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MECHANISM OF BIOSYNTHESIS OF CYCLOARTENOL

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1. The mechanism of cycloartenol biosynthesis in leaves of *Solanum tuberosum* was investigated with the use of [2-^{14}C,(4*R*)-4-$^{3}H_1$]mevalonic acid. 2. The $^{3}H/^{14}C$ atomic ratio in cycloartenol was 6:6, the same as that in squalene; this eliminates lanosterol as a possible biosynthetic precursor of cycloartenol, and indicates that a hydrogen migration from C-9 to C-8 occurs. 3. Chemical isomerization of the cycloartenol to lanosterol ($^{3}H/^{14}C$ ratio 5:6) and parkeol ($^{3}H/^{14}C$ ratio 6:6) confirms the hydrogen migration from C-9 to C-8. 4. Possible mechanisms for the biosynthesis of cycloartenol and parkeol are discussed. 5. The $^{3}H/^{14}C$ ratio for 24-methylenecycloartanol was 6:6, demonstrating that the hydrogen atom at C-24 is retained during alkylation of the cycloartenol side chain.

It has been suggested (Ardenne, Osske, Schreiber, Steinfelder & Tümmler, 1965; Benveniste, Hirth & Ourisson, 1966*a*,*b*; Goad, 1967) that cycloartenol may replace lanosterol as the first product of squalene cyclization in phytosterol biosynthesis. It was therefore decided to examine the mechanism of cycloartenol biosynthesis from squalene.

A mechanism for the cyclization of squalene to lanosterol was proposed by Woodward & Bloch (1953) and Eschenmoser, Ruzicka, Jeger & Arigoni (1955) (also see Ruzicka, 1953, 1959) and is shown in Scheme 1. In this Scheme lanosterol is produced by cyclization of squalene in the chair–boat–chair–boat conformation (I), followed by rearrangement to produce structure (II), which by a series of hydrogen and methyl migrations and proton loss from C-9 (sterol numbering) gives lanosterol (III). Conclusive evidence for the methyl migrations has been obtained in lanosterol biosynthesis (Maudgal, Tchen & Bloch, 1958; Cornforth, Cornforth, Horning, Pelter & Popják, 1959; Cornforth, Cornforth, Pelter, Horning & Popják, 1959). With [2-^{14}C,(4*R*)-4-$^{3}H_1$]MVA* as substrate, Cornforth *et al.* (1965) demonstrated the hydrogen migrations and also that the tritium atom at C-9 was eliminated during lanosterol synthesis by liver preparations.

A similar chair–boat–chair–boat cyclization of squalene (Richards & Hendrickson, 1964; Goad, 1967) could also produce cycloartenol (IV) (Scheme 1), but instead of being lost the C-9 hydrogen migrates to C-8, followed by migration of the methyl group (C-19) from C-10; this migration is intercepted by loss of a proton from the migrating methyl group, with formation of a 9β,19-cyclopropane ring. The availability of [2-^{14}C,(4*R*)-4-$^{3}H_1$]MVA has now allowed the hydrogen migration from C-9 to C-8 to be experimentally investigated. Since potato leaves have been reported to be a rich source of cycloartenol (Schreiber & Osske, 1962), this material was used for incorporation of the labelled MVA.

* Abbreviation: MVA, mevalonic acid.

EXPERIMENTAL

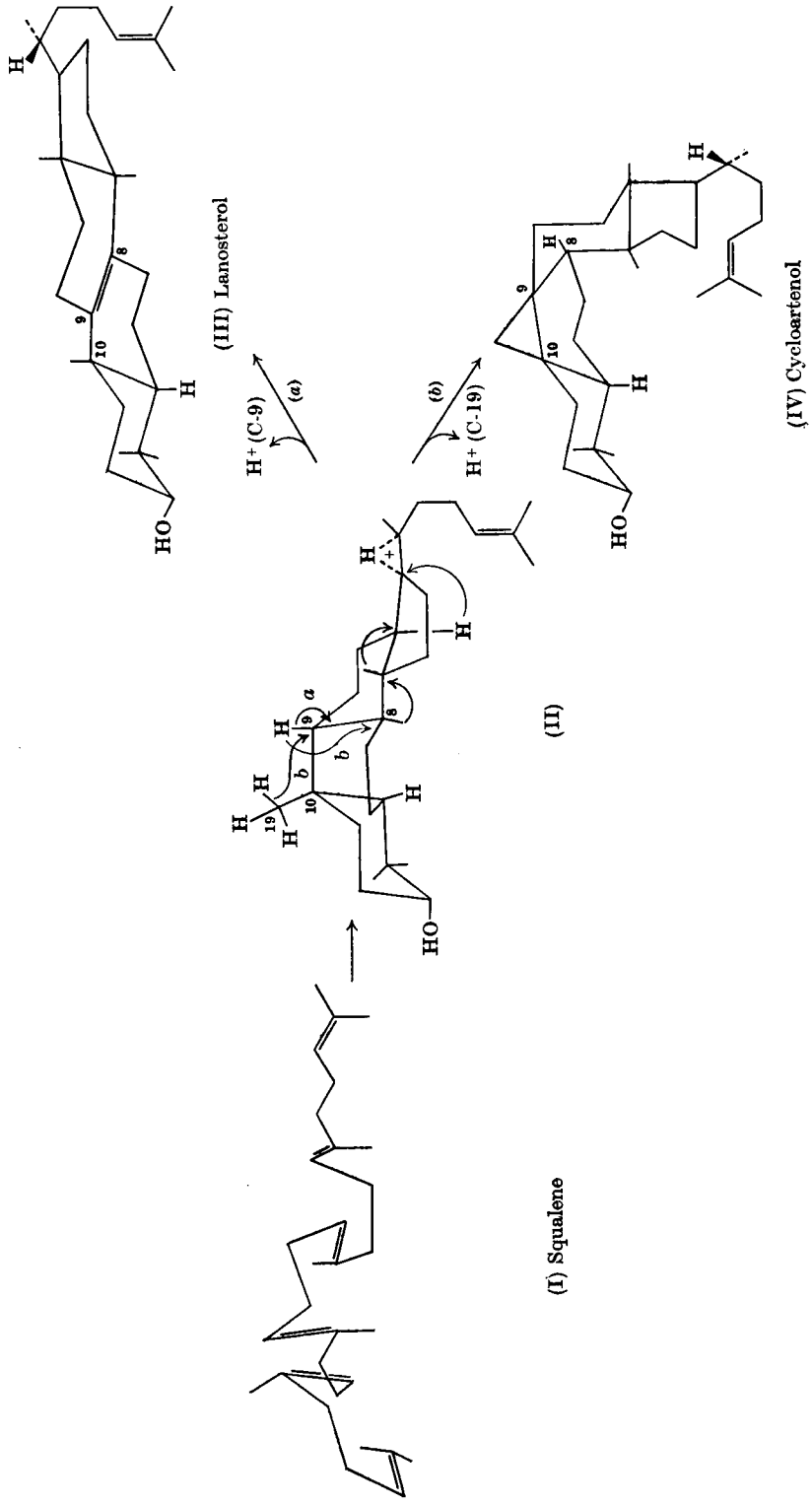
Nomenclature. Trivial names are used. Systematic names are as follows: lanosterol, lanosta-8,24-dien-3β-ol; parkeol, lanosta-9(11),24-dien-3β-ol; cycloartenol, 9,19-cyclo-9β-lanost-24-en-3β-ol; 24-methylenecycloartanol, 24-methylene-9,19-cyclo-9β-lanostan-3β-ol; β-sitosterol, stigmast-5-en-3β-ol; stigmasterol, stigmasta-5,22-dien-3β-ol; cholesterol, cholest-5-en-3β-ol; campesterol, 24α-methylcholest-5-en-3β-ol; brassicasterol, ergosta-5,22-dien-3β-ol.

