco seedling growth when added in combination with the retardants. The exceptions were a considerably delayed reversal of a slight inhibition of stem elongation induced by 10 µg of Amo 1618 per plant and a partial reversal of the marked inhibition of stem elongation induced by 3 µg of Phosfon D per plant. The results with CCC are especially intruiging as GA_3 in combination with the retardant actually enhanced the growth retardation of both leaf and stem growth by the retardant.

Another point of interest was the variable effects of GA_3 on leaf growth observed in the three experiments. In the Amo 1618 experiment (Results C.1.a.), GA_3 alone had no effect on leaf length but caused some slight stimulation in leaf width (figure 44); in the CCC experiment (Results C.1.b.) the reverse was observed, i.e. after several applications GA_3 caused some slight stimulation of leaf length (figure 46) but not leaf width. In the Phosfon D experiment (Results C.1.c.) GA_3 caused a slight stimulation of both leaf length (figure 49) and leaf width (figure 50) after only a few applications. This

data suggests that GA_3 can stimulate leaf growth of tobacco seedlings (though only slightly when compared with stem elongation) and points to an inherent variability in the system.

2. Effect of Exogeneous GA_3 on Growth Retardation Induced by a Single Application of Retardant

In this experiment a single application of either Amo 1618 (100 μg/plant), CCC (100 μg/plant) or Phosfon D (30 μg/plant) either alone, or in combination with GA_3 (30 μg/plant), was made to 21 day-old tobacco seedlings (Methods 3.a.) and measurements of leaf and stem growth were taken each alternate day for 8 days (Methods 2 and 4).

a. Effect on Amo 1618-treated seedlings

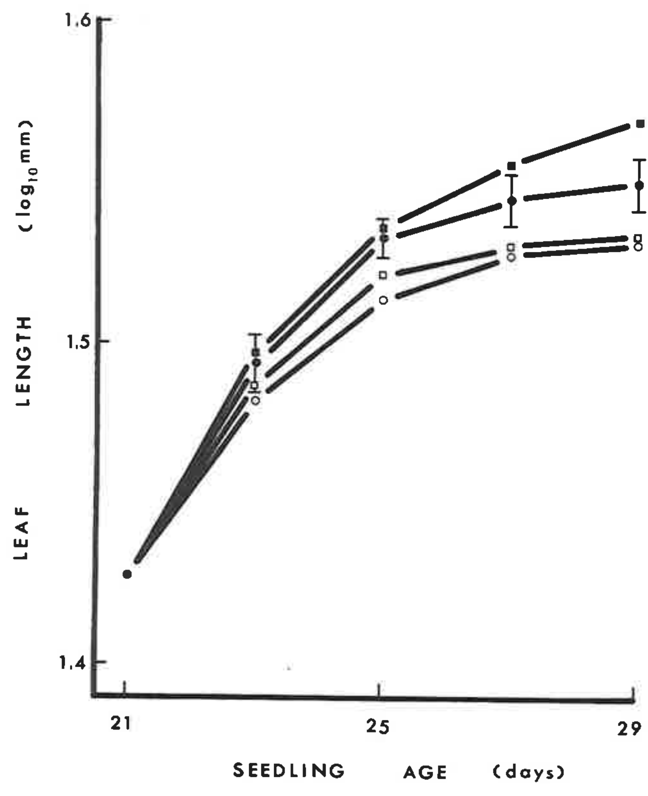
Neither Amo 1618 nor GA_3 had any effect on their own, or in combination, on either leaf length or leaf width of tobacco seedlings (figure 52). The retardant did, however, cause a significant inhibition of stem elongation (figure 53) which was completely reversed by GA_3. After several days the GA_3 plus Amo 1618-treated seedlings were the same height as those treated with GA_3 alone, i.e. quite significantly greater than controls. 48 hr after application of the growth substances, stem height was retarded by Amo 1618 quite significantly and although GA_3 prevented the inhibition of stem elongation induced by the retardant it did not significantly stimulate stem elongation in the same time period (figure 53).

b. Effect on CCC-treated seedlings

Once again, neither CCC nor GA_3 had any significant effect on leaf growth (figure 54) and apart from a significant reduction in stem height 48 hrs after application, CCC at this level had little

Figure 52. The effect of a single application of GA_3 and/or Amo 1618 on leaf growth of tobacco seedlings.

The effects of 100 μg of retardant alone (○) or in combination with 30 μg of GA_3 (□) on leaf length and leaf width are compared with control (●) and GA_3-treated (■) seedlings.


1.6
1.5
1.4
LEAF LENGTH ($\log_{10}$ mm)
21
25
29
SEEDLING AGE (days)

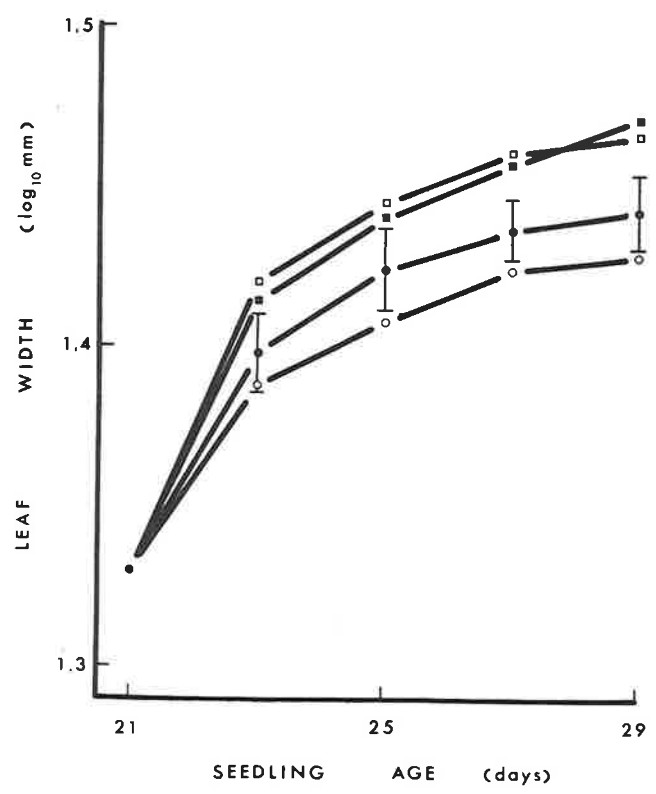

1.5
1.4
1.3
LEAF WIDTH ($\log_{10}$ mm)
21
25
29
SEEDLING AGE (days)

Figure 53. The effect of a single application of GA_3 and/or Amo 1618 on stem elongation of tobacco seedlings.

The effects of 100 μg of retardant alone (○) or in combination with 30 μg of GA_3 (□) on stem height are compared with control (●) and GA_3-treated (■) seedlings.

