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AMO 1618 AND STEROL BIOSYNTHESIS IN TISSUES AND SUB-CELLULAR FRACTIONS OF TOBACCO SEEDLINGS

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Key Word Index—*Nicotiana tabacum*; Solanaceae; AMO-1618; squalene-2,3-epoxide; sterol biosynthesis; gibberellin.

Abstract—Amo 1618 inhibited the incorporation of MVA-[2-^{14}C] into sterols (in particular the 4-desmethylsterols) and promoted its accumulation in squalene-2,-3epoxide in intact seedlings, tissues from treated seedlings, and sub-cellular, membraneous fractions of treated 21-day-old tobacco seedlings. The stem tissues appeared to have a greater sterol requirement (on a per gram fresh weight basis) than leaf tissues and incorporation of radioactivity into stem sterols was more susceptible to inhibition by Amo 1618 than incorporation into leaf tissue.

INTRODUCTION

The mechanism(s) and mode(s) of action of plant growth retardants remain an interesting though unresolved area of investigation. Most workers support the view than Amo 1618, Phosfon, CCC, etc., produce their physiological effects on stem growth through an inhibition of gibberellin biosynthesis [1]. Some other evidence has accumulated, however, suggesting that at least some of the dwarfing syndrome induced by these compounds may be due to an inhibition of sterol biosynthesis [2–4]. In this view, decreased or altered sterol production alters membrane function, possibly affecting protein synthesis and, thus, growth.

Retardant effects on the gross incorporation of radioactive precursors into sterols have been demonstrated in rootless and intact tobacco seedlings [3, 4], but no attempt has been made yet to ascertain the extent of the Amo 1618 effect on different tissues or subcellular fractions. The present work describes the retardant-induced patterns of incorporation of mevalonic acid-[2-^{14}C] (MVA) into sterols and sterol precursors in 21-day-old tobacco seedlings. Leaf, stem and root tissues and subcellular fractions of each were assessed.

RESULTS

When 21-day-old seedlings were treated with 100 μg Amo 1618 in the presence and absence of MVA-[2-^{14}C], the retardant inhibited incorporation into all sterol fractions (particularly 4-desmethylsterols) and caused accumulation of squalene-2,3-epoxide in each of the tissues of the plant (Fig. 1 and Table1). The patterns of incorporation into the various types of compounds in the three tissues are illustrated in Fig. 1 and it is obvious that the gross effect is the same in each of them.

Incorporation of MVA into sterols in the roots was considerably below that of stems and leaves. It is not clear whether the low level of incorporation in the roots was due to low sterol synthesis in this tissue or to the possibility that little MVA-[2-^{14}C] actually reached the roots. In either case the root data in this and subsequent experiments showed the Amo 1618 effect but was generally too low to be reliable; the values exert little influence on the total incorporation patterns.

Incorporation in the control stems in a cpm basis was slightly less than in the leaves, but considerably greater than in the leaves when calculated on a per unit fr. wt basis. Within both the stem and the leaves, accumulation of radioactivity was greatest in the 4-desmethylsterols.

The leaves accounted for *ca* 79% of the total seedling fresh weight and 59% of labelled desmethylsterols, the stem for *ca* 7 and 38% respectively, and the roots *ca* 14 and 3% respectively. Amo 1618 treatment inhibited desmethylsterol biosynthesis more strongly in stems (*ca* 45%) than in leaves (22%) or roots (28%). If an even distribution of MVA and Amo 1618 throughout the stems and leaves is assumed, the data suggest that sterol biosynthesis in stems was more susceptible than in other tissues to inhibition (presumably at the squalene-2,3-epoxide cyclase step) by the retardant.

In both leaves and stems of untreated plants the level of incorporation into squalene-2,3-epoxide was much below the other types of compounds investigated. In the presence of Amo 1618, however, there was a pronounced increase in squalene-2,3-epoxide radioactivity in all tissues, with effects in the stem particularly noteworthy.

In a further attempt to delineate differential effects of the retardant, the 4-desmethylsterol fraction obtained from each tissue was analyzed by GLC (Fig. 2); the values observed with each sterol are presented in Table 2. Similar (though not identical) patterns were obtained for the mass peaks of the four major sterols in each of the tissues; marked differences again showed up between the roots on the one hand, and the stem and leaves on the other hand. In both stems and leaves the major radio-

