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AMO 1618 EFFECTS ON INCORPORATION OF ^{14}C-MVA AND ^{14}C-ACETATE INTO STEROLS IN *NICOTIANA* AND *DIGITALIS* SEEDLINGS AND CELL-FREE PREPARATIONS FROM *NICOTIANA*

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Key Word Index—*Nicotiania*; *Digitalis*; Solanaceae; cell free homogenates; sterol biosynthesis; HMG-CoA reductase; squalene-2,3-epoxide; AMO 1618.

Abstract—Incorporation of radioactivity from acetate-[^{14}C] and MVA-[^{14}C] into sterols and sterol precursors in tobacco was inhibited by Amo 1618; differing patterns of accumulation were obtained with the two precursors, suggesting more than one point of inhibition. This was borne out with cell-free preparations with which it was demonstrated that both HMG-CoA reductase and squalene-2,3-epoxide cyclase were inhibited, the latter more strongly than the former. GLC analysis of gross sterol and hydrocarbon fractions confirmed previous indications that incorporation of radioactivity into individual sterols was inhibited by Amo 1618. Finally, incorporation of MVA-[^{14}C] into sterols and sterol precursors of *Digitalis* was significantly altered by the retardant, thus expanding the generality of the relationship between sterol (particularly 4-desmethylsterol) biosynthesis inhibition and retardant effect.

INTRODUCTION

Previous results [1, 2] demonstrated that treatment of tobacco seedlings, from which the roots were removed, with Amo 1618 inhibited the incorporation of mevalonic acid-[^{14}C] (MVA) into sterols. More recently it was also demonstrated that Amo 1618 inhibited MVA-[^{14}C] incorporation into sterols in roots, stems and leaves of intact tobacco seedlings; stem synthesis was most influenced by the retardant application [3]. The same work also indicated that MVA-[^{14}C] incorporation into sterols in sub-cellular fractions was inhibited by Amo 1618.

The present work explores the relative effects of Amo 1618 on acetate-[^{14}C] and MVA-[^{14}C] incorporation when these sterol precursors were used with tobacco and *Digitalis* seedlings and cell-free preparations of tobacco.

RESULTS

Roots of three- to four-week-old seedlings were removed and the derooted seedlings were treated with either 10 μCi acetate-[1-^{14}C] or 5 μCi DL-MVA-[2-^{14}C] in the presence or absence of 1 mg/ml Amo 1618. After the 24 hr incorporation period, sterols and sterol intermediates were extracted from the seedlings, separated into the various fractions by TLC and assayed for radioactivity.

The distribution of radioactivity on TLC showed that the retardant greatly reduced the incorporation of acetate into all of the sterol fractions, had little effect on incorporation into the squalene fraction, and produced only a slightly elevated level of radioactive label in the squalene-2,3-epoxide fraction. The averaged data from three experiments are indicated in Table 1; overall, 1 mg/ml Amo 1618 produced about 75% inhibition of incorporation of acetate-[^{14}C] into the measured components of the sterol pathway.

The averaged data from three other experiments conducted under identical conditions to those above, showed that 1 mg/ml Amo 1618 also markedly inhibited the incorporation of MVA-[^{14}C] into the same components (Table 1). In this case, however, there was also a much greater accumulation of radioactivity in squalene-2,3-epoxide. GLC analysis of the sterol (Fig. 1) and hydrocarbon fractions (Fig. 2) showed a similar pattern, in that incorporation of MVA into each of the four main 4-desmethylsterols (cholesterol, campesterol, stigmasterol and sitosterol) was inhibited strongly by the retardant; incorporation into 4,4'-dimethylsterols was reduced to a slightly lesser degree, and incorporation into the squalene and, particularly, squalene-2,3-epoxide was increased. Incorporation of MVA into 4-desmethylsterols was inhibited by *ca* 90%, inhibition of incorporation into 4,4'-dimethylsterols was *ca* 70–80%, and radioactivity associated with squalene-2,3-epoxide was increased *ca* 5-fold (Table 2). Thus, 1 mg/ml Amo 1618 inhibited incorporation of acetate as well as MVA, though the retardant-induced pattern of distribution of radioactivity was not the same with the two precursors. Inhibition of incorporation was observed in the individual sterols as well as in the total sterol fractions.