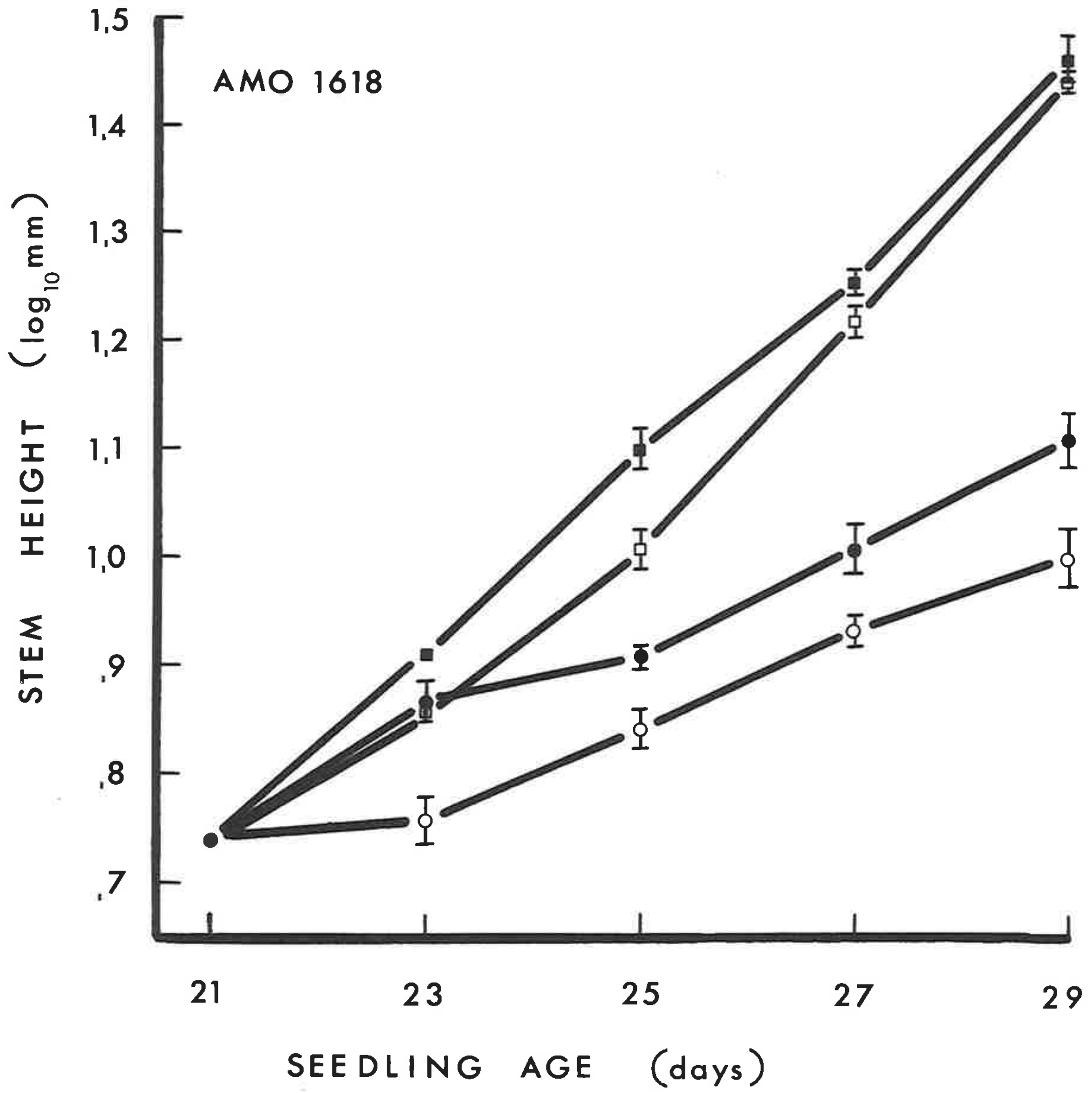
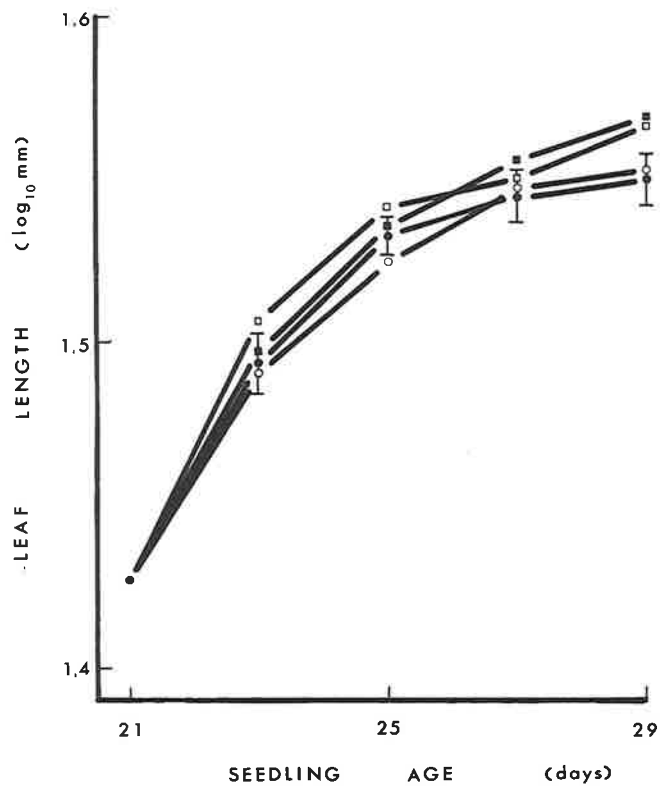
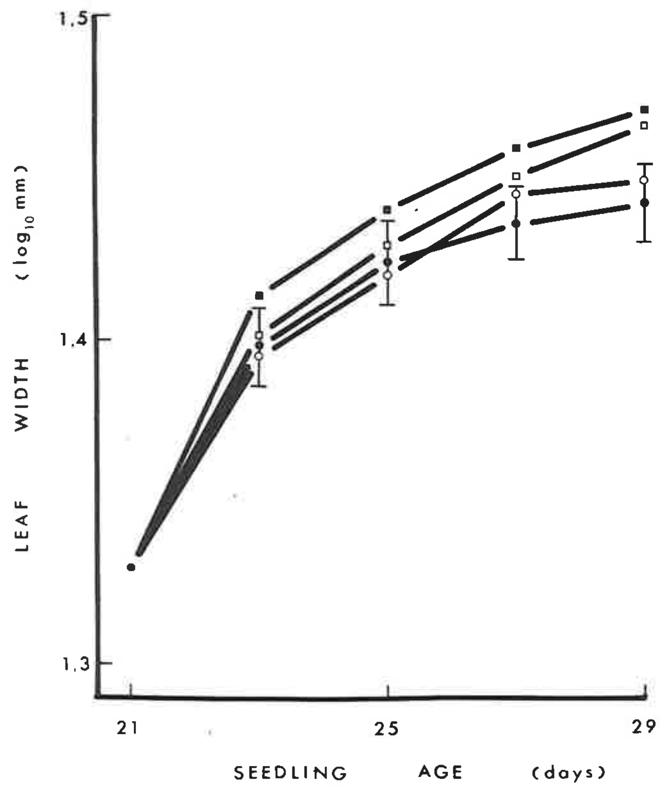

AMO 1618
STEM HEIGHT (log10 mm)
1,5
1,4
1,3
1,2
1,1
1,0
,9
,8
,7
21
23
25
27
29
SEEDLING AGE (days)

Figure 54. The effect of a single application of GA_3 and/or CCC on leaf growth of tobacco seedlings.

The effects of 100 μg of retardant alone (○) or in combination with 30 μg of GA_3 (□) on leaf length and leaf width are compared with control (●) and GA_3-treated (■) seedlings.


1,6
1,5
1,4
LEAF LENGTH (log$_{10}$ mm)
21
25
29
SEEDLING AGE (days)


1,5
1,4
1,3
LEAF WIDTH (log$_{10}$ mm)
21
25
29
SEEDLING AGE (days)

effect on stem elongation (figure 55). Although the retardant did not reduce stem elongation, GA_3 did not stimulate stem elongation above control values until 8 days after application when added in combination with the retardant. Further, the GA_3 plus retardant-treated plants did not attain the same stem height as plants treated with GA_3 alone [c.f. Amo 1618-treated plants (Results C.2.a.)] within 8 days from the time of application. This data suggests that Amo 1618 and CCC might act differently when applied in combination with GA_3. The data might reflect the different effectiveness of the retardants on gibberellin and/or sterol biosynthesis in higher plants or be a reflection of different rates of degradation of the retardants.

c. Effect on Phosfon D-treated seedlings

Apart from one value for leaf length 4 days after application, Phosfon D also caused no retardation of leaf growth (figure 56). The retardant did, however, induce a marked reduction in stem elongation (figure 57) which was completely reversed by GA_3 application. After several days the GA_3 plus retardant-treated seedlings approached the same stem height as seedlings treated with GA_3 alone.

Each of the retardants, at the levels tested, induced a significant reduction in stem elongation within 48 hr of application whereas GA_3 did not induce any significant stimulation of stem growth during the same time period. A similar observation was also made on seedlings treated with continuous application of the retardants (Results C.1.) and one might imply from this data, therefore, that the

Figure 55. The effect of a single application of GA_3 and/or CCC on stem elongation of tobacco seedlings.