Radiochemicals. [2-^{14}C]MVA and [(4*R*)-4-$^{3}H_1$]MVA were purchased from The Radiochemical Centre, Amersham, Bucks., and mixed to produce 3*RS*-[2-^{14}C,(4*R*)-4-$^{3}H_1$]MVA.

Solvents. Diethyl ether was dried over sodium wire and then distilled over reduced iron to remove peroxides. Light petroleum (b.p. 40–60° and 60–80°) was dried over sodium wire and redistilled.

Thin-layer chromatography. Thin-layer chromatography was carried out on (*a*) silica gel (Kieselgel G; E. Merck A.-G., Darmstadt, Germany) and (*b*) silica gel impregnated with 10% (w/w) $AgNO_3$. In (*a*), Rhodamine 6G was incorporated into the plates during their preparation and compounds were located by examination under ultraviolet light (Avigan, Goodman & Steinberg, 1963). In (*b*), the developed plates were sprayed with a solution of Rhodamine 6G in acetone and the compounds were again located under ultraviolet light.

Compounds were chromatographed as bands together with spots of appropriate marker compounds. After



Scheme 1. Cyclization of squalene to lanosterol and cycloartenol.

location, bands were scraped off and eluted with dry diethyl ether. Occasionally traces of Rhodamine 6G were also eluted and were removed by chromatography on an alumina (Brockmann grade III) column (2g.) developed with 50% (v/v) diethyl ether in light petroleum (b.p. 40–60°).

Gas–liquid chromatography. A Varian–Aerograph 1522 B instrument with flame-ionization detectors and on-column injection was used. Stainless-steel columns (6ft. × ⅛in.) were packed with 80–100 mesh silane-treated Chromosorb W coated with (*a*) 1% QF–1, (*b*) 1% SE–30, (*c*) 3% XE–60 or (*d*) 3% SE–30. The coating procedure was carried out by a filtration technique (Horning, Vanden Heuvel & Creech, 1963). Owing to slight variability of retention times, authentic sterols were normally chromatographed separately on the same day as the unknown compounds for identification purposes.

Preparative gas–liquid chromatography was carried out on a 3% XE–60 column, fitted with an effluent splitter, and compounds were collected as they were eluted from the column in glass capillary tubes at room temperature. The collected samples were rendered free of any traces of stationary phase by thin-layer chromatography on silica gel. Chloroform was used as the developing solvent for free sterols and 40% (v/v) benzene in light petroleum (b.p. 60–80°) for acetates.

Plant material and incubation with labelled substrate. Potatoes (*Solanum tuberosum* var. Majestic) were grown for about 1 month. A sample (5g.) of leaves was chopped into strips 1–2mm. wide with a razor blade and transferred to two 50ml. conical flasks, each containing 1ml. of 3*RS*-[2-^{14}C,(4*R*)-4-$^{3}H_1$]MVA (total: 0·68μmole containing 25μc of ^{3}H and 2·5μc of ^{14}C). Incubation in the stoppered flasks was carried out for 4hr. at 25° with illumination by two daylight-emission lamps (Cryselco, 20w, 2ft.) placed 25cm. above the flasks. A further 0·5ml. of water was added to each flask after 2hr. and again after 3hr.

Extraction and separation of terpenoids. After 4hr. the contents of the flasks were combined and saponified directly before extraction by refluxing for 1½hr. with 60ml. of ethanol, 6ml. of water and 7g. of KOH. After the debris had been filtered off, the ethanolic extract was diluted with water (180ml.) and the non-saponifiable material extracted with diethyl ether in the usual manner.