The differences in incorporation patterns observed when acetate and MVA were supplied to tobacco seedlings in the presence of 1 mg/ml Amo 1618 supported earlier reports [4, 5] in which similar effects were obtained with a rat liver system. The most likely point for a pre-MVA inhibiting effect was on HMG-CoA reductase [6, 7] and the effect of the retardant on the

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Table 1. Effect of 1 mg/ml Amo 1618 on the incorporation of acetate-[1-^{14}C] and DL-MVA-[2-^{14}C] into sterols and sterol intermediates* of three-week-old rootless tobacco seedlings

Product	Incorporation of acetate-[1-^{14}C]			Incorporation of L-mevalonate-[2-^{14}C]		
	Control	Amo-1618 treated	% Inhibition or stimulation	Control	Amo-1618 treated	% Inhibition or stimulation
	cpm/g fr. wt					
4-Desmethylsterols	124106	12592	−89.9	248300	24700	−90.1
4-Methylsterols	7517	1357	−81.9	61676	16206	−73.7
4,4'-Dimethylsterols	36801	9910	−73.1	41648	22858	−45.1
Squalene-2,3-epoxide	3475	7001	+101.1	4894	44249	+804.0
Squalene	18732	16052	−14.3	13014	16487	+26.7
Total	190361	46912	−75.4	369532	124500	−66.3

* Non-saponifiable lipid extract separated by TLC dichloromethane–Et_2O (96:4). All values are averages of three experiments.

cell-free conversion of both acetate-[1-^{14}C]- and HMG-[3-^{14}C]-CoA into MVA was analyzed. In a total cell-free homogenate (1000 *g* supernatant) from three-week-old seedlings, Amo 1618 inhibited acetate incorporation into MVA (extracted as the lactone) by 52% (Table 3a). In a cell-free system consisting of microsomes (P_{200}) and soluble enzymes (E_{40}^{80}), also prepared from 21-day-old tobacco seedlings, the retardant inhibited conversion of both acetate and HMG-CoA into MVA. Incorporation of radioactivity from acetate was reduced by about 42%, and from HMG-CoA by about 50 and 65% (Table 3b).

The latter system was also used to assess the effects of 1 mg/ml Amo 1618 on cell-free incorporation of radioactivity from MVA-[^{14}C]- and squalene-[^{14}C] into sterols. Unfortunately, the preparations lacked the ability to convert MVA to sterols and only synthesized squalene and squalene-2,3-epoxide (Table 4a). Similarly, other cell-free preparations consisting of various combinations of particulate (chloroplastidic, mitochondrial, microsomal) and soluble fractions also proved unable to convert MVA to sterols and were, in fact, even less able to utilize the precursor (unpublished results). Amo 1618 caused no significant change in the pattern of hydrocarbon production from MVA in the cell-free system (Table 4a).

When squalene was employed as precursor in the cell-free system, some incorporation into sterols and squalene-2,3-epoxide was observed. Addition of 1 mg/ml Amo 1618 to the incubation mixture resulted in about 79% reduction in squalene incorporation into 4,4'-