The effects of 100 µg of retardant alone (○) or in combination with 30 µg of GA_3 (□) on stem height are compared with control (●) and GA_3-treated (■) seedlings.

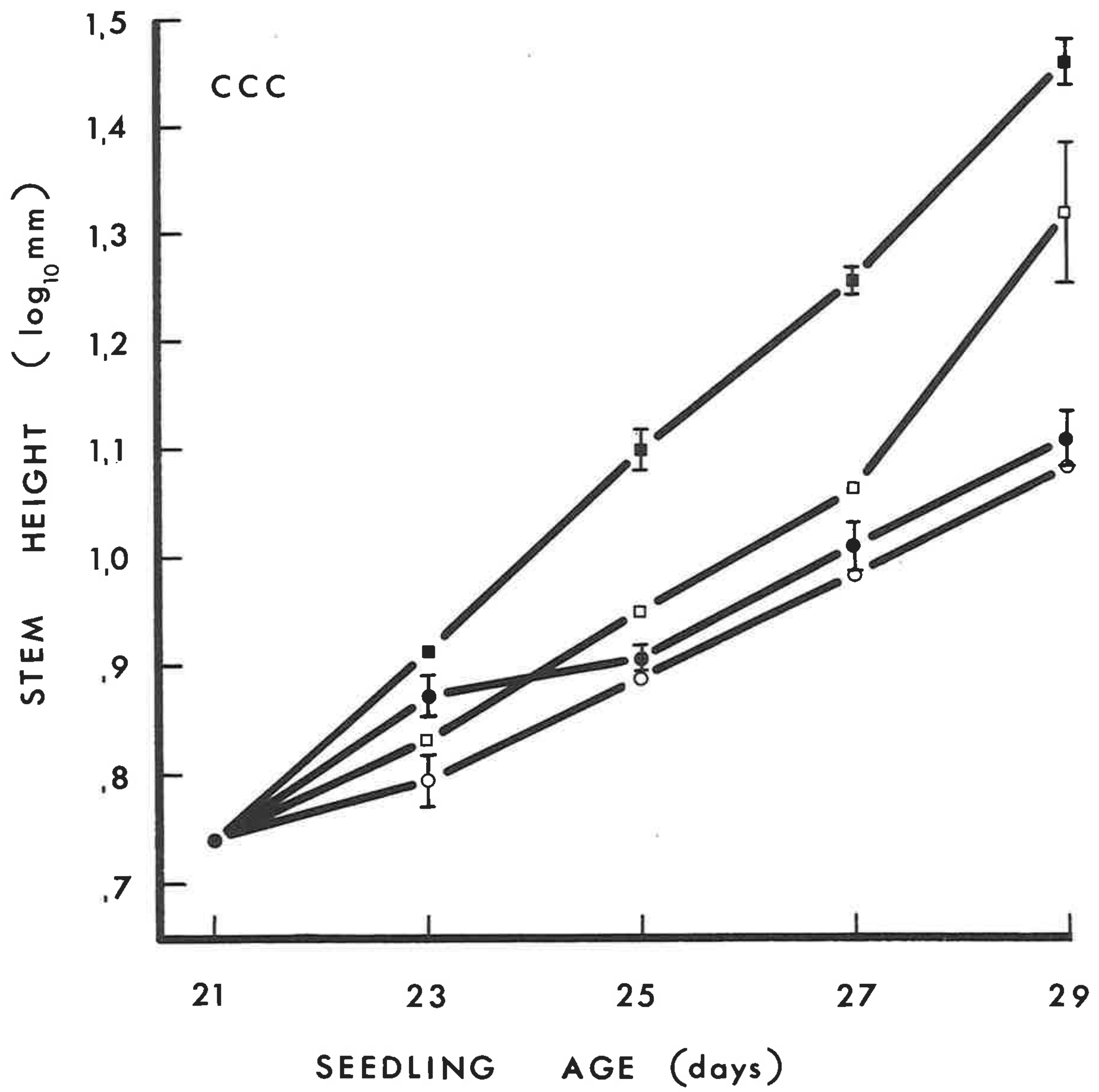
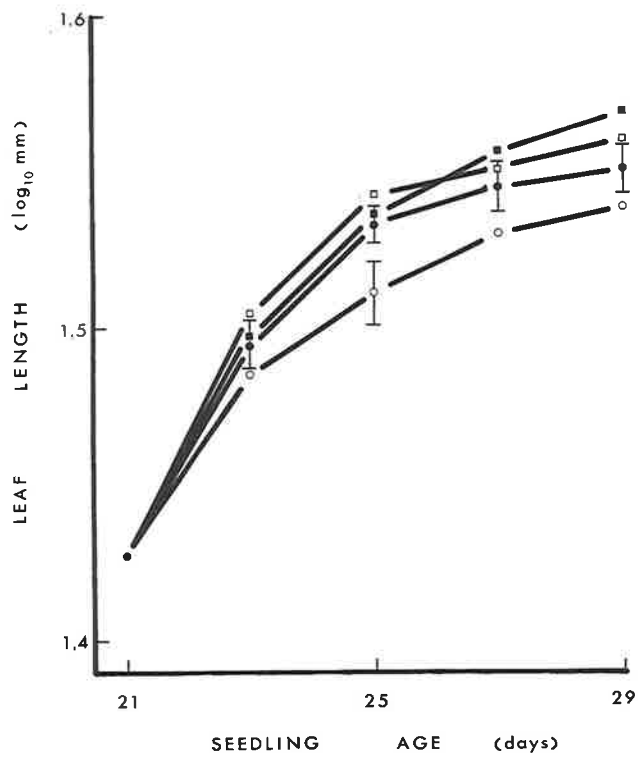

CCC
STEM HEIGHT (log10 mm)
1,5
1,4
1,3
1,2
1,1
1,0
,9
,8
,7
21
23
25
27
29
SEEDLING AGE (days)

Figure 56. The effect of a single application of GA_3 and/or Phosfon D on leaf growth of tobacco seedlings.

The effects of 30 μg of retardant alone (○) or in combination with 30 μg of GA_3 (□) on leaf length and leaf width are compared with control (●) and GA_3-treated (■) seedlings.


1.6
1.5
1.4
LEAF LENGTH (log$_{10}$ mm)
21
25
29
SEEDLING AGE (days)