A mixture of cycloartenol and 24-methylenecycloartanol (12mg.) plus 4α-methyl sterols (2mg.) and phytosterols (5mg.) previously isolated from potato leaves were added to the non-saponifiable material (40mg.) as carrier. The 3β-hydroxy sterols were precipitated from the non-saponifiable material by the method of Windaus (1909). To precipitate as much as possible of the 4,4′-dimethyl sterols, the volume of solvents normally used was approximately halved. A boiling solution of 300mg. of digitonin in 6ml. of aq. 90% (v/v) ethanol was added to a boiling solution of the non-saponifiable material in 8ml. of aq. 95% (v/v) ethanol and the precipitation completed at 0–4° overnight. The precipitated digitonides were centrifuged and washed twice with diethyl ether only. Ethanol was avoided as a solvent because of the solubility of triterpenoids with a 4,4′-dimethyl grouping in this solvent (Ohta & Shimizu, 1957). The precipitation procedure was repeated to precipitate more of the 4,4′-dimethyl sterols, and the sterols were regenerated from the combined digitonides by treatment with pyridine (Schoenheimer & Dam, 1933). Excess of digitonin was removed from the 'sterol-free' non-saponifiable material by addition of excess of diethyl ether, followed by centrifugation to remove precipitated digitonin and washing with water. The 'sterol-free' non-saponifiable material was dried over anhydrous Na_2SO_4 and evaporated to dryness.

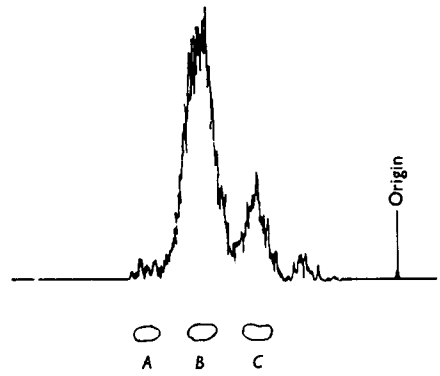


Fig. 1. Radioscan of a thin-layer chromatogram on $AgNO_3$-impregnated silica gel of the potato 4,4′-dimethyl sterols. Markers: *A*, β-amyrin acetate; *B*, cycloartenyl acetate; *C*, 24-methylenecycloartanyl acetate.

The digitonin-precipitable 'sterols' (15mg.) were separated into 4,4′-dimethyl sterols, 4α-methyl sterols and major sterols (4-demethyl sterols) by thin-layer chromatography on silica gel with chloroform as developing solvent.

The 4,4′-dimethyl sterols were acetylated with acetic anhydride and pyridine, and the acetates (7mg.) chromatographed on $AgNO_3$-impregnated silica-gel plates developed with 50% (v/v) benzene in light petroleum (b.p. 60–80°). A radioscan (Desaga scanner) of such a plate is shown in Fig. 1. The bands corresponding to the acetates of cycloartenol (R_F 0·53) and 24-methylenecycloartanol (R_F 0·46) were eluted and rechromatographed on the same system to effect further purification. A portion of the cycloartenyl acetate was also subjected to preparative gas–liquid chromatography after the addition of carrier lanosteryl acetate and the two peaks were collected and assayed for ^{3}H and ^{14}C.

Purification of squalene. The 'sterol-free' non-saponifiable material (37mg.) was chromatographed on a Brockmann grade III alumina column (5g.) developed with 50ml. of light petroleum (b.p. 40–60°). Squalene was purified from the light petroleum fraction by thin-layer chromatography on silica gel with light petroleum (b.p. 60–80°) for development.

Opening of cyclopropane ring of cycloartenol. A portion of the labelled cycloartenyl acetate was isomerized by treatment with dry HCl (Barton, 1951; Bentley, Henry, Irvine & Spring, 1953) to give the acetates of lanosterol and parkeol. During this reaction, the Δ^{24}-bond was protected by formation of the dibromide (Schreiber & Osske, 1964).

The doubly labelled cycloartenyl acetate diluted with unlabelled cycloartenyl acetate (8mg.) was dissolved in carbon tetrachloride (3ml.) and a solution of bromine in carbon tetrachloride (0·2ml. of bromine/50ml.) was added drop by drop until a faint yellow colour persisted. The

reaction mixture was allowed to stand for 30min., evaporated to dryness under N_2 and redissolved in diethyl ether. The ethereal solution was washed successively with 1% (w/v) Na_2SO_3 solution and water, dried over anhydrous Na_2SO_4 and evaporated to dryness.

A vigorous stream of dry HCl was passed through a solution of the 24,25-dibromocycloartenyl acetate in dry chloroform (2ml.) at room temperature for 45min. The reaction mixture was then carefully evaporated to dryness under N_2. The reaction product was dissolved in diethyl ether (5ml.) containing 2 drops of acetic acid and stirred for 5hr. with 20mg. of zinc dust. The mixture was finally filtered and the ethereal solution evaporated to dryness.

A preliminary separation of the reaction products was effected by thin-layer chromatography on $AgNO_3$-impregnated silica gel (see the Results section).

Radioassays. ^{14}C and ^3H counting was carried out on a Beckmann LS200B liquid-scintillation counter. Samples were dissolved in 10ml. of scintillator containing 5·0g. of 2,5-diphenyloxazole and 0·3g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene. The counting efficiencies for ^{14}C and ^3H were 63% and 50% respectively.