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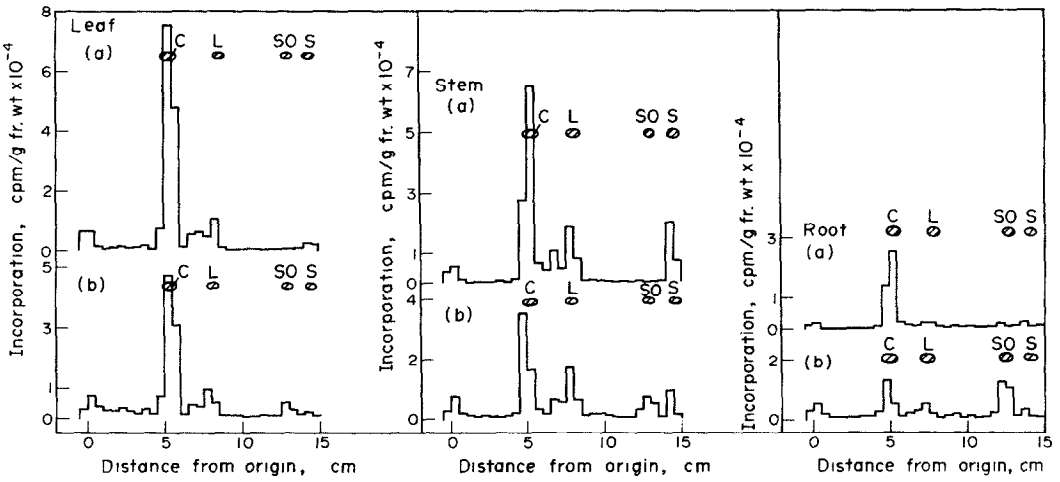

Fig. 1. Incorporation of MVA-[2-^{14}C] into sterols and sterol precursors in leaf, stem and root tissues of 21-day-old tobacco seedlings in the absence (a) and presence (b) of 100 μg Amo 1618 per plant. Radioactivity scan of non-saponifiable lipid extracts separated by TLC on Si gel. C = cholesterol, L = lanosterol, SO = squalene-2,3-epoxide and S = squalene standards.

active sterol was sitosterol, whereas campesterol and stigmasterol shared the greatest portion of radioactive label in roots. Amo 1618 inhibited incorporation into cholesterol in all three tissues, into all sterol in roots, and mainly sitosterol in leaves, and stigmasterol and sitosterol in stems.

When the amount of Amo 1618 applied to each seedling was increased from 100 to 300 μg, the total inhibition of incorporation into sterols almost tripled (Table 3). The effect was most obvious with the 4-desmethyl- and 4-methylsterol fractions. Little effect was observed in the 4,4-dimethylsterol fraction. Squalene-2,3-epoxide accumulated at both concentrations of Amo 1618, but to a somewhat diminished extent at the higher Amo 1618 level. Furthermore, cyclization of squalene-2,3-epoxide was more strongly inhibited in the stems than in the leaves. As with the previous results, the roots were less severely affected (assuming the values are reliable) by Amo 1618 than the stem and leaves, and the stems again exhibited, on a per unit fr. wt basis, considerably

Table 1. Effect of 100 μg Amo 1618/plant on the incorporation of MVA-[2-^{14}C] into sterols and sterol precursors* of leaves, stems and roots of 21-day-old tobacco seedlings

Tissue	Treatment	4-desmethylsterol	4-methylsterol	4,4-dimethylsterol	Squalene-2,3-epoxide	Squalene	Total
				cpm			
	Control	66524	5219	10533	977	3289	86542
Leaves	Amo 1618	51902	4537	8254	2549	1583	68825
	% change	−22.0	−13.2	−21.6	+160.9	−51.9	−20.5
	Control	43449	7681	11648	919	12246	75943
Stems	Amo 1618	24102	6570	9598	5422	6902	52594
	% change	−44.5	−14.5	−17.6	+490.0	−43.6	−30.7
	Control	3320	320	795	153	131	4719
Roots	Amo 1618	2409	247	222	1728	235	4841
	% change	−27.4	−22.8	−72.1	+1029.4	+79.4	+2.6
	Control	113293	13220	22976	2049	15666	167204
Total	Amo 1618	78413	11354	18074	9699	8720	126260
	% change	−30.8	−14.2	−21.3	+373.4	−44.3	−24.5
				cpm/g fr. wt			
	Control	130362	10227	20641	1915	6445	169590
Leaves	Amo 1618	86072	7524	13688	4227	2652	114163
	% change	−34.0	−26.4	−33.7	+120.7	−58.9	−32 7
	Control	1010442	178628	270884	21372	284791	1766117
Stems	Amo 1618	422842	115263	168386	95123	121088	922702
	% change	−58.2	−35.5	−37.8	+345.1	−57.5	−47.8
	Control	36726	3540	8794	1692	1449	52201
Roots	Amo 1618	32554	3338	3000	23351	3176	65419
	% change	−11.4	−5.7	−65.9	+1280.1	+119.2	+25.3
	Control	176003	20538	35694	31833	24377	259755
Total	Amo 1618	106830	15469	24624	13214	11880	172017
	% change	−39.3	−24.7	−30 0	+315	−51.2	−33.8

* Compounds separated by TLC of the total non-saponifiable lipid extract.