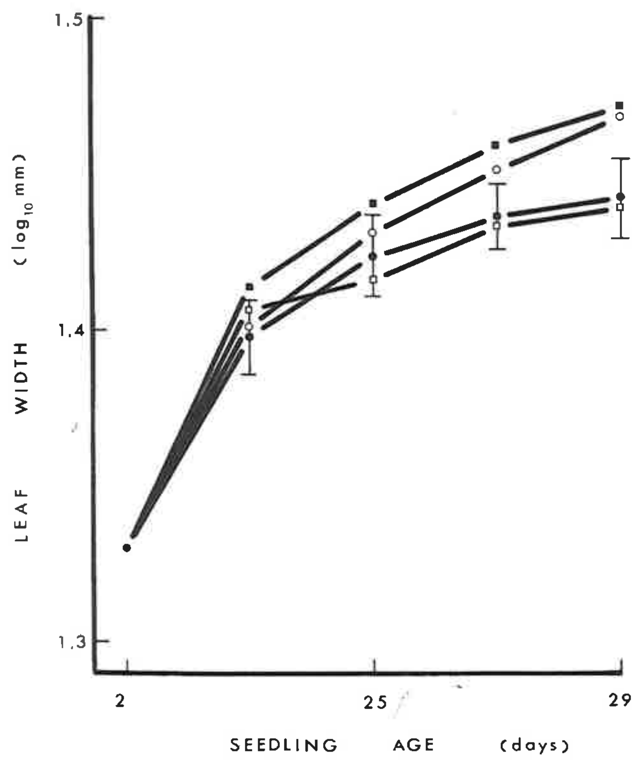
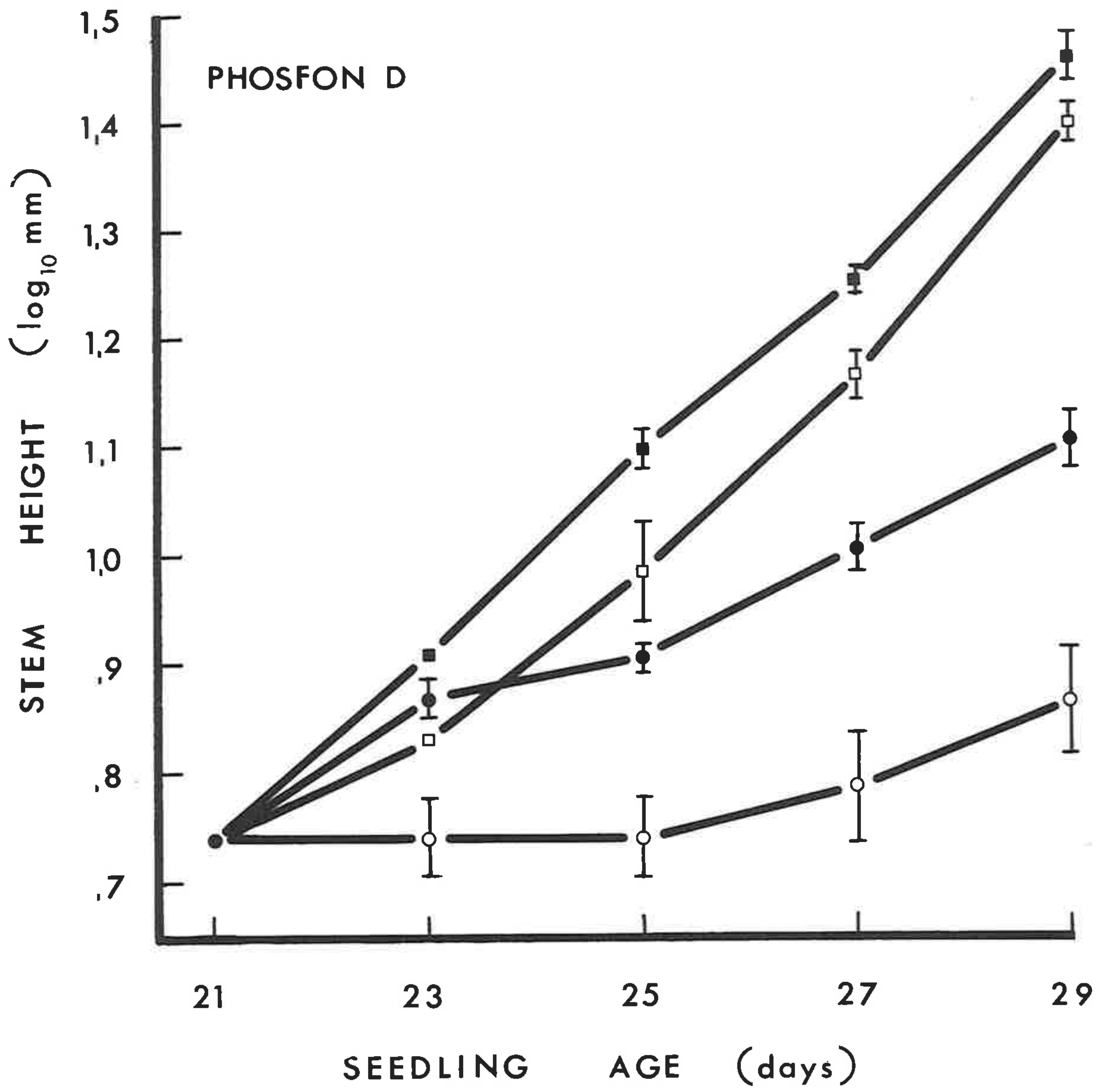

1.5
1.4
1.3
LEAF WIDTH (log$_{10}$ mm)
2
25
29
SEEDLING AGE (days)

Figure 57. The effect of a single application of GA_3 and/or Phosfon D on stem elongation of tobacco seedlings.

The effects of 30 μg of retardant alone (○) or in combination with 30 μg of GA_3 (□) on stem height are compared with control (●) and GA_3-treated (■) seedlings.



PHOSFON D
STEM HEIGHT ($\log_{10}$ mm)
1,5
1,4
1,3
1,2
1,1
1,0
,9
,8
,7
21
23
25
27
29
SEEDLING AGE (days)

retardants' effects on stem elongation precede the effects of GA_3, i.e. their effects are observed sooner after application.

Amo 1618, and to a greater degree Phosfon D, continued to retard stem elongation throughout the test period whereas the retardation of stem elongation induced by CCC disappeared by 4 days after application of the retardant. This could reflect simply a low order of effectiveness of CCC or perhaps a more rapid break-down of this retardant by the seedlings. An interesting observation was that CCC, although unable to inhibit stem elongation, was still able to prevent GA_3-induced stimulation of stem growth.

3. Effect of GA_3 on Sterol Biosynthesis in Tobacco Seedlings

a. Effect of GA_3 on sterol biosynthesis in rootless seedlings

Twenty one-day-old rootless tobacco seedlings grown under low light intensity were treated with several concentrations of GA_3 in the presence of DL-2-^{14}C-mevalonic acid (Methods 5) and after the 24 hr incorporation period sterols and sterol intermediates were extracted from the seedlings as total non-saponifiable lipids [Methods 7.a.(i)] and separated by T.L.C. of the extract (Methods 8.a.). Each of the sterol and hydrocarbon fractions thus obtained were assayed for radio-activity (Methods 11).

Examination of the incorporation products revealed that GA_3 had little to no effect on the incorporation of mevalonate into sterols (Table 21). When calculated on an absolute basis (Table 21A) the incorporation of precursor into 4-desmethylsterols showed a small apparent stimulation (ca. 39%) at 3 µg/ml of GA_3 but was unaffected by higher concentrations of the hormone. The incorporation into hydro-carbon fractions (squalene and squalene-2,3-epoxide) was stimulated slightly by all gibberellin concentrations. On a per gram fresh weight basis (Table 21B), the mevalonate incorporation into 4-desmethylsterols decreased slowly (to a maximum of ca. 31% inhibition at 30 µg/ml GA_3) as the concentration of gibberellic acid increased. Once again, incorporation into squalene and squalene-2,3-epoxide was slightly elevated at all concentrations of GA_3.

Table 21. The effect of exogeneous gibberellic acid on the incorporation of DL-2-^{14}C-mevalonic acid into sterols and sterol precursors of rootless tobacco seedlings.

(Sterols and hydrocarbon fractions were separated by T.L.C. of the total non-saponifiable lipid extract).

Gibberellin Concen-tration (μg/ml)	Incorporation of L-2-^{14}C-mevalonate				
	4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
A.			c.p.m.		
0	65,904	10,391	9,928	1,290	1,639
3	91,508	10,754	15,936	2,436	3,817
10	70,879	14,548	13,563	2,712	3,000
30	67,618	10,032	7,907	2,243	2,112
B.			c.p.m./gm F.W.		
0	179,087	28,237	26,080	3,505	4,454
3	153,989	17,804	29,057	4,034	6,320
10	145,070	30,753	32,261	5,722	6,330
30	123,340	21,902	17,264	4,897	4,612

b. Effect of GA_3 on the inhibition of sterol biosynthesis induced by Amo 1618

In this experiment twenty one-day-old rootless tobacco seedlings were treated with a single concentration of Amo 1618 (500 µg/ml) in the presence of various concentrations of GA_3 ranging from 0 to 30 µg/ml. All of the experimental procedures were exactly as outlined above (Results C.3.a.).

