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Inhibition of Sterol Biosynthesis by 2-Isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine Carboxylate Methyl Chloride in Tobacco and Rat Liver Preparations

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ABSTRACT

2-Isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methyl chloride, 90%, applied to rootless tobacco (*Nicotiana tabacum* cv. Samson) seedlings inhibits the incorporation of ^{14}C-mevalonate into sterols. Concomitantly, the retardant causes the accumulation of squalene-2,3-epoxide, an intermediate in sterol biosynthesis. The results with tobacco are identical to those produced by the retardant in cell-free rat liver preparations.

The physiological manifestations and potential economic advantages of plant growth retardants have created much interest in explanations of their mechanism(s) of action. Although retardant effects have been explored in such diverse systems as fungi (5, 8), higher plants (4, 7), insects (3, 18), and mammals (9, 11), no one set of results satisfactorily accounts for the great diversity of organismic responses.

When a group of compounds produces different results in different systems, it is important to assess carefully whether the effects recorded with one species are in any way pertinent to those recorded for other species. This paper presents evidence which strongly supports the contention that at least one retardant effect observed in cell-free rat liver systems has direct relevance to the effects of the retardant on higher plants. It is also the first report of the inhibition of sterol biosynthesis in plants by a growth retardant.

MATERIALS AND METHODS

Plant Tissue. *Nicotiana tabacum* (cv. Samson) seeds were germinated in potting compost and grown in a glasshouse at 18 to 24 C. Immediately before an experiment, when the seedlings were 2 to 3 cm in length (usually 3–4 weeks after germination), they were removed, thoroughly washed, and the roots were excised. A number of rootless seedlings (totaling about 300 mg in weight) were placed in a solution of 5 μc DL-2-^{14}C-mevalonic acid lactone in 11 mM phosphate buffer, pH 6.5, in 5-ml Petri dishes. Amo 1618,[1] at a final concentration of 1 mg/ml, was added to one set of Petri dishes, and the pH was readjusted to 6.5. Final volume in all treatments was 2 ml. The seedlings were placed around the edge of the dishes so that only the cut stems were immersed in the solution. The Petri dishes containing the seedlings were placed in a closed, clear plastic container and left under constant illumination (230 ft-c) for 24 hr at a constant temperature of 18 C.

Extraction. After incubation, the seedlings were removed with forceps and homogenized in three successive 5-ml portions of ethanol-benzene (4:1), with an Ultra-turrax blender. The combined extracts were filtered through Whatman No. 1 filter paper and reduced to dryness *in vacuo* in a rotary evaporator.

The residue was taken up in 5 ml of water, and the lipids were extracted with diethyl ether (4 × 5 ml). The combined ether extracts were washed with water (2 × 5 ml), and the ether fraction was evaporated *in vacuo*. The lipid residue was redissolved in 20 ml of hexane and extracted with 80% (v/v) methanol (3 × 20 ml) to remove acidic and polar lipids. The hexane was then removed *in vacuo*, and the residue was saponified under reflux with 5% (w/v) KOH in 85% (v/v) methanol (5 ml) for 60 min. All nonsaponifiable lipid material was then extracted with diethyl ether (3 × 10 ml), and the combined extracts were washed with water (5 ml). The ether was removed *in vacuo*, and the lipid residue was dissolved in benzene.

Thin Layer Chromatography. Nonsaponifiable lipid fractions were spotted on thin layer plates in an atmosphere of dry nitrogen. An aliquot of the benzene solution was spotted on a Silica Gel G plate (20 cm × 5 cm × 0.3 mm) and developed twice in 4% diethyl ether in methylene chloride. Cholesterol, lanosterol, squalene-2,3-epoxide, and squalene standards were spotted and developed simultaneously on the same plates and their R_F values determined after I_2-vapor staining.