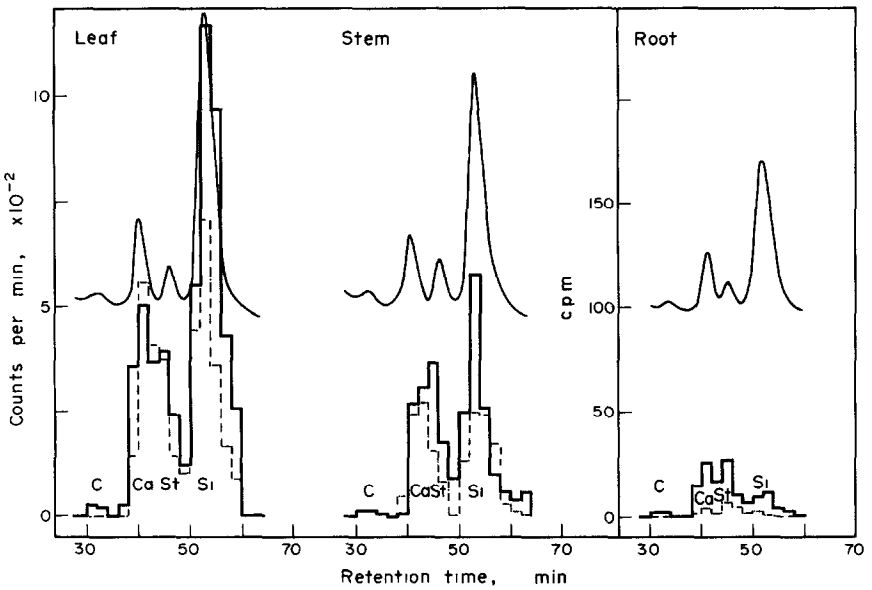

Fig. 2. GLC and radioactivity analyses of 4-desmethylsterol fractions from leaf, stem and root tissue of 21-day-old control and treated (100 μg Amo 1618 per plant) tobacco seedlings. The 4-desmethylsterol fractions eluted from TLC of the nonsaponifiable lipid extracts were injected into a GLC column of 2.5% OV-101. Fractions of the eluates of control (solid lines) and Amo 1618-treated plants (broken lines) were collected and counted. The upper solid line curves show the size and position of the mass peaks of the control extracts. C = cholesterol, Ca = campesterol, St = stigmasterol and Si = sitosterol.

greater evidence of sterol biosynthesis than the leaves (2.3 times) or the roots (50 times). Even after Amo 1618 treatment, the rate of incorporation into stem sterols was 1.7 times greater than into leaf sterols, also suggesting that stem sterol biosynthesis was more severely affected than leaf sterol biosynthesis.

Table 2. Effect of 100 μg Amo 1618/plant on the incorporation of MVA-[2-^{14}C] into 4-desmethylsterols* of leaves, stems and roots of 21-day-old tobacco seedlings

Tissue	Treatment	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Total
			cpm			
	Control	489	11867	10400	31900	54656
Leaves	Amo 1618	ND†	10000	9056	15078	34134
	% change	−100	−15.7	−12.9	−52.7	−37.5
	Control	244	8500	5011	14911	28666
Stems	Amo 1618	ND	7100	1822	8244	17166
	% change	−100	−16.5	−63.6	−44.7	−40.1
	Control	111	356	522	300	1289
Roots	Amo 1618	ND	156	189	89	434
	% change	−100	−56.2	−63.8	−70.3	−66.3
	Control	844	20723	15933	47111	84611
Total	Amo 1618	ND	17256	11067	23411	51734
	% change	−100	−16.7	−30.6	−50.3	−38.9
			cpm/g fr.wt			
	Control	958	23254	20380	62251	106843
Leaves	Amo 1618	ND	16584	15018	25005	56607
	% change	−100	−28.7	−26.3	−59.8	−47.0
	Control	5680	177907	116537	346770	646894
Stems	Amo 1618	ND	125220	32138	145405	302763
	% change	−100	−29.6	−72.4	−58.1	−53.2
	Control	1229	3933	5777	3319	14258
Roots	Amo 1618	ND	2094	2542	1196	5832
	% change	−100	−46.8	−56.0	−64.0	−59.1
	Control	1311	32194	24752	73188	131445
Total	Amo 1618	ND	23509	15078	31895	70482
	% change	−100	−27.0	−39.1	−56.4	−46.4

* Sterols separated by GLC of the 4-desmethylsterol fractions from TLC plates.
† Not detected.

