The Stereospecific Biosynthesis of Plant Sterols and α - and β -Amyrin

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1. A preparation of pea seedlings has been obtained that will incorporate $[2-^{14}\text{C}]$ mevalonate into squalene, α - and β -amyrin and the phytosterols. 2. The $^{14}\text{C}/^3\text{H}$ ratio in α - and β -amyrin biosynthesized in the presence of $[2-^{14}\text{C}, 4R-^3\text{H}]$ -mevalonate is the same as in the starting material and in squalene; this gives experimental support to the mechanism for the cyclization of squalene proposed by Ruzicka for the formation of these pentacyclic triterpenoids. 3. The $^{14}\text{C}/^3\text{H}$ ratio for β -sitosterol was 5:3, the same as that in cholesterol in liver. 4. As the absence of ^3H from C-3 in β -sitosterol was demonstrated ^3H must be present in the side chain and thus the H at C-24 is not lost during alkylation of the side chain; it probably migrates to C-25.

In the biosynthesis of terpenoids the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate involves the expulsion of a proton from what was originally C-4 of MVA* (eqn. 1). Similarly the condensation of isopentenyl pyrophosphate with dimethylallyl pyrophosphate to form geranyl pyrophosphate (C_{10}) also involves the loss of a proton from C-4 of MVA (eqn. 2). The same mechanism is involved in subsequent chain elongation to farnesyl pyrophosphate (C_{15}) and geranylgeranyl pyrophosphate (C_{20}). Cornforth, Cornforth, Donninger & Popják (1966) have synthesized $[2-^{14}\text{C}, 4R-^3\text{H}]$ MVA and $[2-^{14}\text{C}, 4S-^3\text{H}]$ -MVA and used them to show that, in squalene (the C_{30} precursor of sterols produced by the dimerization of farnesyl pyrophosphate) synthesized by liver, the elimination of each proton arising from C-4 of MVA is stereospecific and that all the eliminations have the same stereospecificity; the

$4R$ -hydrogen† is retained, but the $4S$ -hydrogen is eliminated. At the suggestion of Dr J. W. Cornforth and Dr G. Popják, the same problem that arises during the biosynthesis of carotenoids was examined; Goodwin & Williams (1966) showed that the stereochemistry of hydrogen elimination during the formation of phytoene (the C_{40} precursor of carotenoids) in carrot slices was the same as that for squalene in liver; it was also shown that the $4R$ -hydrogen was specifically retained in the squalene synthesized by carrots.

The mechanism proposed for the conversion of squalene synthesized from $[2-^{14}\text{C}, 4R-^3\text{H}]$ MVA into cholesterol via lanosterol (eqn. 3) involves the loss of one of the six labelled carbon atoms and three tritium atoms (from C-3, C-5 and C-9) so that the $^{14}\text{C}/^3\text{H}$ ratio should change from 6:6 in squalene to 5:3 in cholesterol. This expected change has

† The $4R$ -hydrogen is that hydrogen which when replaced by tritium confers the R configuration on C-4.

* Abbreviation: MVA, mevalonic acid.

been demonstrated in liver preparations (Cornforth *et al.* 1965). The present investigation was undertaken partly to see if the same mechanism is involved in the synthesis of plant sterols such as stigmasterol (I) and β -sitosterol (II). Plants have a much greater triterpenoid biosynthetic capacity than animals; they can, for example, synthesize pentacyclic triterpenoids such as α -amyrin (III) and β -amyrin (IV). Ruzicka's group (Eschenmoser, Ruzicka, Jeger & Arigoni, 1955) have suggested that β -amyrin is formed from squalene by elimination of a proton from C-12 with simultaneous transfer of hydride ions from C-13 to C-18 and from C-18 to C-19 (eqn. 4). Further steps are necessary to produce α -amyrin by the same basic mechanism (eqn. 5). If this basic mechanism is functioning *in vivo* then α -amyrin and β -amyrin synthesized from $[2\text{-}^{14}\text{C}, 4\text{R-}^3\text{H}]\text{MVA}$ would have

the same $^{14}\text{C}/^3\text{H}$ ratio as the squalene formed at the same time.