Identification of sterols of potato leaves. Before the experiment with radioactive material was carried out, an extraction of potato leaves was made to identify some of the sterol components. A sample (1100g.) of leaves was crushed and then saponified directly by refluxing for 3hr. with 1400ml. of ethanol, 140ml. of water and 168g. of NaOH. The non-saponifiable material (4·43g.) was extracted from the filtered mixture with diethyl ether in the usual manner. The sterols were obtained by double digitonin precipitation and the regenerated sterols (0·23g.) chromatographed on a Brockmann grade III alumina column (20g.). The following fractions (200ml. volumes) were collected: (I) light petroleum (b.p. 40–60°); (II) 2% (v/v) diethyl ether in light petroleum; (III) 6% (v/v) diethyl ether in light petroleum; (IV) 9% (v/v) diethyl ether in light petroleum; (V) 20% (v/v) diethyl ether in light petroleum. 4,4′-Dimethyl sterols, 4α-methyl sterols and the major sterols were mostly present in fractions (III), (IV) and (V) respectively. The 4,4′-dimethyl and major sterols were further purified by thin-layer chromatography as described above for the experiment with radioactive material. These sterols were then

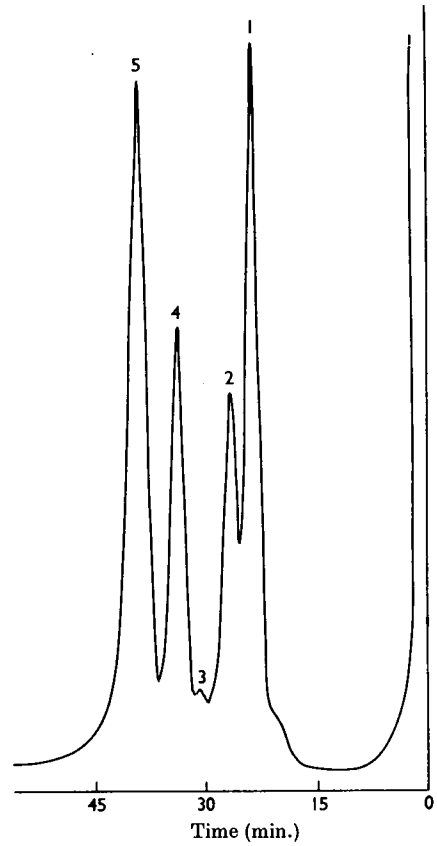


Fig. 2. Gas–liquid chromatography of the major sterols from potato leaves on 3% SE-30. Retention times relative to cholestane were: peak 1, 1·72; peak 2, 2·25; peak 3, 2·61; peak 4, 2·83; peak 5, 3·28. Relative retention times of authentic sterols were: β-sitosterol, 3·30; stigmasterol, 2·87; campesterol, 2·67; cholesterol, 1·94; brassicasterol, 2·21.

Table 1. $^3H/^{14}C$ *ratios in the triterpenes isolated from potato leaves after incubation with* [2-^{14}C,(4*R*)-4-$^3H_1$]-*MVA, and in lanosteryl acetate and parkeyl acetates obtained by hydrogen chloride isomerization of the cycloartenyl acetate*

Substance	Radioactivity (counts/min.) ^3H	Radioactivity (counts/min.) ^{14}C	$^3H/^{14}C$ radioactivity ratio	$^3H/^{14}C$ atomic ratio*
Squalene (sample 1)	35115	5160	6·805	
Squalene (sample 2)	31329	4621	6·780	
Cycloartenyl acetate (isolated by thin-layer chromatography)	11396	1644	6·932	6·12:6
Cycloartenyl acetate (isolated by preparative gas–liquid chromatography)	5752	824	6·981	6·17:6
24-Methylenecycloartanyl acetate	10852	1569	6·917	6·11:6
4-Demethyl phytosterols	8583	1910	4·494	3·31:5
Lanosteryl acetate†	5541	1003	5·524	4·88:6
Parkeyl acetate†	15132	2243	6·746	5·96:6

* Based on the average of the $^3H/^{14}C$ ratios obtained for squalene.

† Formed from cycloartenyl acetate by HCl isomerization (see the Experimental section).

analysed by gas–liquid chromatography. The 4,4′-dimethyl sterols were shown to consist of predominantly cycloartenol (approx. 98%), as reported by Schreiber & Osske (1962). A very minor peak (approx. 1%) was observed with retention data identical with those of 24-methylenecycloartanol. The 4-demethyl sterol mixture was complex, but had components with retention times on an SE-30 column corresponding to β-sitosterol, stigmasterol, campesterol, brassicasterol and, possibly, cholesterol (Fig. 2).

RESULTS

After incorporation of the [2-^{14}C,(4*R*)-4-$^{3}H_1$]MVA into potato leaves, the squalene, cycloartenyl acetate and 4-demethyl sterols were isolated as described in the Experimental section and the $^{3}H/^{14}C$ ratios determined (Table 1). The cycloartenyl acetate had a $^{3}H/^{14}C$ ratio identical with that of squalene. Moreover, cycloartenyl acetate obtained by preparative gas–liquid chromatography also had a $^{3}H/^{14}C$ ratio in close agreement and corresponded to a $^{3}H/^{14}C$ atomic ratio of 6:6, i.e. the same as squalene (Cornforth, Cornforth, Donninger & Popják, 1966). There was negligible activity in the lanosteryl acetate isolated during this preparative gas–liquid-chromatographic separation. The $^{3}H/^{14}C$ ratio for the 4-demethyl sterols represented a $^{3}H/^{14}C$ atomic ratio of 3:5 as found previously in cholesterol from liver (Cornforth *et al.* 1965) and in phytosterols (Rees, Mercer & Goodwin, 1966).