In complete accordance with previous data (Results A and B), the retardant strongly inhibited the incorporation of mevalonate into all of the sterol fractions (in particular the 4-desmethylsterols) and caused a marked accumulation of radioactivity associated with squalene-2,3-epoxide (Table 22). GA_3 at the concentrations tested had no apparent effect on the inhibition of sterol biosynthesis induced by the retardant (Table 22).

c. Effect of GA_3 on sterol biosynthesis in intact tobacco seedlings.

A single application of either 300 µg of Amo 1618, 30 µg of GA_3, or 300 µg of Amo 1618 plus 30 µg of GA_3 was made to the stem apex of twenty one-day-old tobacco seedlings and 0.5 µCi of DL-2-^{14}C-mevalonate was also applied to the apex of each test seedling. Ten seedlings were used for each treatment, including controls to which only ^{14}C-mevalonate and 0.05% (w/v) tween-20 solution were applied. After application of the above test compounds plants were allowed to

Table 22. The effect of GA_3 on the inhibition of sterol biosynthesis induced by Amo 1618-treatment of rootless tobacco seedlings.

(Sterols and sterol intermediates were separated by T.L.C. of the total non-saponifiable lipid extract).

Treatment	Incorporation of L-2-^{14}C-mevalonate				
	4-desmethyl-sterols	4-methyl-sterols	4,4'-dimethyl-sterols	squalene-2,3-epoxide	squalene
A.			c.p.m./gm F.W.		
Control	387,360	54,101	51,457	4,870	18,427
500 μg/ml Amo 1618	103,968	27,563	35,791	192,794	44,121
500 μg/ml Amo 1618 + 1 μg/ml GA_3	105,510	26,225	32,556	196,238	25,234
500 μg/ml Amo 1618 + 3 μg/ml GA_3	102,719	23,979	28,782	209,951	23,381
500 μg/ml Amo 1618 + 10 μg/ml GA_3	116,995	28,280	33,489	253,003	25,923
500 μg/ml Amo 1618 + 30 μg/ml GA_3	122,827	24,761	29,172	154,897	18,709
B.			c.p.m.		
Control	175,086	24,453	23,258	2,201	8,329
500 μg/ml Amo 1618	48,000	12,982	16,857	90,805	20,780
500 μg/ml Amo 1618 + 1 μg/ml GA_3	48,534	12,063	14,975	90,269	11,607
500 μg/ml Amo 1618 + 3 μg/ml GA_3	44,682	10,430	12,520	91,328	10,170
500 μg/ml Amo 1618 + 10 μg/ml GA_3	42,586	10,293	12,189	92,093	9,435
500 μg/ml Amo 1618 + 30 μg/ml GA_3	56,500	11,390	13,419	71,252	8,606

stand in growth cabinets for 24 hr under conditions of high light intensity and constant temperature (Methods 1). After the illumination period the seedlings were divided up into leaf, stem and root tissues, each tissue fraction was weighed and sterols were extracted from the tissues [Methods 7.a.(ii).] and examined by T.L.C. (Methods 8.a.).

Sterol esters were separated from squalene-2,3-epoxide as previously described (Results B.5.).

In leaf tissue the incorporation of mevalonate into 4-desmethylsterols was inhibited by GA_3 by 61.7% (Table 23A), whereas Amo 1618 application decreased the mevalonate incorporation by 81.8%. GA_3 also inhibited the incorporation of precursor into sterol esters (85.6%) and Amo 1618 reduced incorporation into sterol esters to less than 0.2% of control. Incorporation of mevalonate into all of the sterols and sterol intermediates was reduced by GA_3 application but perhaps the greatest percentage inhibition was into squalene (73.5%).

In stem tissues a slightly different pattern emerged. Although GA_3 inhibited mevalonate incorporation into 4-desmethylsterols (61.9%) equally as well in stems (Table 23B) as it did in leaves, the radioactivity associated with sterol intermediates from squalene through to 4-methylsterols showed a slight increase over controls. It would appear that GA_3 exerts different effects on sterol biosynthesis in stems to that in leaves. Sterol ester formation in stems was only inhibited by 25.8%.

Table 23. The effect of GA_3 and Amo 1618 on the incorporation of mevalonate into sterols and their precursors in intact tobacco seedlings.

Sterols and sterol intermediates were extracted from leaf, stem and root tissues of treated intact tobacco seedlings and separated by T.L.C. of the total un-saponifiable lipid extract.

Tissue	Incorporation Product	Incorporation of L-2-^{14}C-mevalonate			
		Control	30 µg GA_3	300 µg Amo 1618	30 µg GA_3 + 300 µg Amo 1618
A.		c.p.m.			
Leaf	4-desmethylsterols	551,043	211,207	100,375	120,480
	4-methylsterols	15,785	9,259	8,624	5,957
	4,4'-dimethylsterols	12,105	6,470	9,938	4,650
	squalene-2,3-epoxide	2,125	1,656	4,013	1,660
	squalene	24,427	6,471	8,018	4,633
	sterol esters	28,510	4,112	59	34

B.	4-desmethylsterols	102,423	39,010	14,309	23,027
	4-methylsterols	5,875	7,309	2,492	3,173
	4,4'-dimethylsterols	5,224	6,487	2,664	4,681
Stem	squalene-2,3-epoxide	1,129	1,372	2,870	2,294
	squalene	3,040	4,239	1,026	2,227
	sterol esters	4,072	3,023	N.D.	123
C.	4-desmethylsterols	399	305	242	372
	4-methylsterols	32	59	97	22
	4,4'-dimethylsterols	37	67	114	32
Root	squalene-2,3-epoxide	37	37	88	6
	squalene	0	0	57	22
	sterol esters	N.D.	N.D.	N.D.	N.D.
D.	4-desmethylsterols	653,865	250,522	114,926	143,879
	4-methylsterols	21,692	16,627	11,213	9,152
	4,4'-dimethylsterols	17,366	13,024	12,716	9,363
Total	squalene-2,3-epoxide	3,291	3,065	6,971	3,960
	squalene	27,467	10,710	9,101	6,882
	sterol esters	32,582	7,135	59	157

N.D. = non detected

The levels of incorporation into sterols and sterol intermediates in roots (Table 23C) were very low (as in previous experiments) and it is therefore not possible to draw any firm conclusions from this data.

GA_3 only partially reversed the inhibitory effect of Amo 1618 on 4-desmethylsterol biosynthesis in whole seedlings (Table 23D) and this reversal of the retardant effect was more pronounced in stems than in leaves (Table 23). This partial reversal by GA_3 was only observed for the 4-desmethylsterol fraction in leaves, however, as incorporation into all other sterol and hydrocarbon fractions was depressed to below Amo 1618 levels in the presence of the hormone; only in stems was a partial GA_3-induced reversal of Amo 1618 inhibition evident in all of the sterol and sterol intermediate fractions (Table 23). It should be noted that these data are not completely comparable with the growth data of Figure 53 since the Amo 1618 level in this experiment was three times greater (300 μg vs. 100 μg/plant).

4. Discussion

a. Growth studies

It must be pointed out initially that a greater-than-desirable level of variability exists in these experiments. In the experiments with CCC (figure 46) and Phosfon D (figure 49) GA_3 alone caused a significant increase in leaf length, but in the Amo 1618 experiment (figure 43) it had no effect on leaf length. Again, in the CCC experiment (figure 47), GA_3 had no effect on leaf width but a significant increase in this parameter was recorded in the experiments with Amo 1618 (figure 44) and Phosfon D (figure 50). Finally, there appeared to be a difference between these three experiments in the magnitude of stimulation of stem growth by GA_3 (figures 45, 48 and 51). Overall, however, the data suggests that multiple applications of GA_3 may lead to a small but significant increase in leaf growth (in particular leaf length) and a more pronounced (but variable) stimulation of stem growth of intact tobacco seedlings. A single application of the hormone was able to stimulate only stem elongation and not leaf growth.