Table 3. Effect of 300 μg Amo 1618/plant on the incorporation of MVA-[2-^{14}C] into sterols and sterol precursors* of leaves, stems and roots of 21-day-old tobacco seedlings

Tissue	Treatment	4-desmethyl-sterol	4-methylsterol	4,4-dimethyl-sterol	Squalene-2,3-epoxide	Squalene	Total
				cpm			
Leaves	Control	551043	15785	12105	2125	24427	605485
	Amo 1618	100375	8624	9938	4013	8018	130968
	% change	−81.8	−45.4	−17.9	+88.8	−67.2	−78.4
Stems	Control	102423	5875	5224	1129	3040	117691
	Amo 1618	14309	2492	2664	2870	1026	23361
	% change	−86.0	−57.6	−49.0	+154	−66.3	−80.2
Roots	Control	399	32	37	37	0	505
	Amo 1618	242	97	114	88	57	598
	% change	−39.4	+203.0	+208.0	+137.0	—	+18.4
Total	Control	653865	21692	17366	3291	27467	723681
	Amo 1618	114926	11213	12716	6971	9101	154927
	% change	−82.4	−48.3	−26.8	+111	−66.9	−78.6
				cpm/g/fr.wt			
Leaves	Control	658355	18859	14462	2539	29184	723399
	Amo 1618	118647	10194	11747	4743	9478	154809
	%change	−81.9	−45.9	−18.8	+86.8	−67.5	−78.6
Stems	Control	1542515	88479	78675	17003	45783	1772455
	Amo 1618	236904	41258	44106	47517	16987	386772
	% change	−84.6	−53.4	−43.9	+179.0	−62.9	−78.2
Roots	Control	5573	447	517	517	0	7054
	Amo 1618	3218	1290	1516	1170	758	7952
	% change	−42.3	+188.0	+193.0	+126.0	—	+12.7
Total	Control	670631	22248	17811	3375	28171	742236
	Amo 1618	117033	11419	12949	7099	9268	157767
	% change	−82.5	−48.7	−27.3	+110.0	−67.1	−78.7

* Compounds separated by TLC of the total non-saponifiable lipid extract.

The higher (300 μg) level of Amo 1618 was applied to seedlings which were separated into leaf, stem and root tissues, each of which was then further sub-divided into three sub-cellular fractions, a 20000 *g* (mitochondria-enriched) pellet, a 200000 g (microsome-enriched) pellet, and a 200000 g supernatant fraction. In general, the incorporation of MVA into free sterols and sterol intermediates in the sub-cellular oganelles of tobacco seedlings was inhibited by Amo 1618 in much the same way as already described for intact, and tissues of intact, tobacco seedlings (Table 4).

The roots contained little radioactivity in any sterol or precursor in any of the three fractions. In addition, the supernatant fractions also contained little radioactivity in any compound except squalene-2,3-epoxide extracted from treated leaves. It would seem that the greater Amo 1618-induced accumulation of radioactivity in squalene-2,3-epoxide in the 200000 *g* pellet and in the supernatant, as compared with the 20000 *g* pellet, might reflect the greater participation of microsomal and soluble enzyme systems in sterol biosynthesis (particularly the mevalonate to squalene-2,3-epoxide steps) than mitochondrial enzymes.

The 20000 *g* pellet accounted for 69.2% of the total radioactivity associated with 4-desmethylsterols (by far the most radioactive fraction), the 200000 *g* pellet for 30.8% and the supernatant for only 0.03%. The retardant effect on the desmethylsterols was greater in the 20000 *g* pellet than in the 200000 *g* pellet where it was also strong. Incorporation into the 4-methyl and 4,4-dimethylsterol components of the 20000 *g* pellet was significantly inhibited by Amo 1618, but, in contrast, the 4-methyl- and 4,4-dimethylsterol fractions of the 200000 *g* pellet seemed little, if at all, affected. Overall incorporation into 20000 *g* pellet of the leaves and stems was inhibited more strongly than incorporation into the 200000 *g* pellet.

Once again, the stem tissue fractions reflected both a greater inhibition by Amo 1618 of incorporation of MVA into 4-desmethylsterols, and a greater accumulation of radioactivity in squalene-2,3-epoxide than the other tissues. It would appear that the retardant acted preferentially in the stem, or, perhaps, that sterol biosynthesis proceeds at a greater rate in stems than in leaves.

A summary of incorporation into each sterol fraction of the intracellular organelle fractions of the tissues revealed that the retardant reduced incorporation into 4-desmethylsterols by 79.5%, 4-methylsterols by 45.4%, 4,4-dimethylsterols by 40.2%, squalene by 54.6%, and increased incorporation in squalene-2,3-epoxide by 57% (Table 5).