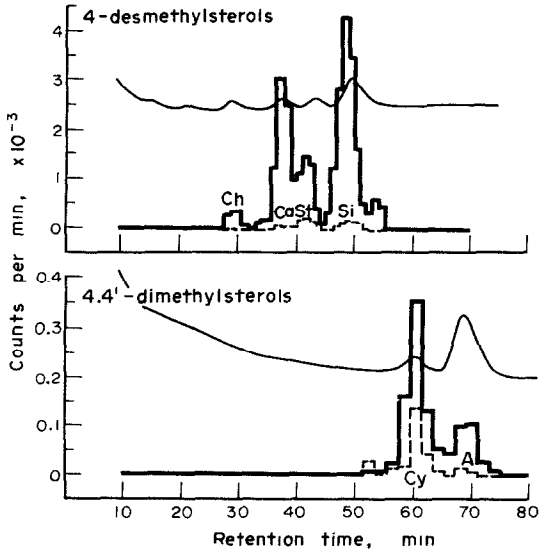

Fig. 1. Effect of 1 mg/ml Amo 1618 on the incorporation of MVA-[2-^{14}C] into 4-desmethylsterols and 4,4'-dimethylsterols of 21-day-old rootless tobacco seedlings. Distribution of radioactivity from GLC analysis of control (solid lines and Amo 1618-treated (broken lines) non-saponifiable lipid fractions after TLC is shown. The curves are of mass peaks of sterols in control extracts. (Ch = cholesterol, Ca = campesterol, St = stigmasterol, Si = sitosterol, Cy = cycloartenol, A = β-amyrin).

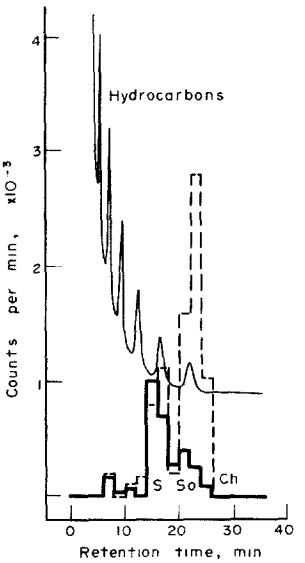

Fig. 2. Effect of 1 mg/ml Amo 1618 on the incorporation of MVA-[2-^{14}C] into hydrocarbons of 21-day-old rootless tobacco seedlings. Distribution of radioactivity from GLC analysis of control (solid lines) and Amo 1618-treated (broken lines) non-saponifiable lipid fractions after TLC is shown. The curve shows mass peaks in control extract. (S = squalene, SO = squalene-2,3-epoxide, Ch = cholesterol).

Table 2. GLC analysis of 4-desmethylsterols and 4,4′-dimethylsterols labelled from MVA-[2-^{14}C] 1 mg/ml Amo 1618-treated and control rootless tobacco seedlings*

Sterol fraction	Product identified	Incorporation into Control	Amo 1618-treated	% Inhibition or stimulation
			cpm	
4-Desmethylsterols	Cholesterol	575	45	−92.2
	Campesterol	7145	219	−96.9
	Stigmasterol	4351	498	−88.6
	Sitosterol	14109	811	−94.3
	Total	26180	1573	−94.0
4,4′-Dimethylsterols	Cycloartenol	695	221	−68.2
	Unknown	235	47	−80.0
	Total	930	268	−71.2
	Squalene	1749	2023	+15.7
	Squalene-2,3-epoxide	931	5638	+506.0
	Total	2680	7661	+186.0

* Equal aliquots from TLC were analysed by GLC and fractions from the GLC eluate were assayed for radioactivity.

Table 3. The effect of 1 mg/ml Amo 1618 on the biosynthesis of mevalonate in cell-free preparations of tobacco seedlings*. (a) The effect on acetate-[1-^{14}C] conversion to mevalonate in a whole cell-free homogenate from tobacco seedlings. (b) The effect on acetate-[1-^{14}C] and HMG-[3-^{14}C]-CoA, conversion to mevalonate in a microsomal-rich fraction from a cell-free homogenate of rootless tobacco seedlings.

Expt.	Precursor	Incorporation into mevalonate Control	Amo 1618-treated	% inhibition
			cpm	
a	acetate-[1-^{14}C]	7217	3482	51.8
b	acetate-[1-^{14}C]	9511	5507	42.1
b (i)	HMG-[3-^{14}C]-CoA	1291	650	49.7
(ii)†	HMG-[3-^{14}C]-CoA	1090 ± 415	378.6 ± 26.7	65.3

* In all incubations boiled enzyme blanks were less than 15% of the control values.
† Values are means of triplicate determinations and standard deviations.

dimethylsterols and a 7-fold increase in incorporation into squalene-2,3-epoxide (Table 4b), thus demonstrating the inhibition of squalene-2,3-epoxide cyclase activity at the cell-free level.