The three retardants (Amo 1618, CCC and Phosfon D) demonstrated the ability to inhibit stem elongation whether applied repeatedly in small doses or singly as one larger dose, and GA_3 was able to completely reverse the effect induced by the retardants. Multiple application of retardants led to a reduction in the rates of growth of both parameters of leaf growth (viz. length and width) and, with one exception (GA_3 and

10 μg Amo 1618 effects on leaf width) GA_3 was ineffective in reversing the retardant-induced reductions in leaf growth. In one, apparently anomalous, treatment GA_3 actually enhanced the inhibition of leaf length induced by 25 μg of CCC. Taken as a whole, the data suggests that leaf growth and stem growth are controlled differently.

The interaction between GA_3 and CCC differed from that between GA_3 and Amo 1618, or GA_3 and Phosfon D on stem growth. Multiple applications of GA_3 were able to reverse the effects of low levels of Amo 1618 (figure 45), and partially reverse the effects of Phosfon D (figure 51) but had little effect on the retardation of stem growth induced by CCC (figure 48). With single applications, GA_3 did not produce the same magnitude of stimulation of stem elongation in the presence of CCC (figure 55) as it did in the presence of Amo 1618 (figure 53) or Phosfon D (figure 57) which, by themselves, were able to induce a much greater reduction in stem growth than CCC. The results reinforce those of the previous section (Results B) which indicated that CCC acts on tobacco seedling growth in a different manner than Amo 1618 and Phosfon D.

Finally, the often reported ability of GA_3 to cause stem growth in excess of untreated controls, and even to cause greater-than-control stem growth in plants treated with a single application of retardants, is closely demonstrated in these experiments also. In view of the fact that application of exogeneous sterols to retardant-treated plants can also restore, but not exceed control rates of stem growth (Results

B.4.), it is tempting to question the role of gibberellins in the control of "normal" vegetative stem growth. In this connection it is of interest that the involvement of gibberellins as controlling agents in the growth of dwarf species of plants has also recently been questioned (Jones, 1973).

b. Sterol biosynthesis

Two experiments were carried out in which GA_3 was applied to rootless seedlings, either alone (Table 21) or in conjugation with Amo 1618 (Table 22). In neither case was there evidence of a significant response to the hormone (although in Table 21, when applied alone, there was a very slight indication of a concentration-dependant inhibition of mevalonate incorporation into 4-desmethylsterols when calculated on a per gram fresh weight basis).

When applied to intact seedlings, however, GA_3 caused a considerable decrease (on both an absolute and a per gram fresh weight basis) of mevalonate incorporation into all the sterol and sterol precursor fractions (Table 23). The difference in GA_3 action on sterol biosynthesis in rootless and intact seedlings is unclear, particularly since three factors (viz. light intensity, method of application of the hormone, and the presence or absence of roots) differed in the two types of experiments. Unfortunately it has not been possible to explore these factors further.

The pronounced inhibition of exogeneous radioactive mevalonate incorporation into sterols, resulting from GA_3 treatment of intact seedlings, seems likely to be due to isotope dilution. The mobilization of non-radioactive stored reserves (carbohydrates) would lead to a greater availability of precursors, such as acetyl CoA and isopentenyl pyrophosphate, for sterol biosynthesis. This possibility is not contradicted by an examination of total 4-desmethylsterol levels of GA_3-treated seedlings. Although the data are not presented, there was no appreciable reduction in the sterol level in spite of the reduction in mevalonate incorporation and the apparently reduced rate of sterol biosynthesis. GA_3 has been shown to increase reducing sugar and decrease starch levels in green tobacco leaves (Lee and Rosa, 1969). Such a mechanism was proposed by Paleg (1965) to possibly play a significant role in the control of elongation growth.

When GA_3 and Amo 1618 were supplied together to intact seedlings there was no consistent effect of GA_3 on the inhibition of mevalonate incorporation produced by Amo 1618 (Table 23). The combined effects of both compounds were neither additive nor synergistic on the one hand, and, on the other, there was no reliable evidence of a GA_3-induced reversal of the retardant's action. This suggests that GA_3 and Amo 1618 produce their effects on apparent sterol biosynthesis in different ways.

A comparison of the effect of GA_3 on mevalonate incorporation into 4-desmethylsterols in leaf and stem tissues did produce one

interesting indication. Although the hormone inhibits incorporation into 4-desmethylsterols in both tissues, it does not have the same effect on the other sterol fractions. In the leaf incorporation is reduced, while incorporation into other sterol fractions in the stem is not influenced. The data indicate that, although the pathway of biosynthesis in both tissues may be the same, the controls operating on the pathway in both tissues are likely to be different.

Contrary to the results with the effects of retardants on sterol biosynthesis and growth, from which a simple direct correlation was obtained, no correlation between GA_3 effects on sterol biosynthesis and growth were demonstrated in these experiments. Although it was suggested above that GA_3 was likely to increase the availability of endogeneous non-radioactive isoprenoid precursors, and thus lead to isotopic dilution and an apparent inhibition of sterol biosynthesis, at least two other explanations are possible. GA_3 may increase the further metabolism of sterols, thus lowering apparent biosynthesis of those fractions examined. Many different steroids, derived from the 4-desmethylsterols, exist in plants (Heftmann, 1971; Knights, 1973) and if any of them possess hormonal activity of their own, the relationship between the effects of GA_3 on growth and sterol biosynthesis would be even more difficult to unravel. Finally, GA_3 could alter the sterol spectrum. For example, in stem tissues of tobacco it was noted that the decreased incorporation into 4-desmethylsterols was accompanied by an increased incorporation into other sterol fractions (in particular the 4-methylsterols). In germinating hazel seeds it was shown that

GA_3 similarly influenced the incorporation of 2-^{14}C-MVA into various sterol fractions (Shewry and Stobart, 1974). Such changes in sterol spectrum may be related to changes in the permeability of constituent membranes and thus GA_3 could exert its effects on growth by inducing sterol changes which affect the permeability of specific membranes in the plant cell.

IV. GENERAL DISCUSSION

The tobacco seedlings used in these studies responded to retardant treatment in the "classical" manner, i.e. stem elongation was retarded by each of the retardants tested and the degree of retardation was concentration dependant. Leaf growth was much less affected by the retardants and only multiple application of the retardants led to significant reductions in leaf growth. Exogeneous GA_3 was able to completely reverse the effects of the retardants on stem growth. These results suggest that tobacco seedlings are an excellent system in which to study the modes, and mechanisms, of action of the plant growth retardants.

Marth et al. (1953) showed that N. tabacum plant growth was retarded by spray application of Amo 1618 whereas Cathey and Stuart (1961) report that Amo 1618 caused no growth retardation in N. tabacum when applied as a soil amendment. In the work presented in this Thesis all of the retardants were applied as single drops of a solution to the stem apex and Amo 1618 was effective in reducing stem elongation of the N. tabacum (cv. Turkish Samson) seedlings used in these studies. The method of application of retardants seems important, therefore, in assessing the effectiveness of retardants on plants of the same species.

Amo 1618, Phosfon D, and CCC all inhibited sterol biosynthesis in tobacco seedlings and tobacco seedling preparations in much the same way as reported for rat liver preparations (Paleg and Seamark, 1968; Paleg, 1970a; Paleg, 1970b). Amo 1618 and Phosfon D were both potent

inhibitors of sterol biosynthesis in tobacco and in rat liver preparations. CCC was an effective, although less potent, inhibitor of sterol biosynthesis in tobacco and was also a poor inhibitor of cholesterol biosynthesis in rat liver homogenates (Paleg and Seamark, 1968; Paleg, 1970a). A similarity between the sites of action of the retardants Amo 1618 and Phosfon D was also apparent in both systems. Amo 1618 inhibited sterol biosynthesis at both pre-mevalonate and post-mevalonate sites in tobacco seedlings and also in rat liver preparations (Paleg, 1970b). An identical post-mevalonate site of inhibition by Amo 1618 has been demonstrated in rootless tobacco seedlings (Douglas and Paleg, 1972) and rat liver homogenates (Paleg, unpublished data), viz. the cyclization of squalene-2,3-epoxide. Inhibition of squalene-2,3-epoxide cyclase activity by Amo 1618 appeared to occur in intact tobacco seedlings, rootless tobacco and digitalis seedlings, leaf, stem, and root tissues of intact tobacco seedlings, and in cell-free preparations from intact tobacco seedlings. Phosfon D apparently inhibited the demethylation reactions involved in the conversion of lanosterol to cholesterol in rat livers and in the conversion of 4,4'-dimethylsterols to 4-desmethylsterols in tobacco seedlings.