An examination of sterol esters from the intra-cellular tissue fractions (Table 6) revealed that the vast majority (99.1%) of radioactive sterol esters were associated with leaf tissue and that the 20000 *g* pellet (mitochondria-enriched) accounted for the major portion (74.1%) of this amount. The stem tissue accounted for the remaining 0.9% of sterol esters and most of this was also in the mitochondrial-enriched pellet. No radioactive sterol esters were detected in either the supernatant fraction of leaves or stems, or in any fraction from root tissue. Amo 1618 strongly inhibited the incorporation of MVA into sterol esters in all cases and the level of inhibition was,

Table 4. Effect of 300 μg Amo 1618/plant on the incorporation of MVA-[2-^{14}C] into sterols and sterol precursors* of 20000 *g* (P_{20}), 200000 *g* (P_{200}) pellets and 200000 *g* (S_{200}) supernatant fractions of leaves, stems and roots of 21-day-old tobacco seedlings

Tissue	Treatment	4-desmethyl-sterol	4-methylsterol	4,4-dimethyl-sterol	Sualene-2,3-epoxide	Squalene	Total
			P_{20} fraction (cpm)				
Leaves	Control	689579	53160	41625	16011	80651	881026
	Amo 1618	105223	18792	12717	19734	19788	176254
	% change	−84.7	−64.7	−69.5	+23.3	−75.5	−80.0
Stems	Control	182556	6798	4610	1606	7356	202926
	Amo 1618	19674	4759	3023	3245	9994	40695
	% change	−89.2	−30.0	−34.4	+102.1	+35.9	−80.0
Root	Control	516	201	178	96	292	1238
	Amo 1618	249	103	62	137	83	634
	% change	−51.7	−48.8	−65.2	+42.7	−71.6	−50.6
Total	Control	872651	60159	46413	17713	88299	1085235
	Amo 1618	125146	23654	15802	23116	29865	217583
	% change	−85.7	−60.7	−66.0	+30.5	−66.2	−80.0
			P_{200} fraction (cpm)				
Leaves	Control	340206	27482	30512	11102	40739	450041
	Amo 1618	115441	23558	29280	14567	24751	207597
	% change	−66.1	−14.3	−4.0	+31.2	−39.3	−53.9
Stems	Control	48007	1447	1372	458	2011	53295
	Amo 1618	13809	1505	1333	4779	3074	24500
	% change	−71.2	+4.0	−2.9	+943.4	+52.9	−54.0
Roots	Controls	453	161	221	117	241	1193
	Amo 1618	101	2	44	81	57	285
	% change	−77.1	−98.8	−80.1	−30.8	−76.4	−76.1
Total	Control	388666	29090	32105	11677	42991	504529
	Amo 1618	129351	25065	30657	19427	27882	232382
	% change	−66.7	−13.8	−4.5	+66.4	−35.2	−53.9
			S_{200} fraction (cpm)				
Leaves	Control	248	1832	577	560	2283	5500
	Amo 1618	223	862	729	4448	2704	8966
	% change	−10.1	−53.0	+26.3	+694.3	+18.4	+63.0
Stems	Control	45	169	82	47	59	402
	Amo 1618	110	169	150	91	199	719
	% change	+144.4	—	+82.9	+93.6	+237.3	+78.9
Roots	Control	28	13	1	39	34	115
	Amo 1618	17	65	26	72	45	225
	% change	−39.3	+400.0	—	+84.6	+32.4	+95.7
Total	Control	321	2014	660	646	2376	6017
	Amo 1618	350	1096	905	4611	2948	9910
	% change	+9.0	−45.6	+37.1	+613.8	+24.1	+64.7

* Compounds separated by TLC of un-saponified lipid extract of the sub-cellular fractions. Squalene-2,3-epoxide obtained after TLC of the non-saponifiable lipid extracts.

generally, greater than that observed for 4-desmethylsterols in the identical fractions. It is likely that the inhibition obseved in this case was due to decreased sterol biosynthesis rather than to any inhibition of the esterifying enzyme systems.

As indicated earlier, incorporation results with root tissues were very low and are considered not completely reliable. In spite of that, however, agreement between the two experiments in which the higher Amo 1618 level was used is good, in terms of the inhibiting effects of the retardant on both total incorporation percentages into the individual sterol classes, and total incorporation percentages in the sterols of the three types of tissues. For instance, in control tissue, on a fr. wt basis, 86 and 88% of total radioactivity in the latter two experiments occurred in the desmethylsterols, 4.1 and 4.2% as 4-methylsterols, and 3.3 and 3.7% as 4,4-dimethylsterols. These proportions were altered by Amo 1618 treatment to 54 and 66%, 10.4 and 9.3%, and 8.1 and 10.2% respectively. Thus, at the end of 24 hr of retardant treatment there was a change in the apparent rate of passage of radioactivity from one sterol group to another.