These experiments confirmed the ability of Amo 1618 to inhibit terpenoid biosynthesis at a pre-MVA step in tobacco. They also indicated that all of the inhibition observed with acetate-[^{14}C] could be accounted for by an inhibition of HMG-CoA reductase activity, which was not as marked as the inhibition of squalene-2,3-epoxide cyclase activity with the same Amo 1618 concentration and an identical cell-free preparation from 21-day-old tobacco seedlings. It seems reasonable to conclude that the cyclase may be more sensitive than the reductase, though both are inhibited.

Finally, to ascertain whether Amo 1618 was also able to inhibit sterol biosynthesis in a species other than *Nicotiana*, its effect on *Digitalis* was examined. A series of Amo 1618 concentrations was tested for effects on sterol biosynthesis in 21-day-old rootless *Digitalis* seedlings and all experimental conditions employed were exactly as used in studies of tobacco [1, 2]. The results are presented in Fig. 3. Similarities between these results and those obtained previously with tobacco were the decreased incorporation of MVA into 4-desmethyl-

Table 4. The effect of 1 mg/ml Amo 1618 on the incorporation of MVA-[2-^{14}C] and squalene-[^{14}C] into sterols and sterol intermediates* in cell-free preparations of tobacco seedlings. (a) DL-mevalonate-[2-^{14}C] incorporation. (b) squalene-[^{14}C] incorporation

Incorporation product	Incorporation of ^{14}C-precursor Control	Amo 1618-treated	% Inhibition or stimulation
(a)			
4-Desmethylsterols	0	0	—
4-Methylsterols	0	0	—
4,4′-Dimethylsterols	0	0	—
Squalene-2,3-epoxide	10216	12392	+21.3
Squalene	1020	986	−3.3
(b)			
4-Desmethylsterols	10721	1112	−89.6
4-Methylsterols	4443	988	−77.8
4,4′-Dimethylsterols	9278	1970	−78.8
Squalene-2,3-epoxide	1079	9112	+744

* Sterols and hydrocarbons separated by TLC of the non-saponifiable lipid fraction.

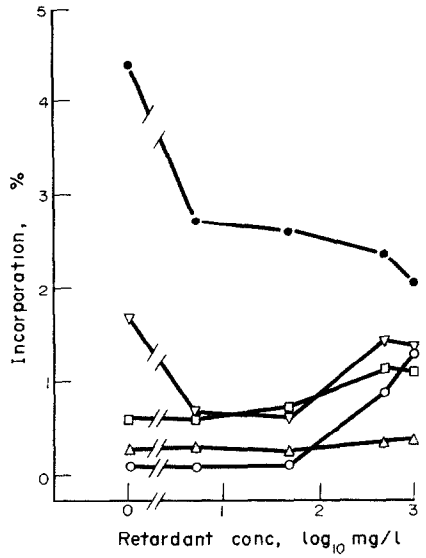

Fig. 3. Effects of several concentrations of Amo 1618 on percent incorporation of MVA-[2-^{14}C] into sterols and sterol intermediates in 21-day-old rootless seedlings of *Digitalis*. (● = 4-desmethylsterols, ▽ = 4,4′-dimethylsterols, □ = 4-methylsterols, ○ = squalene-2,3-epoxide, △ = squalene).

sterols and the increased incorporation into squalene-2,3-epoxide as retardant concentration was increased. An extensive interpretation of the data is not warranted since a more detailed study of *Digitalis* was not undertaken. However, Amo 1618 was effective in altering patterns of MVA-[^{14}C] incorporation into sterols and sterol precursors in *Digitalis*.