Taken as a whole, the data suggest that the retardants inhibit sterol biosynthesis in tobacco seedlings in essentially the same manner as they do in rat liver homogenates. Further, the implications from this finding are that, by analogy, other retardants reported to inhibit cholesterol biosynthesis in rat liver preparations may also inhibit sterol biosynthesis in tobacco seedlings and, in the same way, the

plant growth retardants therefore, may be described as general inhibitors of sterol biosynthesis.

It should be made clear that throughout this Thesis the sterol and sterol precursor fractions examined were only tentatively identified by co-chromatography with authentic standards on T.L.C. and, at best, G.L.C. For this reason, the work presented here represents only a preliminary investigation of the effects of the plant growth retardants on sterol biosynthesis in higher plants. A more thorough examination involving positive identification of the various sterol compounds would be necessary to acquire a detailed understanding of the mechanisms of action of the retardants as inhibitors of sterol biosynthesis. Nevertheless, the data shows quite clearly that Amo 1618 at least acts as an inhibitor of sterol biosynthesis in tobacco seedlings even at the sub-cellular level.

The proposed site(s) of inhibition of sterol biosynthesis of the retardants Amo 1618, Phosfon D, and CCC in tobacco seedlings are as follows*:

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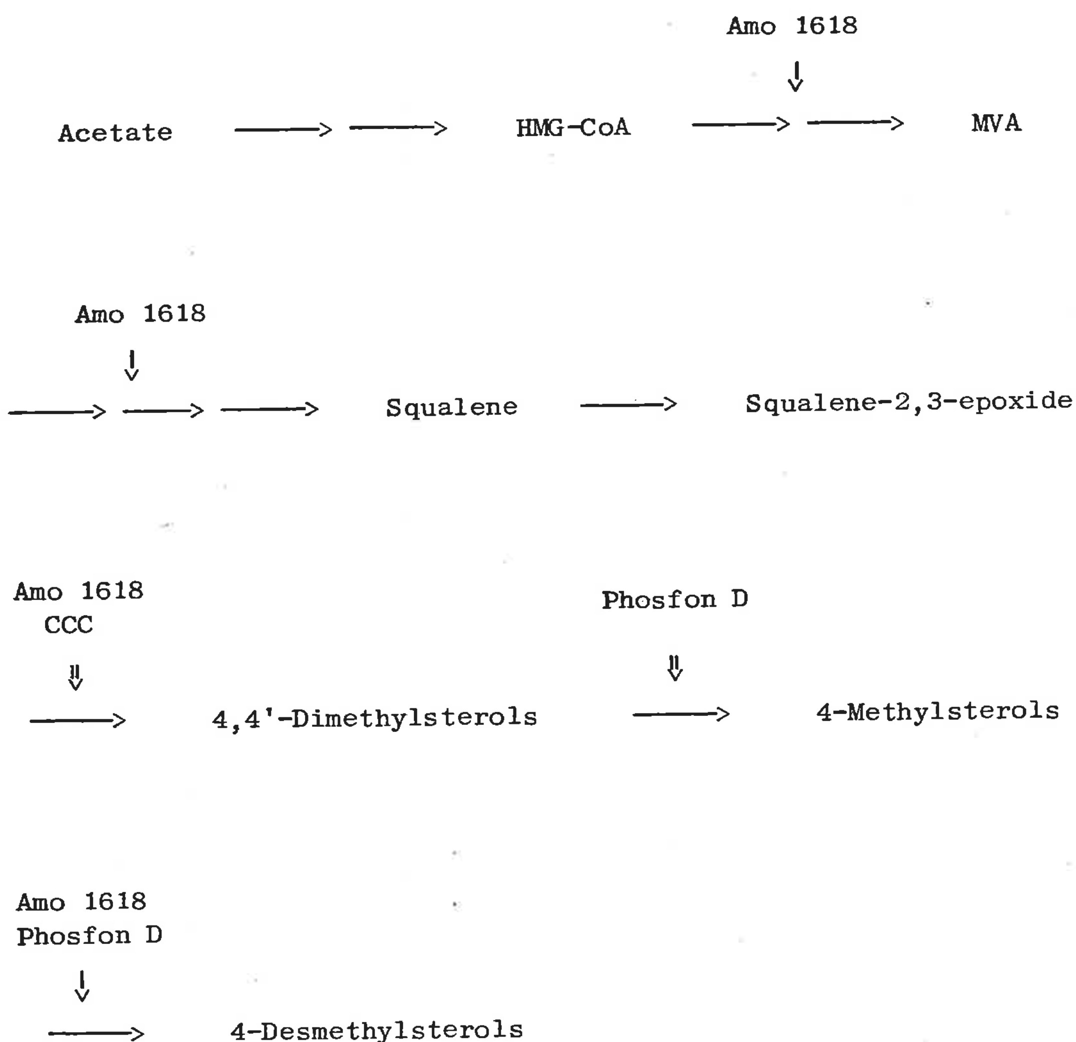
* Major site(s) of inhibition (⇓) and other site(s) of inhibition (↓) observed in tobacco seedlings are denoted.



The same order of potency of the three retardants (viz. Phosfon D > Amo 1618 > CCC) observed in tobacco sterol biosynthesis has also been reported for the effects of these retardants on (-)-kaurene biosynthesis in E. macrocarpa endosperm preparations (Dennis et al., 1965). It would appear, therefore, that the retardants may exert similar inhibitory effects on sterol biosynthesis and gibberellin biosynthesis.

Thus it would be very difficult, at this time, to attribute the effects of the retardants on growth of higher plants to an inhibition of only one of these two pathways. Further, if, as the data presented here and in the literature suggests, the retardants inhibit both gibberellin and sterol biosynthesis, it remains to be seen whether it is the reduced levels of sterols or gibberellins which brings about the primary effects on growth. The fact that sterol application to retardant-treated plants completely reversed the retardant-induced inhibition of stem elongation certainly suggests that the retardants are not specific inhibitors of gibberellin biosynthesis and supports the conclusion that the retardants inhibit sterol biosynthesis in higher plants. One interesting observation from this work was that CCC appeared to be a more potent inhibitor of sterol biosynthesis in tobacco seedlings than of (-)-kaurene (and thus presumably gibberellin) biosynthesis in other plants. The same order of inhibition of 2-^{14}C-MVA incorporation into 4-desmethylsterols of tobacco seedlings was observed for 3 μg/ml Amo 1618 and 50 μg/ml CCC. In cell-free extracts of pea seeds almost 1,000-fold greater concentrations of CCC were required to produce the same order of inhibition of (-)-kaurene synthesis from 2-^{14}C-MVA as Amo 1618 (Anderson and Moore, 1967); and in E. macrocarpa endosperm preparations, CCC was reported to have no inhibitory effect on (-)-kaurene biosynthesis (Dennis et al., 1965).

The accumulation products (viz. squalene-2,3-epoxide, 4,4'-dimethylsterols, 4-methylsterols etc.) resulting from an inhibition of sterol biosynthesis by the retardants could, themselves, play a part

in retardant-induced growth inhibition. For example, it is possible that they may compete with the sterols (4-desmethylsterols) for active sites within the cell thereby resulting in, for example, membranes which have reduced structural and/or functional capacity. Alternatively such intermediates may be shunted into the production of compounds such as glycosidic sterols and glycosidic steryl esters which could have intrinsic effects on properties such as membrane permeability.