In particular, incorporation into 4-desmethylsterols in leaves and stems was inhibited more strongly than incorporation into the other two sterol classes. This suggests that the second demethylation step is directly inhibited by the retardant and is, in fact, one point of Amo 1618 action.

Uniformity of the results with squalene and squalene-2, 3-epoxide was not as good although it is obvious that accumulation of radioactivity in the epoxide was considerably heightened by retardant treatment of all three tissues at both concentrations. There seems little

Table 5. Summation of effects of 300 μg Amo 1618/plant on incorporation of MVA-[2-^{14}C] into sterols and sterol precursors of sub-cellular fractions (see Table 4) of leaves, stems and roots of 21-day-old tobacco seedlings

Tissue	Treatment	4-desmethyl-sterol	4-methylsterol	4,4-dimethyl-sterol	Squalene-2,3-epoxide	Squalene	Total
				cpm			
Leaves	Control	1030033	82474	72714	27673	123673	1336567
	Amo 1618	220887	43212	42726	38749	47243	392817
	% change	−78.6	−47.6	−41.2	+40.0	−61.8	−70.6
Stems	Control	230608	8414	6064	2111	9426	256623
	Amo 1618	33593	6433	4506	8115	13267	65914
	% change	−85.4	−23.5	−25.7	+284 4	+40.7	−74.3
Roots	Control	997	375	400	252	567	2591
	Amo 1618	367	170	132	290	185	1144
	% change	−63.2	−54.7	−67.0	+15.1	−67.4	−55.8
Total	Control	1261638	91263	79178	30036	133666	1595781
	Amo 1618	254847	49815	47364	47154	60695	459875
	% change	−79.8	−45.4	−40.2	+57.0	−54.6	−71.2
				cpm/g fr.wt			
Leaves	Control	334426	26777	23608	8984	40153	433948
	Amo 1618	64738	12664	12522	11356	13846	115126
	% change	−80.7	−52.7	−47.0	+26.4	−65.5	−73.5
Stems	Control	1048218	38245	27563	9595	42845	1166466
	Amo 1618	158457	30344	21254	28278	62580	300913
	% change	−84.9	−20.7	−22.9	+194.7	+46.1	−74.2
Roots	Control	3379	1271	1355	854	1922	8781
	Amo 1618	1125	521	404	889	567	3506
	% change	−66.7	−59.0	−70.2	+4.1	−70.5	−60.1
Total	Control	350942	25386	22024	8355	37181	443889
	Amo 1618	64518	12611	11991	11938	15366	116424
	% change	−81.6	−50.3	−45.6	+42.9	−58.7	−73.8

doubt that cyclization of squalene-2,3-epoxide was inhibited, and on both an absolute and a fr. wt basis, the retardant effect on the stem was more pronounced than on the leaf. The Amo 1618-induced inhibition of incorporation into squalene was not as reproducible as with the sterol groups, but the results generally support the conclusion that there was, in fact, an inhibition, and, thus also a pre-squalene site of Amo 1618 action.

Table 6. Effect of 300 μg Amo 1618/plant on incorporation of MVA-[2-^{14}C] into sterol esters* from sub-cellular fractions of leaves, stems and roots of 21-day-old tobacco seedlings

Tissue	Treatment	Incorporation of mevalonate-[2-^{14}C] into sterol esters from P_{20}	P_{200}	S_{200}
			cpm	
Leaves	Control	83167	28076	ND
	Amo 1618-treated	11297	2625	ND
% Inhibition		86.4	90.6	—
Stems	Control	1021	20	ND
	Amo 1618-treated	21	0	ND
% Inhibition		97.9	100.0	—
Roots	Control	ND	ND	ND
	Amo 1618-treated	ND	ND	ND
% Inhibition		—	—	—

* The sterol ester-squalene-2,3-epoxide regions of TLC plates, on which the total un-saponified lipid extracts were developed, were saponified and the free sterols liberated were separated from squalene-2,3-epoxide by TLC.

ND = none detected.

DISCUSSION

The individual and groups of compounds examined in this and previous reports [3, 4] have a definite biosynthetic relationship [5–9]. Squalene is oxidized to squalene-2,3-epoxide which in turn is cyclized to produce 4,4-dimethylsterols (e.g. cycloartenol, 24-methylenecycloartenol, lanosterol, etc.). Demethylation leads to the 4-methylsterols (e.g. cycloeucalenol, obtusifoliol, etc.) and a second demethylation results in the 4-desmethylsterol group (cholesterol, campesterol, sitosterol, stigmasterol, etc.). The desmethylsterols are present insignificant amounts and are the major components of both free and esterified sterol fractions found in tobacco [8].