DISCUSSION

Several aspects of the effect of Amo 1618 on sterol biosynthesis have been explored. It seems apparent that the retardant has more than one potential site of action in tobacco since different patterns of product accumulation were found following incubation of rootless seedlings with radioactive acetate and MVA. With acetate, the retardant caused a relatively uniform (73–90%) inhibition of incorporation into all sterol fractions of the rootless seedlings, and only a doubling in the squalene-2,3-epoxide level. Inhibition of incorporation of MVA, however, varied between 46 and 90% in the sterol fractions, and an 800% increase in radioactivity in the epoxide was observed.

In addition, when cell-free preparations of tobacco were incubated with either acetate-[^{14}C]- or HMG-[^{14}C]-CoA, incorporation into MVA was reduced by 50% indicating that the retardant clearly has the capacity to inhibit HMG-CoA reductase. The second site of inhibition was demonstrated when squalene-[^{14}C] was supplied to the cell-free preparation. In the presence of the retardant, radioactivity in squalene-2,3-epoxide increased more than 7-fold, and decreased in the sterols by 75–90%. Thus, together with the earlier demonstration of an inhibition of (−)-kaurene biosynthesis [8], Amo 1618 has now been shown to inhibit three distinct steps in isoprenoid biosynthetic pathways in plants.

Multiple sites of action of sterol biosynthesis inhibitors is not uncommon. Holmes and di Tullio [9] investigated SK & F 525, SK & F 3301 and triparanol, and reported that all three inhibited the sterol biosynthetic pathway in rats at more than one site, both *in vivo* and *in vitro*, and that there was a correlation between *in vivo* and *in vitro* sites of action. Multiple sites of inhibition of sterol biosynthesis have also been reported with triparanol and *Chlorella* [10]. In this respect it is of interest, also, that the product of squalene-2,3-epoxide cyclase activity in plants and animals is different. In plants the first cyclized sterol is cycloartenol [11] while lanosterol is the cyclized metabolite of squalene-2,3-epoxide in animals [12]. Though the cyclase enzymes must therefore be different, both are inhibited by Amo 1618 [2].

Inhibition by Amo 1618 of the enzyme HMG-CoA reductase is of considerable interest. The enzyme has been studied in some detail in animal [13], yeast [14] and bacterial [15] systems and is thought to be a key regulatory enzyme responsible for, among other aspects, the physiological regulation of the hepatic cholesterogenic pathway [6, 16]. No work of a similar nature has been conducted on the enzyme in higher plants although Brooker and Russell [17] have reported phytochrome regulation of HMG-CoA reductase in *Pisum sativum*. The enzyme from peas prepared according to Brooker and Russell [17] seems to differ in some properties from that of tobacco since it did not respond to Amo 1618 (unpublished results).

The failure of cell-free tobacco preparations to utilize MVA-[2-^{14}C] in sterol biosynthesis paralleled the findings of Staby *et al.* [18] who showed that excised shoots, but not cell-free preparations from shoots of Wedgwood iris converted MVA-[2-^{14}] to sterols. The cell-free extracts were able, however, to convert the precursor to squalene, farnesol, and geranylgeraniol. Benveniste *et al.* [19] also found that MVA was converted to squalene but not sterols in a cell-free system from tobacco tissue cultures. There is no simple explanation for the fact that, in the present work, MVA-[^{14}C] was converted to squalene-2,3-epoxide but not to sterols, whereas, in an identical preparation, squalene-[^{14}C], a precursor of squalene-2,3-epoxide, was converted to sterols. Compartmentation of isoprenoid biosynthesis has been demonstrated by Rogers *et al.* [20] and the explanation of the above anomaly may lie in the differential solubilities of squalene and MVA and the ease with which the two compounds can traverse membranes of organelles.