The hydrogen chloride isomerization of cycloartenyl acetate was first carried out on non-radioactive material to characterize the products before application of the method to the doubly labelled material. A preliminary separation of the reaction products together with the acetates of lanosterol and parkeol as markers was carried out by thin-layer chromatography on silver nitrate-impregnated silica gel developed with 50% benzene in light petroleum (b.p. 60–80°). Three bands were eluted corresponding to the acetates of parkeol (R_F 0·47) (band *A*), lanosterol (R_F 0·53) (band *B*) and a very minor band (*C*) of slightly higher mobility than lanosterol. Each band was examined by gas–liquid chromatography with QF–1 as stationary phase. The results are shown in Fig. 3.

Lanosteryl acetate was purified from band *B* (peak 1*b*) by preparative gas–liquid chromatography on a 3% XE–60 column and was shown to co-chromatograph with the authentic compound on QF–1. Mass spectrometry showed a mass peak at *m/e* 468 and other peaks at *m/e* 453 [$M^+ - CH_3$] and 393 [$M^+ - (CH_3 + \text{acetate})$]. Only a very minor peak was observed at *m/e* 286; this ion is characteristic of cycloartenol and is due to loss of ring A in 9β,19-cyclopropane sterols (Benveniste *et al.* 1966*a*,*b*; Audier, Beugelmans & Das, 1966; Aplin & Hornby, 1966). Band *A* material was pure (Fig. 3) and had the same retention time as authentic

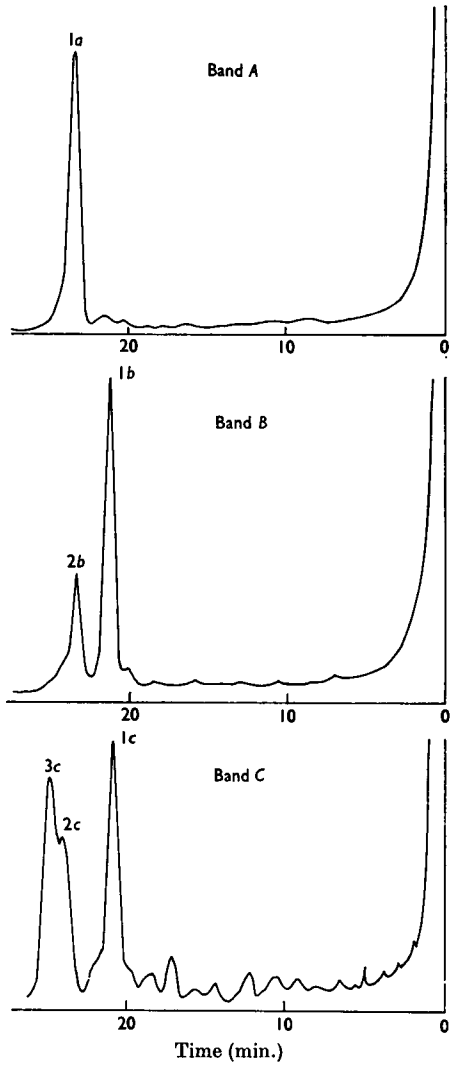
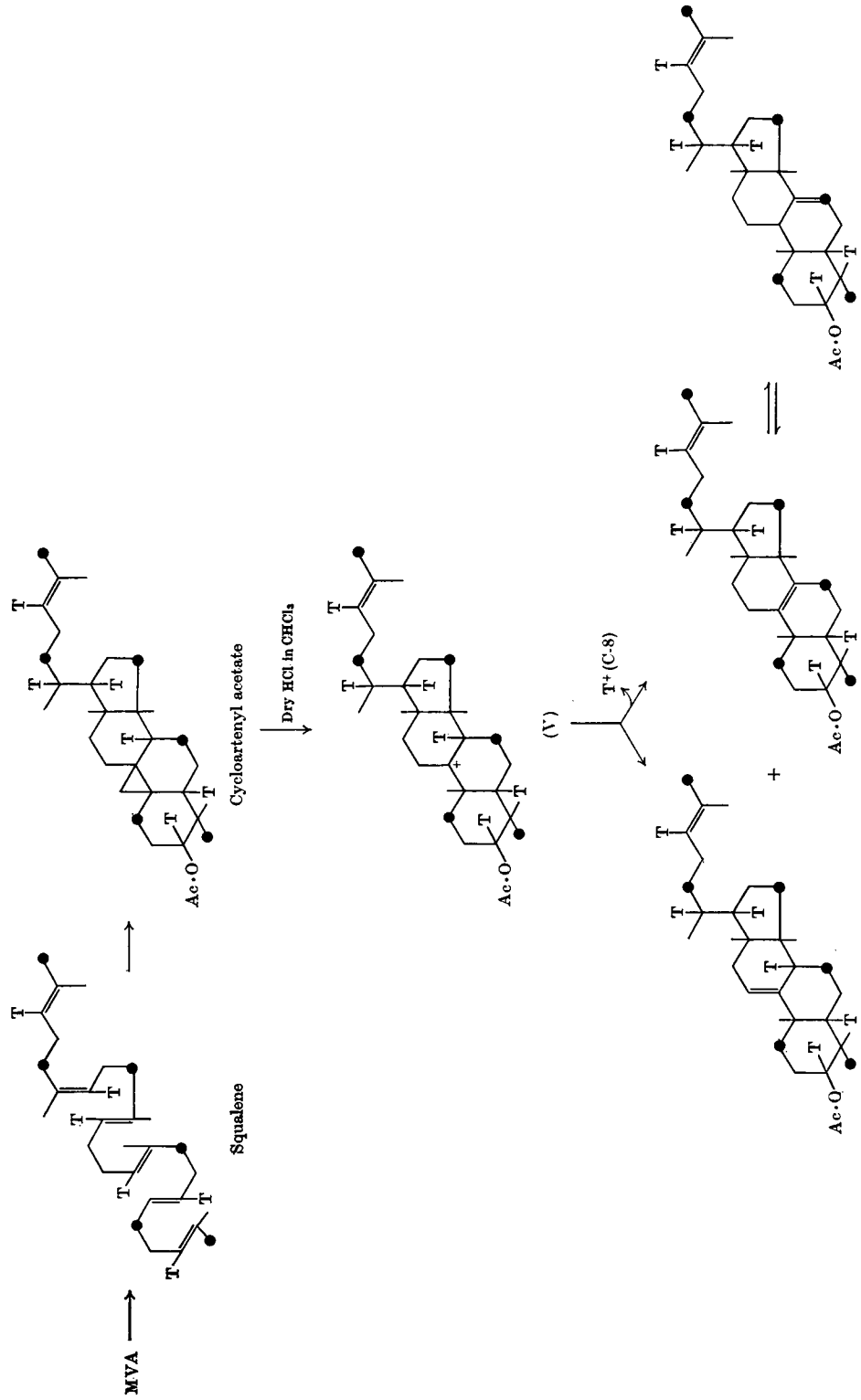