We have developed a system from peas that synthesizes squalene, α -amyrin and β -amyrin and phytosterols from MVA, and have devised analytical methods for preparing these compounds radioactively pure. Through the kindness of Dr Cornforth and Dr Popják, who kindly gave us $[2\text{-}^{14}\text{C}, 4\text{R-}^3\text{H}]\text{MVA}$, we have been able to test directly the validity of Ruzicka's proposed cyclizing mechanisms. A brief report of some of this work has already been published (Rees, Mercer & Goodwin, 1965).

EXPERIMENTAL

Seed germination and incubation with labelled substrate. Seeds (50) of *Pisum sativum* (var. Onward) were germinated in a 10 cm. Petri dish in the presence of the labelled MVA

in 3 ml. of water. To avoid excessive evaporation of water during the initial stages of germination the Petri dish was placed in a larger Pyrex dish containing filter paper moistened with water and loosely covered with aluminium foil. In this way all the MVA is absorbed in about 3-5 hr. (Baisted, Capstack & Nes, 1962). Germination was then continued for 117 hr., during which time the peas were kept damp by the addition of water when necessary. Previous work had shown that maximum activity is found in the amyirin fraction at this time (Baisted *et al.* 1962). At the end of the germination period, shoots 1-2 cm. long had developed.

Extraction and separation of terpenoids. At the end of the incubation the seedlings were washed with water and extracted with redistilled acetone (3 × 90 ml.) in an MSE homogenizer; the residue was then refluxed (30 min.) with acetone (100 ml.), cooled and filtered. The combined acetone extract was diluted with water (3 vol.) and extracted with redistilled diethyl ether (3 × 300 ml.). The combined ether extracts were washed free from acetone with water, dried overnight over anhydrous Na₂SO₄, distilled to small volume and then evaporated to dryness under N₂. The residue was saponified and the unsaponifiable matter extracted by the method described in detail by Goodwin (1955). The unsaponifiable fraction was then chromatographed on an alumina (Brockmann grade III) column (20 g.) and 200 ml. volumes of the following fractions were collected: light petroleum (b.p. 40-60°) (I); light petroleum containing 2% (v/v) of diethyl ether (II); light petroleum containing 4% (v/v) of diethyl ether (III); light petroleum containing 6% (v/v) of diethyl ether (IV); light petroleum containing 14% (v/v) of diethyl ether (V); diethyl ether (VI).

Fraction I. This contained squalene (Davies, Jones & Goodwin, 1963; Mercer, Davies & Goodwin, 1963); it was purified by chromatography on a Brockmann grade II

alumina column, from which the squalene was eluted with light petroleum. This fraction was then chromatographed on a Kieselgel G thin-layer plate sprayed with Rhodamine 6G (Avigan, Goodman & Steinberg, 1963) with light petroleum as developing solvent. The dried adsorbent layers were lightly sprayed with aq. 0.3% Rhodamine 6G solution and dried for 30 min. at 105° before use. The squalene spot was made visible under u.v. light; the spot was scraped off and eluted with chloroform.

Fraction II. This was discarded.

Fractions III and IV. These were combined for examination. The material was chromatographed on a Rhodamine 6G-impregnated Kieselgel G thin-layer plate together with lanosterol, β -sitosterol and the amyirins as markers and with chloroform as developing solvent. The system does not separate amyirins from 4-dimethylsterols but does run the mixture clear of tocopherols, 4 α -methyl sterols and sterols. The crude amyirin band (R_f 0.43) was made visible with u.v. light, scraped off and eluted with chloroform. It was rechromatographed on the same system, eluted and acetylated by dissolving the residue in pyridine (2 ml.), adding acetic anhydride (4 ml.) and keeping overnight under N₂. The mixture was then poured into ice-cold HCl (5N) in a separating funnel and left for 30 min. to allow the excess of acetic anhydride to decompose. The mixture was then extracted three times with diethyl ether; the combined extract was washed with water (once) and NaHCO₃ solution until free of acetic acid, and then with water until free of alkali. The ether solution was dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue was then chromatographed on a column (2 g.) of alumina (Brockmann grade III) to separate the acetates from unchanged original material. The acetates were removed with light petroleum (b.p. 40-60°) containing 2% (v/v) of diethyl ether.