After developing the remainder of the benzene solution in the same system, the plates were scanned (Nuclear-Chicago Actigraph II scanner, Model 8415) to determine the location of radioactive incorporation products. All radioactive material in the squalene-2,3-epoxide region was recovered from the thin layer plates by scraping and eluting the silica gel with methylene chloride and, after concentrating, the radioactive material was respotted on a Silica Gel G plate and developed in 5% ethyl acetate in hexane. The plate was then scanned for radioactivity and the squalene-2,3-epoxide standard located by I_2-vapor staining.

Gas-Liquid Chromatography. An aliquot of the radioactive compound which cochromatographed with authentic squalene-2,3-epoxide in both thin layer solvent systems was injected into a gas-liquid chromatography column, and the eluate was col-

[1] Abbreviations: Amo 1618: 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methyl chloride, 90%; CCC: 2-chloroethyl-trimethyl ammonium chloride; SK & F 7997: tris(2-diethylaminoethyl)phosphate hydrochloride.

lected as fractions in anthracene-packed collection tubes. Gas-liquid chromatography was carried out on a Perkin-Elmer Model 811 gas chromatograph fitted with a 2-m × 0.4-cm column packed with 2% SE-30 on Anakrom ABS (80/90 mesh: flux-calcined diatomaceous earth, supplied by Analabs Inc., Hamden, Conn.). The chromatograph was also fitted with a stream splitter and a fraction collector.

The fractions collected on anthracene were placed into scintillation vials, and their radioactivity was determined with a Packard Tricarb liquid scintillation spectrometer Model 3320.

Digitonin Precipitation. In some cases the nonsaponifiable lipid fraction was taken to dryness *in vacuo*, and the residue was dissolved in 3 ml of acetone-diethyl ether (1:1). 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 at room temperature for 3 hr. 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-diethyl ether (1:1, 10 ml), and twice more with two 10-ml portions of diethyl ether. The digitonin complex 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 rpm for 15 min), decanting the supernatant into round-bottomed flasks.

The solvent was removed *in vacuo*, and the residue was dissolved in 0.5 ml of chloroform-methanol (1:1) and spotted on thin layer chromatography plates. Chromatography was carried out in 5% ethyl acetate in hexane and in 25% ethyl acetate in hexane. Radioactive peaks were located by scanning, and sterol standards, run on the same plates, were located with I_2-vapor. The plates were scraped, in 0.5-cm portions, into liquid scintillation vials, and their radioactivity was determined as indicated above.

Chemicals. DL-2-^{14}C-Mevalonic acid was obtained as the lactone form in benzene from Amersham Radiochemical Centre, London, U.K., and Amo 1618 was obtained from Calbiochem, Los Angeles, California.

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

Values expressed as radioactivity in labeled compounds as percentage of radioactivity of original mevalonate per g fresh wt of tissue.

Incorporation Product	Incorporation of L-2-^{14}C-Mevalonate	
	Control	Amo 1618-treated
	%	
4-Demethyl sterols	29.8	3.5
4-Methyl sterols	16.8	4.3
4,4'-Dimethyl sterols	7.5	5.7
Squalene-2,3-epoxide	2.9	14.7
Squalene	2.6	2.8