In view of the sequence of occurrence [9] of these components, it is possible to make certain generalizations about the nature and sites of action of Amo 1618. In leaves and stems Amo 1618 inhibits (a) a pre-squalene step, (b) the cyclization of squalene-2,3-epoxide and (c) the demethylation of 4-methylsterols and hence the formation of 4-desmethylsterols. The most pronounced effect is on the cyclization reaction, and the second most severe effect is the decreased desmethylsterol formation. These findings confirm previous work [3, 4] and agree in detail with the retardant effects on cell-free rat liver cholesterol biosynthesizing systems [2, 10].

The effects of the retardant indicated above are manifested within 24 hr in all of the tissues (leaves, stems and roots) of the 21-day-old tobacco seedling, and in at least two sub-cellular fractions (mitochondria-enriched, microsomal-enriched) of each of the tissues. Inhibition of the incorporation of radioactivity into sterol esters in the two particulate fractions obtained from leaves and stems was also observed, as was an inhibition of incorporation of radioactivity into four desmethylsterols extracted from leaves and stems and analyzed in greater detail.

Thus, in *Nicotiana tabacum* the rapid, reproducible and potent sterol biosynthesis-inhibiting effect of Amo 1618 has been demonstrated in rootless and intact seedlings, tissues of seedlings, and sub-cellular organelles of seedlings. It seems reasonable to conclude that it is a major action of the retardant on this plant.

Several aspects of the Amo 1618 effect are of interset in considering the way in which inhibition(s) of sterol formation could affect growth. Sterol biosynthesis in stem tissue proceeded at a faster rate than in leaf tissue when calculated on a per unit fr. wt basis, and, on both a fr. wt and an absolute basis, was inhibited to a greater extent in stem tissue than in leaves. The pelletable, membranous fractions from the various sub-cellular preparations are the major site of free sterol (desmethylsterol) concentration in seedling tissue [8]. Thus, as a result of a reduced supply of sterols, the mitochondria, Golgi, endoplasmic reticulum, etc., and other membranous fractions will either have their membranes made more slowly or will make 'atypical' membranes. The manifold consequences of such a situation in rapidly growing stems cerainly includes a decreased rate of growth. In fact, if gibberellins are synthesized at least of partly on membranes of leaf or stem tissue, as appears likely [11–13], a decrease in gibberellin content might also be a result of retardant activity.

In addition to a gross decrease in membrane synthesis, several other possible biological consequences of inhibited sterol biosynthesis can be envisaged. For example, changes in the ratio of stigmasterol to sitosterol have been shown [14, 15] to accompany changes in growth and development, but it is not clear whether such changes in the sterol ratios precede or are a consequence of growth changes. The present results suggest that some sterols (e.g. cholesterol and sitosterol) are more strongly affected than others, and, as Grunwald [16] has shown, some sterols may be more important for normal membrane structure and function than others. Elliott and Knight [17] have demonstrated the requirements of *Pythium* and *Phytophthora* for precise structural configurations of sterols before normal reproductive development will occur. The differential effects that may result from retardant application are also demonstrated by the more pronounced inhibition of incorporation into the membranes of the 20000 *g* fraction than into the 200000 *g* membranes. Another alternative biological consequence of inhibited sterol formation may be the effects of the accumulated intermediates; squalene-2,3-epoxide has not been shown to have any biological action but in some tissues it may be active.

If a primary action of Amo 1618 is indeed the inhibition of desmethylsterol biosynthesis, there are several ways which exogenous gibberellin might cause an apparent reversal of the Amo 1618 effect. The hormone might be incorporated into membranes at sites normally occupied by sterols. It might decrease turnover of sterols in existing membranes, or might increase the availability of precursors so that, in spite of the retardant-induced reduction in enzyme activity, total sterol biosynthesis might be normal or even enhanced. The latter hormone effect might be brought about by increased hydrolytic activity or increased membrane permeability, etc.

Finally, notwithstanding the above, the present results do not completely rule out the possibility that gibberellins control sterol biosynthesis and that retardant effects are manifested first through a decrease in gibberellin level, and secondarily through inhibited sterol biosynthesis.