The acetates were then chromatographed on a AgNO_3 -impregnated Rhodamine G-sprayed Kieselgel G thin-layer plate, which was developed for 14 cm. with light petroleum (b.p. 40 – 60°) containing 20% (v/v) of benzene as developer. The plates are prepared by lightly spraying the dried adsorbent layer with a saturated solution of AgNO_3 in aq. 90% (v/v) ethanol, and then drying at 105° for 30 min. The plates were then sprayed with Rhodamine G in the usual way. This system clearly separates α - and β -amyrin from any cycloartenol and lanosterol, R_F values of the two groups being 0.20 and 0.08 respectively. This system did not separate α -amyrin from β -amyrin.

Gas-liquid radiochromatography (see below) showed the presence of a trace of impurity in the α - and β -amyrin fraction from peas. This was removed by chromatography on a Rhodamine G-sprayed plate of kieselguhr G (Merck)-aluminium oxide G (Merck) (4:1, w/w) with light petroleum as developing solvent. The purified α - and β -amyrin spot (R_F 0.22) was removed and eluted with chloroform. As kieselguhr is a very weak adsorbent some Rhodamine 6G was eluted with the amyryns. It was removed by chromatographing the samples on an alumina Brockmann grade III column and collecting the amyryns in the fraction eluted with light petroleum containing 2% (v/v) of diethyl ether; the Rhodamine 6G remained at the top of the column.

Fraction V. This fraction contains the sterols, which were purified by precipitation as digitonides (Windaus, 1909) and regeneration therefrom (Schoenheimer & Dam, 1933). The regenerated sterols were purified by chromatography on a Rhodamine 6G-sprayed Kieselgel G thin-layer plate with chloroform as developer. The sterol band was made visible and eluted in the usual manner. It contained β -sitosterol as the major component plus small and approximately equal amounts of stigmasterol and campesterol.

Fraction VI. This was discarded.

The separation procedures used are summarized in Scheme 1.

Oppenauer oxidation of phytosterols. To ascertain whether or not the tritium at C-3 was lost during the formation of the phytosterols, as in cholesterol biosynthesis, a portion of the doubly labelled phytosterols mixture was oxidized to the 3-oxo- Δ^4 sterol by the Oppenauer method (Meystre, Frey, Neher, Wettstein & Miescher, 1946). During this reaction, the Δ^5 -bond isomerizes to Δ^4 , but this does not affect the tritium-labelling pattern.

Labelled sterol (4.9 mg.) plus 4.7 mg. of purified carrier β -sitosterol were dissolved in 0.1 ml. of redistilled cyclohexanone and 0.35 ml. of dry redistilled toluene. A very

small amount of toluene (approx. 0.2 ml.) was distilled off, and then 6 mg. of aluminium isopropoxide (freshly distilled at 140 – 150° at 12 mm. Hg) in 0.75 ml. of dry toluene was added and the mixture refluxed for 45 min., with the condenser protected with a CaCl_2 tube. Then approx. 1 ml. of a concentrated solution of sodium potassium tartrate was added and the mixture steam-distilled to remove cyclohexanone. The mixture was transferred to a separating funnel and the aqueous layer extracted with diethyl ether (3×20 ml.). The combined ether extracts were washed with sodium potassium tartrate solution, approx. 3N- H_2SO_4 and then with water, dried over Na_2SO_4 , distilled to a small volume and then evaporated to dryness under N_2 .