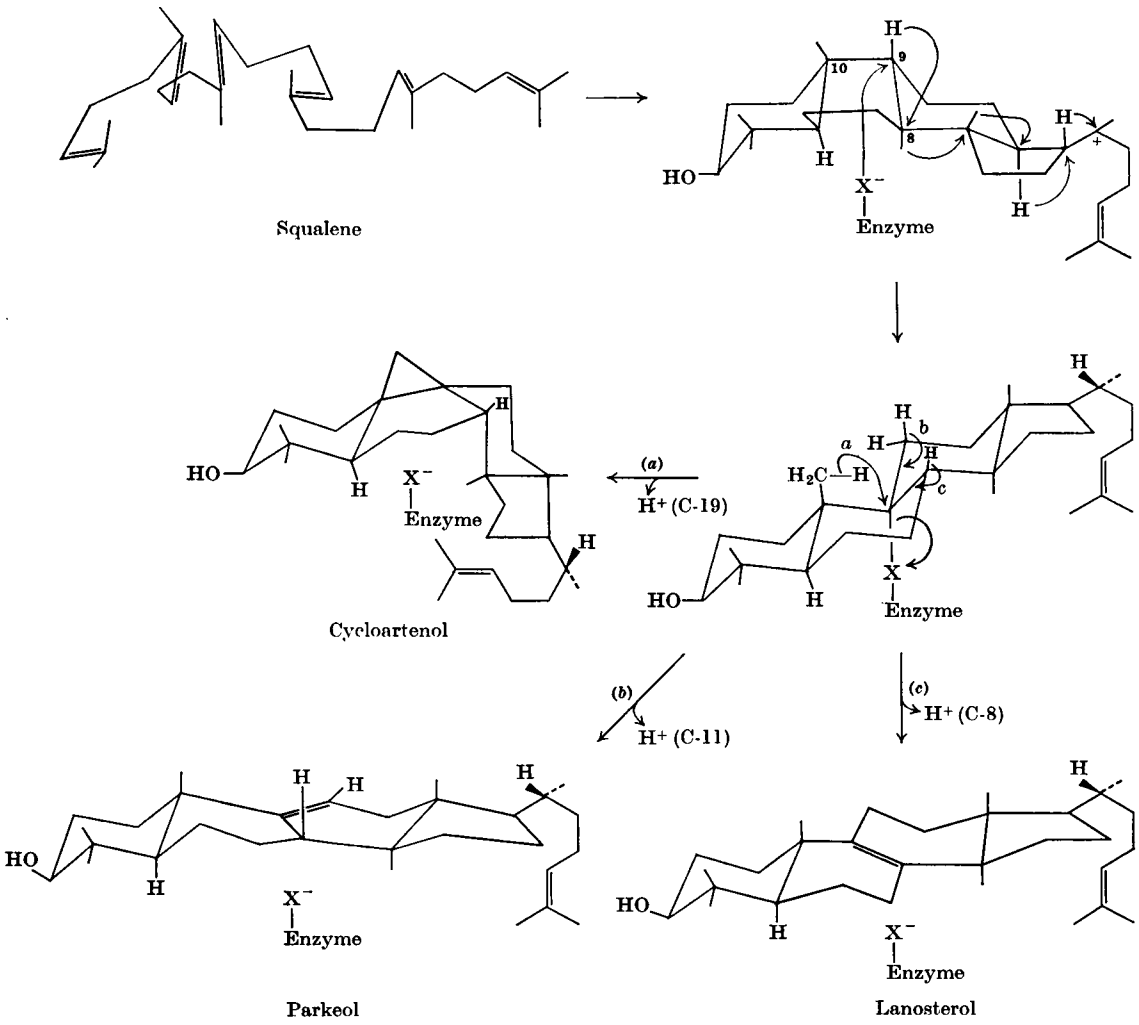
Fig. 3. Gas–liquid chromatography on QF–1 of the reaction products obtained by HCl isomerization of cycloartenyl acetate. The products were separated into the three bands by thin-layer chromatography on $AgNO_3$-impregnated silica gel. Retention times relative to cholestane were: band *A*, peak 1*a*, 6·83; band *B*, peak 1*b*, 6·17; peak 2*b*, 6·79; band *C*, peak 1*c*, 6·14; peak 2*c*, 7·07; peak 3*c*, 7·31. Relative retention times of authentic steryl acetates were: lanosteryl acetate, 6·17; parkeyl acetate, 6·76; cycloartenyl acetate, 7·21.

parkeyl acetate on QF–1. The mass spectrum had peaks at *m/e* 468 [M^+], 453 [$M^+ - CH_3$] and 393 [$M^+ - (\text{acetate} + CH_3)$]. The peak at *m/e* 286 was again negligible.