GLC of the 4-desmethylsterol, 4,4′-dimethylsterol and hydrocarbon fractions indicated that the inhibition observed with TLC, not surprisingly, was also reflected in an actual decrease in incorporation of radioactivity into the individual sterols, and an increase into squalene-2,3-epoxide. It must be emphasized, however, that identification of the individual sterols was at best only presumptive (i.e. through co-chromatography with standards) and should be regarded as tentative. The values for incorporation of MVA-[^{14}C] into the individual sterols (Table 2) indicated that either differential rates of synthesis or metabolism existed for the different sterols. The data were insufficient to distinguish between these possibilities. The mass peaks in Figs 2 and 3 also indicated that the significant differences existed between the amounts of the four major 4-desmethylsterols, and that there seemed little relationship between the incorporation of radioactive MVA into those sterols and the total amounts present.

Finally, it was of interest to note that Amo 1618 caused significant changes in the incorporation of radioactivity from MVA-[^{14}C] into sterols and hydrocarbons of *Digitalis*. Similar plant growth retardant-induced inhibitions of sterol biosynthesis have been reported by Kimura *et al.* [21] in *Pharbitis* and Staby [22] in cell-free preparations from Wedgwood iris. It is probable, therefore, that these effects will be observed with a wide variety of plants and other organisms.

None of the work reported here elucidates the relationship between the effect of Amo 1618 and the biosynthesis and role of gibberellins during growth. It does, however, strengthen the conclusion [2] that the retardant may exert more than one effect in any given situation, and that different varieties of plants may react to retardants in differing ways because of distinct effects on various steps of the many isoprenoid biosynthetic pathways.

EXPERIMENTAL

Treatment of seedlings. 21-Day-old tobacco and *Digitalis* seedlings (4–5 per treatment), germinated and grown under constant illumination (2000 ft.c.) and constant temp. (23 ± 2°), were placed into 5 ml Petri dishes. The cut stems, from which roots were removed, were immersed in 11 mM phosphate buffer (2 ml; pH 6.5) in which the required amount of acetate-[1-^{14}C] (10 μCi) or MVA-[2-^{14}C] (5 μCi) and retardant were dissolved. After 24 hr illumination (300 ft.c.) the rootless seedlings were removed from the dishes, blotted dry, and the sterols extracted as previously described [1]. Sterol fractions were separated by (TLC of the total non-saponifiable lipid extract on Sil gel-G (5 × 20 cm × 0.3 mm). GLC of the sterol fractions was performed on a GC fitted with a stream splitter and fraction collector. 1.5% SE-30 on Anachrom ABS was employed as column packing in glass columns (1.85 m × 0.4 cm). Operating conditions were: column temp. 230°, FID temp. 300°, and carrier gas (N_2) flow rate 80 ml/min.

Preparation of total cell-free homogenate. A known weight (usually 5–10 g) of 21-day-old rootless tobacco seedlings was placed into 2 × its fr. wt of cold 44 mM (KH_2–K_2H) phosphate buffer pH 7.6, containing 126 mM sucrose, 28 mM nicotinamide and 7 mM $MgCl_2.6H_2O$ and homogenized in an Ultraturrax (3 × 30 sec at slow speed). The crude homogenate was filtered through cheese-cloth and centrifuged at 2000 rpm in a bench centrifuge to produce a clear supernatant. All operations were carried out at 0–4°. 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-MVA-[2-^{14}C] or 1.0 μCi of acetate-[1-^{14}C] where applicable. Amo 1618 was added to a final concn of 1 mg/ml where indicated and incubations were carried out for 3 hr at 30° in 5 × 2.5 cm glass vials plugged with cotton wool in a shaking water bath. After incubation, the reaction mixture was treated with 5 ml Me_2CO; sterols were extracted by the same procedure used for rootless seedlings. In the case of acetate-[1-^{14}C] incorporations where MVA was extracted as the lactone, the incubation mixture was treated with 0.5 ml 30% KOH and the mixture left to stand for 15 min prior to mevalonolactone extraction. The mixture was then treated with 10N HCl to lower the pH below 2 and, after allowing to stand the mixture was extracted exhaustively with Et_2O by vigorous shaking. The Et_2O extracts were combined, taken to dryness *in vacuo* and the residue dissolved in 0.2 ml Me_2CO for TLC.