The crude oxidation product was chromatographed together with marker spots of β -sitosterol and Δ^4 -stigmasterol-3-one on a Rhodamine 6G-sprayed Kieselgel G chromatoplate developed with 5% (v/v) of ethyl acetate in benzene. The ultraviolet-absorbing oxo sterol band (R_F 0.29) was clearly separated from any unchanged sterol (R_F 0.17), and was eluted. Any Rhodamine 6G was removed from the sample by chromatography on a 2 g. Brockmann grade II alumina column; the oxo sterols were collected in the fraction eluted with light petroleum containing 14% (v/v) of diethyl ether; the Rhodamine 6G remained at the top of the column.

Gas-liquid chromatography. All experiments were carried out on a Pye Argon Chromatograph. Pilot experiments showed that α -amyrin, β -amyrin and lupeol can all be separated on a column of 5% of H.I.-EFF3A (neopentylglycol adipate) on Gas-Chrom P (80–100 mesh). α -Amyrin and β -amyrin can also be separated on columns of 5% Dow Corning QF-1 silicone and 3% SE-30 methyl silicone rubber gum, but these columns will not resolve α -amyrin and lupeol, nor their acetates. The retention times relative to cholestane of the compounds on the three columns are given in Table 1.

The amyirin sample from pea seedlings was shown to consist of β -amyrin containing approx. 16% of α -amyrin by gas-liquid chromatography, both as the acetates and as the free compounds on 5% QF-1, 5% neopentylglycol adipate and 3% SE-30 columns. It was shown with the neopentylglycol adipate column that there was no lupeol present in peas.

The phytosterol mixture was shown, on an SE-30 column, to contain β -sitosterol as the major component, plus small and about equal amounts of stigmasterol and campesterol.

Scheme 1. Summary of isolation procedure.

Isotope assay. The various fractions were assayed for ^{14}C and ^3H in a Packard Tri-Carb Scintillation Spectrometer.

RESULTS AND DISCUSSION

The pea seeds were germinated for 5 days in the presence of $[2\text{-}^{14}\text{C}, 4\text{R-}^3\text{H}]\text{MVA}$ ($0.176\text{ }\mu\text{C}$ of ^{14}C and $1.869\text{ }\mu\text{C}$ of ^3H). The unsaponifiable matter was separated as indicated in Scheme 1. The separation and purification of squalene and sterols have been recorded previously, but Scheme 1 represents the only satisfactory thin-layer method that we have found for the separation of α - and β -amyrin from lanosterol and cycloartenol (over 30 other systems were examined; H. H. Rees, unpublished work). This method will not separate α -amyrin from β -amyrin or lupeol, but controls with gas-liquid chromatography on 5% neopentylglycol adipate showed that no lupeol was present in the pea seedlings and that about one-sixth of the total

amyrin fraction was α -amyrin. It was this mixture that was assayed for ^{14}C and ^3H ; the fact that it is a mixture which is examined is of no significance; the extra intramolecular rearrangements of the basic cyclized intermediate, which is required to form α -amyrin rather than β -amyrin, does not involve the loss of any tritium atoms when $[2\text{-}^{14}\text{C}, 4\text{R-}^3\text{H}]\text{MVA}$ is the substrate.

The labelling patterns obtained in the various compounds are recorded in Table 2. Values for squalene from liver (kindly supplied by Dr Popják) are included as controls. In the first place the $^{14}\text{C}/^3\text{H}$ ratio is the same in the α - and β -amyrin fractions as in squalene and as in the original mevalonate, i.e. 1:1. Thus no tritium is lost during the cyclization of squalene to β -amyrin and the pathway outlined in eqn. (4) represents the probable mechanism for the synthesis of pentacyclic triterpenoids of the amyryn type.