During the treatment of tobacco seedlings with very high levels of CCC it was noted that the retardant caused chlorosis of leaf tissue as well as pronounced growth retardation. This observation could be explained by an inhibition of chlorophyll biosynthesis by the retardant which has been reported to occur in other plants (Knypl, 1969; Knypl and Chylinska, 1972). The fact that Amo 1618 and Phosfon D did not induce chlorotic lesions at relatively high levels might suggest that these retardants are not inhibitors of chlorophyll biosynthesis in tobacco seedlings and that their sites of action may be more specifically associated with certain other isoprenoid biosynthetic pathways.

In recent years it has become increasingly evident that plant sterols can no longer be thought of as secondary metabolites. As pointed out by Heftmann (1971), work on the biosynthesis and metabolism of steroids suggests that the sterols and steroids which are common to both plants and animals have the same metabolic fate in these organisms. If this is so then one would expect that the same classes of steroid compounds produced by plant and animal systems might also serve similar

functions. Knights (1973), in an excellent review of sterol metabolism in plants, points out that higher plants contain members of all the steroid classes (except apparently the bile acids) and presents convincing evidence that the metabolism of plant sterol fractions demonstrates selectivity toward one or more components of a complex mixture. It would appear, therefore, that sterol metabolism in plants is a non-random process and that the amounts and distribution of individual sterols is of functional significance.

Sterols or their intermediates have been shown, principally by Heftmann and co-workers, to be the starting materials for the biosynthesis of many other steroids (Heftmann, 1971). It is evident, therefore, that one of the roles of sterols in plants must be as precursors of other steroids. Secondly, sterols appear to be a requirement for the growth of many microorganisms (Aaronson, 1965; Elliott, 1969) and undoubtedly serve the same role in higher plants. In this context it is interesting to note that, as with tobacco seedlings, the growth of the bacterium Rhodopseudomonas palustris (Aaronson, 1964), the fungus Phytophthora (Elliott, 1969), and the green alga Chlorella sorokiniana (Chan et al., 1974) is inhibited by sterol biosynthesis inhibitors (e.g. triparanol, and the specific animal steroid biosynthesis inhibitors) and sterol application to the inhibitor-treated organisms completely reversed the growth inhibition. Further, the application of sterols to bacteria, algae, protozoa, and fungi (Aaronson, 1965) showed low structural specificity, similar to the ability of several different sterols to reverse the Amo 1618-induced

retardation of tobacco stem growth (Results B.4.c.). One plausible explanation for this observation in tobacco comes from the fact that interconversion of the sterols occurs in _Nicotiana_ (Tso and Cheng, 1971) and _Digitalis_ (Bennett and Heftmann, 1969), and thus, if a specific sterol were required to reverse the growth inhibition induced by retardants it could most probably be produced as a result of metabolism of the applied sterol. Hendrix _et al_. (1970) propose, similarly, that the phytopathogenic fungi _Pythium_ and _Phytophthora_ could metabolise applied sterols to a common active steroid. In the present experiments, although β-sitosterol (and also cholesterol and stigmasterol) application reversed stem growth inhibition induced by both Amo 1618 and CCC, it was unable to reverse the effect of Phosfon D. In this case, therefore, interconversion of sterols does not appear to supply the answer to reversal of Phosfon D-induced growth inhibition by a specific sterol. It was noted, however, that Phosfon D inhibited sterol biosynthesis later in the sequence than did Amo 1618 or CCC, resulting in an accumulation of both 4,4'-dimethyl- and 4-methyl-sterols. These intermediate sterols could possibly compete with applied sterols for active sites within the cell and thus greatly reduce the possibility of a reversal of growth inhibition by sterol application. A similar finding has been demonstrated in the fungus _Phytophthora_ (Elliott and Knights, 1969). The fungus required sterols, when growing on minimal medium, for the production of oospores and maximal growth rate and sterol precursors effectively inhibited growth and oospore production. The authors concluded that the relative

amounts of available sterols and sterol precursors determines the growth rate and oospore production in Phytophthora.

Heftmann (1971) proposes that sterols may play a hormonal role in plants although this role has not as yet been satisfactorily explored. Helmkamp and Bonner (1953) showed that several of the well-known plant sterols (β-sitosterol, stigmasterol, cholesterol) were without growth-promoting effect when tested on pea embryos and only one of the many steroids tested, estrone, was able to significantly increase the growth of pea embryos. Further, the role of steroids in the flowering of plants remains one of conjecture. None of the sterols used in growth studies on tobacco seedlings (this Thesis) were able to promote the growth of seedlings to above that of controls although some slight increase was noted in stem height of dwarf pea and dwarf bean seedlings (Results B.4.d.). It appears, therefore, that the role of sterols as plant hormones awaits proper and full investigation.

Another function of sterols common to plants, animals and, at least, some fungi, is as membrane components. Sterols have been shown to be largely associated with the membraneous organelles of plant cells (Grunwald, 1970; Brandt and Benveniste, 1972) and to influence the permeability of membranes of red beet (Grunwald, 1968) and barley root (Grunwald, 1971) tissues. Thus, sterols not only play a part in controlling the structural integrity and permeability of plant membranes but, by virtue of their occurrence in most, if not all, plant membranes, must also influence protein synthesis, charge separation,

and active uptake of components by membranes. Inhibition of sterol biosynthesis by the retardants could lead to a reduction in the sterol content of membranes which could, in turn, lead to impaired membrane function and thus, reduced growth.

Sterol biosynthesis has been shown to occur in leaf (Bennett et al., 1963; Goad and Goodwin, 1967; Alcaide et al., 1969), stem (Aexel et al., 1967; Knapp et al., 1969) and root (Kemp et al., 1968; Staby and De Hertough, 1969; Hartmann et al., 1972) tissues of several higher plants and an inhibition of sterol biosynthesis by retardants could therefore explain why growth of each of these tissues has been reported to be inhibited by the retardants. Of particular interest, therefore, is the finding that the retardant Amo 1618 was most potent as an inhibitor of sterol biosynthesis in stems of tobacco seedlings as the stem is the tissue which is most responsive to retardant-induced growth inhibition (see also Introduction).

The relationship between the effects of plant growth retardants and the biosynthesis and role of gibberellins during growth, remains unclear. If the primary action of the retardants is the inhibition of 4-desmethylsterol biosynthesis, there are several ways in which exogeneous GA_3 might cause an apparent reversal of retardant-induced stem growth inhibition without apparently reversing the effect of the retardant on sterol biosynthesis. The hormone might be incorporated into membranes at the site(s) normally occupied by sterols or might decrease the rate of turnover of sterols in existing membranes. It

might increase the availability of alternative sterol precursors (e.g. carbohydrates) so that, although biosynthetic enzyme activity is reduced by retardants, total sterol biosynthesis is normal or even enhanced. Such an effect could be occasioned by increased hydrolytic activity or increased membrane permeability. The former possibility (i.e. the increased mobilization of stored reserves for sterol biosynthesis) is supported to some extent by the data presented in Results C but the incomplete nature of the work does not allow a definitive answer to this problem.

Finally, the frequently reported (Baldev et al., 1965; Reid and Carr, 1967; Reid et al., 1968; Cleland and Zeevaart, 1970, etc.) ability of retardants to depress endogeneous gibberellin levels in plants could be explained by postulating that gibberellins (like sterols) are synthesized on membranes. Particulate fractions have been shown to be responsible for the further oxidation of (-)-kaurene (West et al., 1968; Murphy and West, 1969; Lew and West, 1971), and any impairment of membrane structure and/or function could be reflected in decreased gibberellin levels, concomitant with, but not necessarily causally related to decreased growth.

The data presented in this Thesis does not make it possible to (nor is it meant to) distinguish between the effects of retardants on gibberellin biosynthesis and sterol biosynthesis as being the primary action which leads to growth retardation. Rather, it presents for the first time, a plausible alternative to the effects of retardants

on growth being mediated only through an inhibition of gibberellin biosynthesis and offers a new approach to the way in which retardants exert their effects on plant growth.

In conclusion, it is postulated that, the plant growth retardants are inhibitors of sterol biosynthesis in higher plants and, at least part, if not most of the retardant-induced reduction of stem growth can be accounted for by an inhibition of sterol production.

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