The hydrogen chloride isomerization reaction was then carried out with a portion of the doubly



Scheme 2. Acid rearrangement of cycloartenyl acetate biosynthesized from [2-^{14}C,(4*R*)-4-^{3}H$_1$]MVA according to the mechanism of Bentley *et al.* (1953). For simplicity a Δ^{24}-bond is shown. In practice the HCl isomerization reaction was conducted on the dibromo derivative as described in the Experimental section. Ac, $CH_3 \cdot CO$; ●, ^{14}C; T, ^{3}H.



Scheme 3. Hypothetical scheme for the enzymic formation of cycloartenol, parkeol and lanosterol.

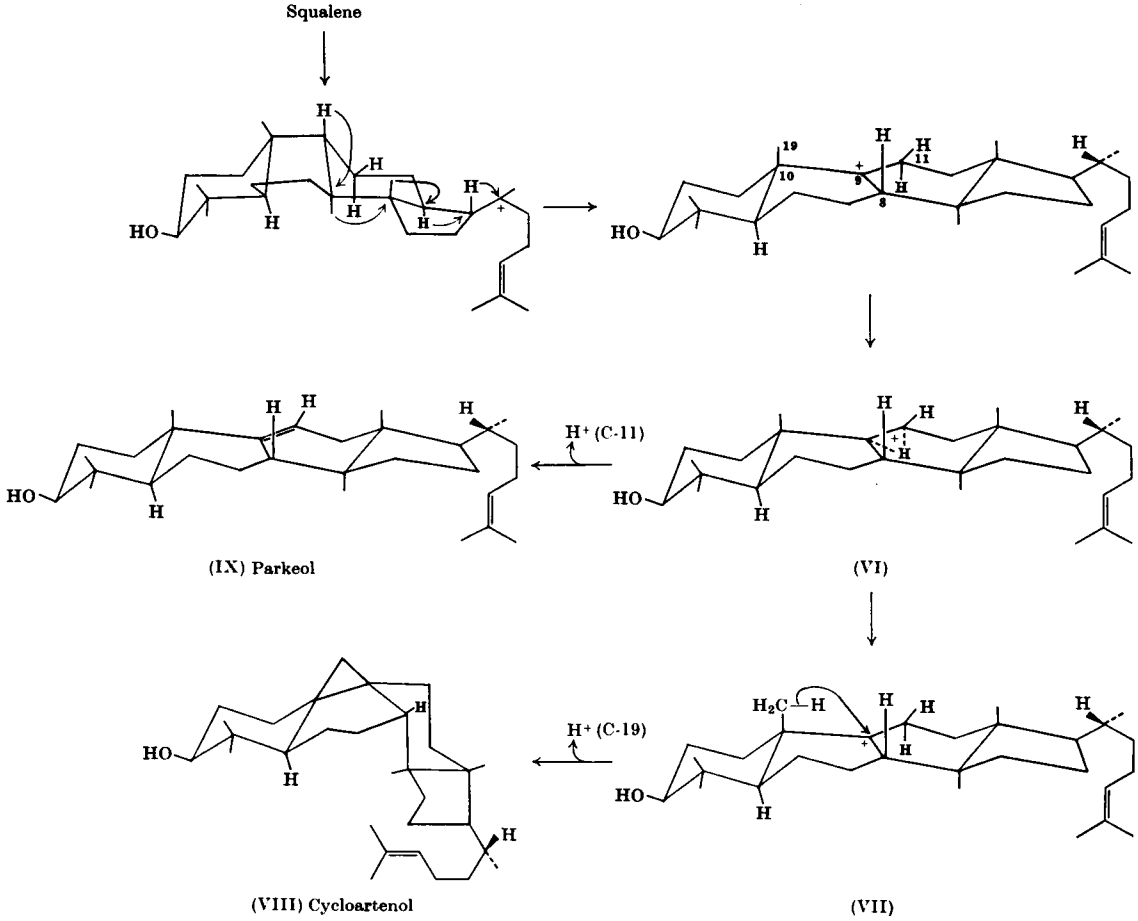
labelled cycloartenyl acetate and the products were isolated as described above. The parkeyl acetate had the same $^3H/^{14}C$ ratio as the starting cycloartenyl acetate (Table 1). However, the $^3H/^{14}C$ ratio of the lanosteryl acetate had decreased and corresponded to a $^3H/^{14}C$ atomic ratio of 5:6.

24-Methylenecycloartanyl acetate isolated by thin-layer chromatography on silver nitrate-impregnated silica gel was also found to have the same $^3H/^{14}C$ ratio as squalene and cycloartenyl acetate (Table 1).

DISCUSSION

The conversion of squalene labelled from [2-^{14}C,(4*R*)-4-$^3H_1$]MVA into a Δ^8-sterol (e.g. lanosterol) would involve elimination of a tritium atom from C-9 (Cornforth *et al.* 1965). It is conceivable that cycloartenol could be formed enzymically in biological systems from lanosterol by protonation at C-8 to give an electron deficiency at C-9, followed by formation of the 9β,19-cyclopropane ring. Such a mechanism, if operative, would result in a $^3H/^{14}C$ atomic ratio of 5:6 when [2-^{14}C,(4*R*)-4-$^3H_1$]-MVA is incorporated into cycloartenol. The observed $^3H/^{14}C$ atomic ratio of 6:6 for both cycloartenol and 24-methylenecycloartanol (Table 1) clearly indicates that there is no loss of tritium during their formation from squalene. This result therefore eliminates lanosterol as a precursor of cycloartenol and 24-methylenecycloartanol.