Preparation of cell-free components. Microsomally-rich, mitochondrial, and soluble enzyme preparations were obtained as in ref. [19] except that tobacco seedlings were used instead of tobacco tissue culture. A known weight of 21-day-old tobacco seedlings was homogenized in 2 × its wt of soln A by a motor-driven glass pestle in a glass homogenizer (45 sec, 2 strokes of the pestle). The homogenate was filtered through cheese-cloth and centrifuged (1000 *g*; 5 min). The resulting supernatant was then centrifuged at 20 000 *g* for 15 min in an MSE Mk I Ultracentrifuge fitted with a fixed angle rotor to produce pellet (P_{20}) and supernatent (S_{20}) fractions. The supernatant (S_{20}) was then centrifuged (200 000 *g*; 90 min) in a Beckman L-65 centrifuge fitted with a Ti-50 rotor to produce a pellet (P_{200}) fraction which was used as the microsomal prepn. The 200 000 *g* supernatant was subjected to 40% $(NH_4)_2SO_4$ fractionation to yield an insoluble enzyme fraction E_0^{40} which was pelleted by centrifugation (10 000 *g*; 5 min). The remaining supernatant was satd with 80% $(NH_4)_2SO_4$ soln to produce a further enzyme fraction E_{40}^{80} which was pelleted by centrifugation (as above). All pelleted fractions thus obtained (in particular P_{200} and E_{40}^{80}) were suspended in soln B, placed into dialysis tubing and dialysed *ca* 18 hr against ice-cold soln B. All operations were conducted at 0–4°. After dialysis an estimation of protein [23] was carried out on the P_{200} and E_{40}^{80} fractions and vols of the two fractions, each equivalent to 0.2 mg of protein, were mixed with soln C (0.125 ml) containing either hydroxymethylglutaryl-[3-^{14}C] co-enzyme A (HMG-CoA; 5 × 10^4 cpm; 1.5 μmol), acetate-[1-^{14}C] sodium salt (6 × 10^5 cpm; 2.3 μmol), or squalene-[^{14}C] (6 × 10^6 cpm). Where required, Amo 1618 was added to incubates at a final concn of 1 mg/ml. For acetate-[1-^{14}C] incorporation studies, the incubation mixture also contained glutathione (20 μmol), MVA (30 μmol) and glucose-6-phosphate (G-6-P; 48 μmol), and incubation was performed in a shaking water bath (30°, 2 hr). The incubation medium for HMG-[3-^{14}C]-CoA treatments included NADP (3 μmol), G-6-P (3 μmol), G-6-P dehydrogenase (1 I.U.), MVA (10 μmol), glutathione (10 μmol) and incubation was for 1 hr at 30°. The reactions were terminated by addition of KOH (30%, 0.5 ml) to each incubate and MVA was extracted as the lactone as described above. The total mevalonolactone extract was applied to Sil gel G TLC plates which were developed once in C_6H_6–Me_2CO (1:1) and the mevalonolactone was located according to ref. [24]. Radioactivity determinations were made by liquid scintillation counting. Squalene-[^{14}C] was prepared by incubating rat liver homogenates in the presence of 1.0 μCi DL-MVA-[2-^{14}C] under a constant stream of N_2. The rat liver homogenates and incubation procedures were carried out as described in ref. [4] except that the incubation was under a constant N_2 stream. After incubation the reaction was stopped by the addition of 30% KOH (2 ml), allowed to saponify for 16 hr at 25°, and the squalene-[^{14}C] extracted into petrol (bp 60–80°). The crude squalene-[^{14}C] was then purified by TLC using, successively, *n*-hexane and then *n*-hexane–EtOAc (19:1) as developing solvents. The final product gave 95–98% recovery of radioactivity in the squalene mass peak area when an aliquot was subjected to GLC.

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