EXPERIMENTAL

Treatment of plant material. Nicotiana tabacum (cv Turkish Samson) seedlings, germinated and cultivated under conditions of constant illumination and temp. as previously described [4], were treated with Amo 1618 (100 or 300 μg/plant: Calbiochem. Calif.) and DL-MVA-[2-^{14}C]-(Radiochemical Centre, Amersham, England) or, in the case of controls, MVA-[2-^{14}C] alone, 21 days after germination. Amo 1618 [in 0.05% Tween-20 soln] and MVA-[2-^{14}C] [in 11 mM (KH_2-K_2HPO_4) phosphate buffer, pH 6.5] were applied as a single 5 μl drop to each stem apex. Controls were treated with a 5 μ drop of 0.05% Tween-20 sol only. In the plant tissue studies, 1.25 μCi of MVA-[2-^{14}C] were administered to each plant and 1 μCi of precursor was supplied to each seedling used in the organelle studies. Two plants per treatment were used in the 100 μg per plant Amo 1618 expt, 4 per treatment in the 300 μg Amo1618 experiment, and 15 per treatment in the sub-cellular organelle studies. 24 hr after MVA-[2-^{14}C] application, the plants were carefully removed from pots (ensuring minimal root damage), washed thoroughly with H_2O blotted dry and the fr. wt determined. For the experiment with 100 μg Amo1618 per plant, the fr. wts of the leaf, stem and root tissues of the two controls were 0.511, 0.043 and 0.090 g respectively, and 0.603, 0.057 and 0.074 g respectively for the tissues of the two Amo 1618-treated plants. The leaf, stem and root tissues of the four control plants (in the first 300 μg Amo 1618 per plant expt) weighed 0.837, 0.066 and 0.072 g respectively and 0.846, 0.060 and 0.075 g respectively from the Amo 1618-treated plants. For tissue and subcellular organelle studies, the plants were divided into leaf, stem and root tissues prior to fr. wt determination.

Preparation of sub-cellular fractions. The method of ref. [18] with minor modifications, was employed for preparation of sub-cellular fractions. Plant tissue was cut into small pieces and homogenized in 2× its own wt of ice-cold sucrose (0.25 M). $MgCl_2.6H_2O$ (4 mM) and glutathione (5 mM) in 0.10 M Tris buffer, pH 7.5. Homogenization was achieved by a motor-driven glass pestle in a glass homogenizer (Contes, U.S.A.) run at slow speed. The crude homogenate was then filtered through 2 layers of cheese-cloth and centrifuged (1000 *g*, 5 min) to yield a pellet and supernatant. The supernatant was then centrifuged (20000 *g* 15 min) in an 8 × 50 ml fixed angle rotor at 4° to yield the mitochondria-rich pellet fraction (P_{20}) and a supernatant. The supernatant was centrifuged at 200000 *g* (90 min) to obtain the microsome-rich pellet (P_{200}) and supernatant (S_{200}). All operations were carried out at 0–4°.

Extraction and estimation of sterols. Sterols and their precursors were extracted from tissues and sub-cellular fractions by the method of ref. [19]. Tissues or organelles were transferred to round-bottomed flasks and gently refluxed, successively, with Me_2CO (2 × 30 ml, each 1 hr) and $CHCl_3$–MeOH (2:1) (1 × 30 ml, 1 hr). The combined organic extracts were taken to dryness *in vacuo* and the residue dissolved in dry C_6H_6 (2 ml) for TLC analysis. The total unsaponified lipid was separated by TLC M Si gel developed 2× in CH_2Cl_2–Et_2O (24:1). Radioactive spots were located by a radioscanner, and sterols located by UV fluorescence after lightly spraying the plate with methanolic 1% berbine–HCl soln. Standards (usually cholesterol, lanosterol, squalene-2,3-epoxide and squalene) were co-chromatographed. Sterols esters, which co-chromatographed with squalene-2,3-epoxide in this TLC system, were assayed by saponifying the band from TLC in methanolic KOH (5% in 85% MeOH) under reflux for 1 hr. The sterols and squalene-2,3-epoxide were then extracted from the mixture with Et_2O (3 × 10 ml), the combined extracts washed with H_2O (3 × 5 ml) and dried over dry Na_2SO_4. After filtering, the extract was evapd to dryness *in vacuo* and the residue taken up in dry C_6H_6 (1 ml). The sterols thus obtained were separated from squalene-2,3-epoxide by TLC as above. After TLC, the Si gel plates were scraped into 0.5 cm bands from the origin to solvent front and each fraction radioassayed by scintillation counting. Separation of the 4-desmethylsterol fraction into its individual sterol components was achieved by GLC with a 9:1 stream-splitter and FID. The glass column (1.84 m × 4 mm) was packed with 2.5% OV-101 on Gaschrom Q (80–100 mesh). Column temp. was 230°, detector temp. 300°, and N_2 carrier gas flow rate 100 ml/min. Fractions of effluent from the GLC column were collected in 1 ml luer-lok syringes plugged with MeOH-soaked glass wool which attached, by special fittings on a heated metal block, to the larger diameter tubing of the stream-splitter. Cholestane was used as int. stand. and peak indentification was by comparison of relative retention times of sterols with those of authentic standards.

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