During the hydrogen chloride isomerization of



Scheme 4. Possible mechanism of formation of cyclopropane ring during biosynthesis of cycloartenol.

cycloartenol to lanosterol there was an elimination of one tritium atom (Table 1). According to Bentley *et al.* (1953), parkeyl acetate and lanosteryl acetate are formed simultaneously by loss of a proton from the carbonium ion (V) (Scheme 2) and the lanosterol acetate subsequently equilibrates to a mixture with the Δ^7-compound. Consideration of these facts indicates that in the present work the tritium atom eliminated during lanosterol formation must have been that present at C-8 of cycloartenyl acetate and demonstrates that a tritium migration from C-9 to C-8 must have occurred during cycloartenol biosynthesis, as outlined in Scheme 1. The $^3H/^{14}C$ atomic ratio of 6:6 (Table 1) for parkeyl acetate, in addition to confirming that the tritium atom is present at C-8 in cycloartenol, also demonstrates that lanosteryl acetate and parkeyl acetate formed during the action of hydrogen chloride on cycloartenyl acetate are not in equilibrium, as shown in Scheme 2.

In the squalene-cyclization mechanism shown in Scheme 1, the closure of the 9β,19-cyclopropane ring by partial migration of the C-10 methyl group is *cis* to the preceding C-9 hydrogen migration; this contravenes one of the basic postulates of the biogenetic isoprene rule of Ruzicka's group (Eschenmoser *et al.* 1955; Ruzicka, 1959), which requires that squalene cyclization proceeds by antiplanar 1,2-rearrangements and 1,2-eliminations. Richards & Hendrickson (1964) suggest that cycloartenol formation cannot therefore be explained without the intervention of some non-bridged intermediate. It is possible that the cyclizing enzyme might neutralize the electron deficiency at C-9 in the intermediate carbonium ion as depicted in Scheme 3. 'X' can be any electron-donating group

on the enzyme. Withdrawal of the enzyme would then allow the further migration of the C-10 methyl group to proceed according to the rules of Wagner–Meerwein rearrangements (Scheme 3). From such an enzyme-bound intermediate parkeol and lanosterol could also be formed as shown in Scheme 3. However, this mechanism is contrary to one of the postulates of the biogenetic isoprene rule of Ruzicka, which suggests that squalene cyclization to the final product proceeds in a non-stop reaction with the formation of no intermediates produced by neutralization of the electron deficiency. This important possibility clearly warrants further investigation. As an alternative mechanism to Scheme 3, the axial hydrogen atom at C-11 may form a transitory bridge between C-11 and C-9 (Scheme 4, VI). Elimination of this hydrogen would then give parkeol (IX) (isolated from *Butyrospermum parkii*; Lawrie, Spring & Watson, 1956). (This mechanism would result in the elimination of the 11α-hydrogen as compared with the 11β-hydrogen if Scheme 3 were operative and may provide a method of distinguishing between these two mechanisms.) Reversion of the bridged hydrogen back to C-11 would leave an electron deficiency again at C-9 (VII), but in this case the C-19 methyl group could now bridge by the accepted Wagner–Meerwein mechanism and hence give cycloartenol (VIII) by hydrogen elimination.

Evidence has been obtained (Goad & Goodwin, 1965; Goad, Hammam, Dennis & Goodwin, 1966; Rees *et al.* 1966; Hammam, 1966) that, during alkylation at C-24 in phytosterol biosynthesis, hydrogen migration from C-24 to C-25 occurs. This migration has also been demonstrated in ergosterol biosynthesis (Stone & Hemming, 1965, 1967; Akhtar, Hunt & Parvez, 1967). The present results reveal that there has been no loss of tritium during 24-methylenecycloartanol formation, and this unequivocally shows that the tritium atom originally at C-24 is retained during alkylation of the cycloartenol side chain. This is in accord with the migration of the C-24 hydrogen to C-25 as required in the alkylation scheme of Castle, Blondin & Nes (1963).

A $^3H/^{14}C$ ratio of 3:5 for the major phytosterol mixture (Table 1) confirms earlier results (Goad & Goodwin, 1965; Rees *et al.* 1966). On the basis of the work of Cornforth *et al.* (1965) on cholesterol biosynthesis from [2-^{14}C,(4*R*)-4-$^3H_1$]MVA, it is reasonable to assume that the three tritium atoms are located at C-17, C-20 and C-25 (in C-24 alkyl sterols). If cycloartenol proves to be the phytosterol precursor as proposed (Ardenne *et al.* 1965; Benveniste *et al.* 1966*a*,*b*; Goad, 1967), opening of the cyclopropane ring will presumably therefore at some stage involve the elimination of the C-8 tritium atom, as for example by formation of a Δ^8-bond. Subsequent modification of the sterol nucleus by analogous pathways to those operative in cholesterol biosynthesis (Clayton, 1965) would then give the $^3H/^{14}C$ ratio 3:5 as observed.

Very recent observations (Corey, Russey & Ortiz de Montellano, 1966; Van Tamelen, Willett, Clayton & Lord, 1966; Benveniste & Massy-Westropp, 1967; Corey & Ortiz de Montellano, 1967; Dean, Ortiz de Montellano, Bloch & Corey, 1967) indicate that squalene is first oxidized to give 2,3-oxidosqualene, which is then subsequently cyclized to give the various triterpenes found in Nature. Preliminary work from our Laboratory (Rees, Goad & Goodwin, 1968) shows that plant-leaf homogenates will also cyclize 2,3-oxidosqualene to give cycloartenol. However, it should be noted that though these observations may require minor modifications to the squalene-cyclization schemes discussed above they do not affect the general conclusions drawn from the present work with regard to the formation of the cyclopropane ring in cycloartenol.

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