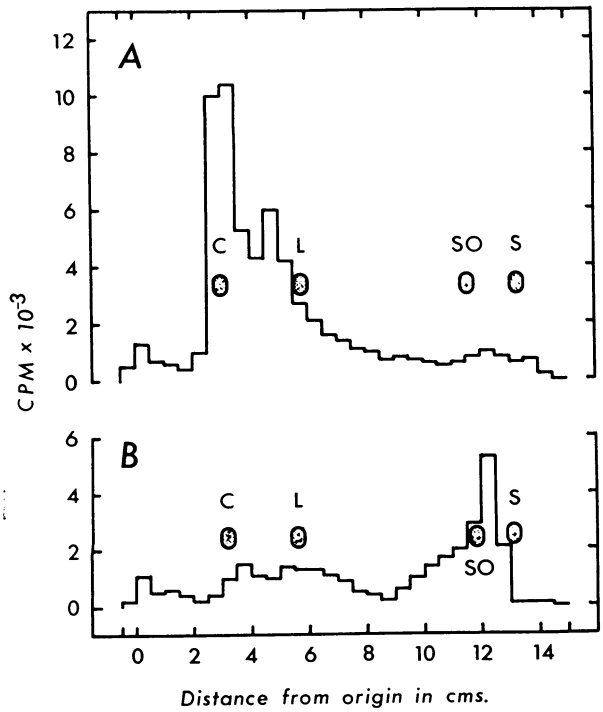

FIG. 1. Distribution of radioactivity on thin-layer chromatograms of non-saponifiable lipid fraction from ^{14}C-mevalonate-fed, A. Control seedlings, and B. Amo 1618-treated seedlings. Developing solvent was 4% diethyl ether in methylene chloride (plates developed twice). C = cholesterol; L = lanosterol; SO = squalene-2,3-epoxide; S = squalene.

RESULTS

Tissue slices and tissue cultures of *Nicotiana tabacum* have been shown previously to incorporate DL-2-^{14}C-mevalonic acid (15) and 2-^{14}C-sodium acetate (2) into triterpenes and sterols. Further, when the mammalian cholesterol synthesis inhibitor SK & F 7997 was administered to tobacco tissue slices, the sterol intermediate, squalene-2,3-epoxide (presumptively identified), was found to accumulate at the expense of sterols; in particular, the demethyl sterols β-sitosterol, stigmasterol, and campesterol were decreased (15, 16).

Similar results are illustrated in Table I, in which the percentage incorporation of the L form of ^{14}C-mevalonate into hydrocarbon triterpenes and sterols is indicated. The compounds were recovered (as indicated above) as nonsaponifiable lipids from the excised *Nicotiana tabacum* seedlings incubated in the presence and absence of the plant growth retardant, Amo 1618. The data indicate marked inhibition of the synthesis of sterol fractions (*e.g.*, more than 8-fold decrease in 4-demethyl sterols) and a 5-fold increase in incorporation into squalene-2,3-epoxide in the presence of 1 mg/ml Amo 1618.

The location of radioactivity in the nonsaponifiable lipid fraction from control seedlings, on a thin layer Silica Gel G plate developed twice in the solvent 4% diethyl ether in methylene chloride, is indicated in Figure 1A. The effect of Amo 1618 on the distribution of radioactivity in a similar fraction from retardant-treated seedlings is illustrated in Figure 1B. It is obvious that there is a marked decrease in incorporation of radioactivity into sterols (in particular the demethyl sterols which cochromatograph with the cholesterol standards), and a large increase in incorporation into the hydrocarbon fraction cochromatographing with squalene-2,3-epoxide.

When the radioactive material accumulating at the squalene-2,3-epoxide region of several chromatograms was eluted and rechromatographed in 5% ethyl acetate in hexane (Fig. 2), a single peak was obtained which was located exactly at the epoxide region (as determined with authentic squalene-2,3-epoxide).

Further evidence of the identity of the accumulated inter-

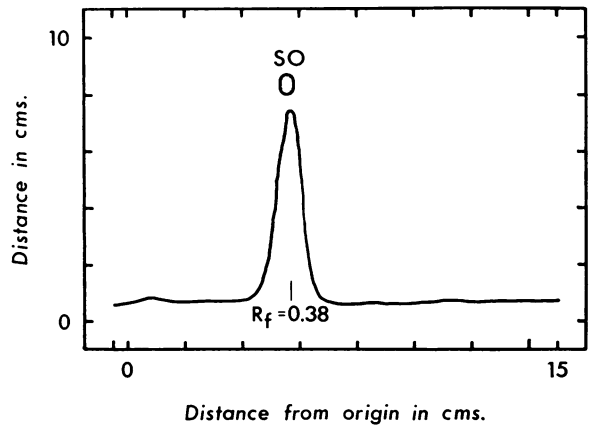

FIG. 2. Scan of distribution of radioactivity from squalene-2,3-epoxide region eluted from thin-layer chromatograms similar to those in Figure 1. Developing solvent was 5% ethyl acetate in hexane.