The observed $^{14}\text{C}/^3\text{H}$ ratio in the purified sterol fraction, mainly β -sitosterol but containing traces of campesterol and stigmasterol, approaches 5:3, the same as that observed in liver cholesterol (Cornforth *et al.* 1965).

If the conversion of squalene into β -sitosterol in plants is strictly analogous to the formation of cholesterol from squalene in animals, then the three tritium atoms would be lost at C-3 (during the formation of 3-oxo compound while the *gem*-dimethyl group was being oxidized, at C-4) (Lindberg, Gautschi & Bloch, 1963), at C-5 and at C-9 (eqn. 6). However, a complication is the ethylation at C-24, which does not occur in animals. A tritium atom could conceivably be lost during this process, and if no tritium was lost from C-3 then the observed $^{14}\text{C}/^3\text{H}$ ratio 5:3 would still be expected. The loss of tritium from C-3 has now

Table 1. *Retention times relative to cholestane of various pentacyclic triterpenoids on different columns*

Abbreviation: NGA, neopentylglycol adipate.

Compound	Stationary phase		
	QF-1	NGA	SE-30
α -Amyrin	5.76	12.94	3.65*
β -Amyrin	5.30	11.24	3.31*
Lupeol		14.28	3.73*
α -Amyrin acetate	9.66		4.94
β -Amyrin acetate	8.58		4.51
Lupeol acetate			5.15

* B. L. Williams (personal communication).

Table 2. $^3\text{H}/^{14}\text{C}$ ratios in squalene, α - and β -amyrin and the phytosterol from pea seedlings incubated with $[2\text{-}^{14}\text{C}, 4\text{R-}^3\text{H}]\text{mevalonic acid}$

Details are given in the Experimental section.

Substance	Radioactivity (disintegrations/min.)		$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio	Mean atomic ratio ($\pm$ S.E.M.)
	^3H	^{14}C		(based on liver squalene)	(based on pea squalene)	
$[2\text{-}^{14}\text{C}, 4\text{R-}^3\text{H}]\text{MVA}$	185729	37714	4.925			
Squalene (liver)	30105	6084	4.948			
Squalene (peas), sample 1	18377	3651	5.033	6.10:6		6.10 $\pm$ 0.08
Squalene (peas), sample 2	4972	971	5.120	6.21:6		6.21 $\pm$ 0.11
α - and β -Amyrin (peas), sample 1	28252	5463	5.172	6.27:6	6.11:6	6.11 $\pm$ 0.03
α - and β -Amyrin (peas), sample 2	51467	10159	5.066	6.14:6	5.99:6	5.99 $\pm$ 0.15
Sterols (<i>ex plate</i>)	3148	1129	2.788	2.82:5	2.75:5	2.75 $\pm$ 0.03
3-Oxo sterols	2292	791	2.898	2.93:5	2.86:5	2.86 $\pm$ 0.06

*Hypothetical intermediates to show positions of ¹⁴C and ³H at various stages of formation of phytosterols.

been clearly demonstrated; the β -sitosterol with a 5:3 ratio was oxidized to the corresponding 3-oxo- Δ^4 sterol, which was found to have the same ¹⁴C/³H ratio as β -sitosterol (Table 2). Therefore, unless an entirely different mechanism for cyclization of squalene exists in plants, it can be concluded that the proton at C-24 is not lost during the alkylation of the side chain. It is, however, almost certainly shifted to C-25; Goad & Goodwin (1965) have demonstrated that fucosterol (24-ethylidene-cholesterol) synthesized by *Fucus spiralis* in the presence of [2-¹⁴C,4R-³H]MVA exhibits a ¹⁴C/³H ratio 5:3. An analogous result has been observed with eburicoic acid (A. S. A. Hammam, unpublished work).

The sterol fraction from carrot-root slices, which consists mainly of β -sitosterol, also show the same ratio, namely 5:3 (experiments carried out by Mr R. J. H. Williams during investigations on the stereospecificity of carotenoid biosynthesis; Goodwin & Williams, 1965, 1966).

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