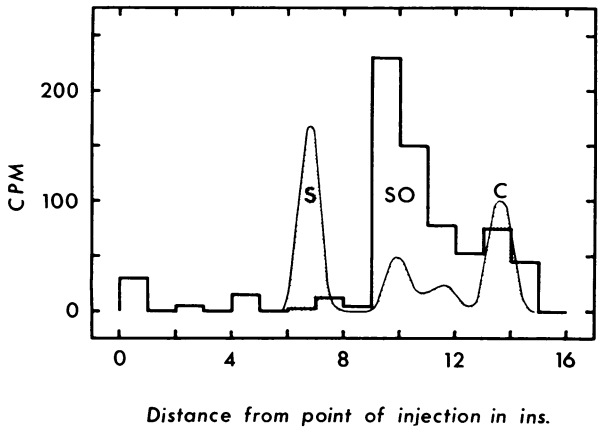

FIG. 3. Distribution of radioactivity in samples collected during gas-liquid chromatography of eluate of squalene-2,3-epoxide region of thin-layer chromatograms from Amo 1618-treated tobacco seedlings. Total counts injected, 5,767 cpm; total counts recovered, 700 cpm (recovery = 12.2%). Rate of chart movement, 24 in/hr. Hatched area represents gas-liquid chromatogram trace.

mediate with squalene-2,3-epoxide was obtained by collecting samples from a gas chromatographic analysis of the radioactive components at the epoxide region on a thin layer chromatogram. The mass peaks for standard squalene, squalene-2,3-epoxide, and cholesterol, and the location of radioactivity, are shown in Figure 3. Approximately 64% of the total recovered radioactivity was associated with the mass peak of genuine squalene-2,3-epoxide.

The nature of the digitonin-precipitable compounds was not explored extensively. However, chromatography of the split digitonide fraction indicated conclusively that Amo 1618 almost completely eliminated incorporation of ^{14}C-mevalonate into sterols which are located at cholesterol to lanosterol regions (Fig. 4). The most marked inhibition (about 99%) occurred in the demethyl sterol region which cochromatographs with cholesterol and β-sitosterol standards. Table II summarizes the effect of Amo 1618 on incorporation of ^{14}C-mevalonate into various sterol fractions.

DISCUSSION

The results above leave little doubt that the plant growth retardant, Amo 1618, causes a marked inhibition of the incorporation of DL-2-^{14}C-mevalonic acid into sterols in the tissues of the tobacco seedling. This conclusion agrees completely with effects of Amo 1618 on mevalonate incorporation into cholesterol by cell-free (10) and slice (12) preparations from rat livers reported earlier.

In addition, Amo 1618 causes the accumulation of a hydrocarbon fraction in tobacco seedlings, which cochromatographs in 2 thin layer and 1 gas-liquid chromatography systems with authentic squalene-2,3-epoxide. This finding is also in complete agreement with unpublished data (Paleg) on the nature of the accumulated Amo 1618-induced intermediate in rat liver preparations. The identity of the accumulated intermediate from cell-free rat liver preparations has been determined by cochromatography in seven thin layer systems, reincubation in the presence and absence of Amo 1618, gas chromatography of natural and acetylated forms, and mass spectrum analysis.

Thus, it has been demonstrated that at least one point of action of the plant growth retardant Amo 1618, in both higher

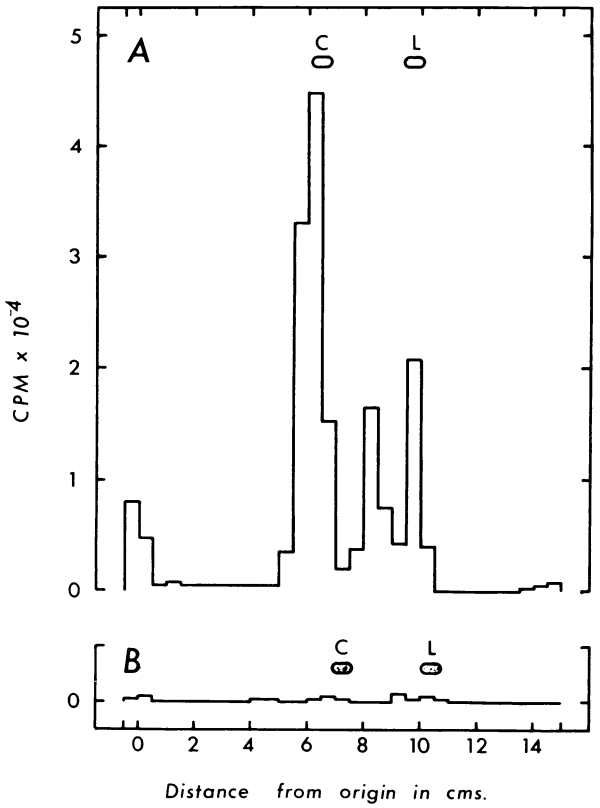

FIG. 4. Distribution of radioactivity on thin layer chromatograms of split digitonide from A. Control seedlings, and B. Amo 1618-treated seedlings fed ^{14}C-mevalonate. Developing solvent was 25% ethyl acetate in hexane.

Table II. *Effect of Amo 1618 on the Incorporation of* DL-2-^{14}C-*Mevalonic Acid into Digitonin-precipitable Sterols of Tobacco Seedlings*

Sterol Fraction	Incorporation		Inhibition by Amo 1618
	Control	Amo 1618-treated	
	cpm/g fresh wt		%
4-Demethyl sterols	152,600	2,350	98.5
4-Methyl sterols	44,000	2,060	95.3
4,4'-Dimethyl sterols	45,500	2,490	94.5

plants and mammals, is at the cyclization step in sterol synthesis at which squalene-2,3-epoxide is converted to cyclized triterpene sterols. It is also of interest to note that Amo 1618 in this work has the same effect as SK & F 7997 on both tobacco seedlings (16) and on cell-free rat liver preparations (10). These results provide a logical explanation for the reports (13, 14) of increases in extractable gibberellins following growth retardant application. Since gibberellins and sterols share a common biosynthetic pathway as far as farnesyl pyrophosphate, the inhibition of the sterol branch would increase the amount of precursor available for the gibberellin branch.

The explanation above leads to the interesting speculation that under some circumstances exogenous gibberellin may be reversing physiological effects due to the growth retardant-induced inhibition of sterol biosynthesis rather than an inhibition of gibberellin biosynthesis. This possibility emphasizes the lack of knowledge concerning both the role of membrane synthesis in growth and the interaction of gibberellin hormones with membrane function.

The cyclization of squalene-2,3-epoxide is the second cyclization reaction in higher plants found to be inhibited by Amo 1618. Shechter and West (17) reported that the cyclization of *trans*-geranyl-geranyl pyrophosphate to copalyl pyrophosphate (both intermediates in the formation of gibberellins) is inhibited, and this suggests that there are similarities in the nature of these two, and possibly other, enzymes that render them sensitive to the action of the retardant.

Finally, a word must be said about a previous report in which it was concluded that sterol biosynthesis in barley was not affected by growth retardant action (1). Barley has been described as one of a number of species that ". . . were so much less responsive than wheat to CCC that they were considered to be non-responsive" (4). Nonetheless, where CCC concentrations are high enough, retardant effects may be observed, even on barley (6). The report (1) demonstrated, at a CCC concentration of 200 μg/l, a 17% inhibition of growth and a 23% inhibition of incorporation of acetate into sitosterol, and at a CCC concentration of 2 g/l, a 47% inhibition of growth and an 83% inhibition of incorporation. It may be concluded that the concentrations of CCC employed and the results obtained support the conclusion that plant growth retardants, in addition to their other suggested potential (10) actions, inhibit sterol biosynthesis in higher plants as they do in cell-free rat liver preparations.

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