The Biosynthesis of Sterols

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1 Introduction

The discovery of the intermediates and the stereochemistry of the enzymic processes by which squalene is biosynthesized from mevalonic acid has been described in a previous Quarterly Review[1] and in other reviews.[2,3] Application of these results to studies of the later stages of triterpene and sterol biosynthesis has proceeded rapidly over the past six years and, together with improved methods of enzyme isolation and chemical synthesis, is providing a detailed picture of the complex reactions involved. This review describes the main areas of recent investigation, with particular reference to the mechanistic and stereochemical results which have been obtained. Some properties of the enzymes mediating various stages of the biosynthetic sequence are also outlined. Although many investigations have been concerned with cholesterol biosynthesis in animals, parallel studies on other organisms have also been reported. Some interesting variations of reaction sequence and mechanism are observed in different species and are summarized here. However, this aspect has been more fully reviewed elsewhere.[4] The uses and limitations of radioactive compounds in the study of biosynthetic sequences have also been discussed in depth elsewhere.[3a] Literature received in England after April 1971 has not been included in the Review, although, for the sake of completion, important recent results have been appended in footnotes.

2 The Conversion of Farnesyl Pyrophosphate to Squalene

A. Stereochemistry.—The work of Cornforth, Popják, and co-workers[1,2] has established the stereochemistry of the processes by which mevalonic acid (MVA) is converted to squalene. In particular, the use of specifically deuteriated and tritiated farnesyl pyrophosphate has revealed the stereochemical result of the

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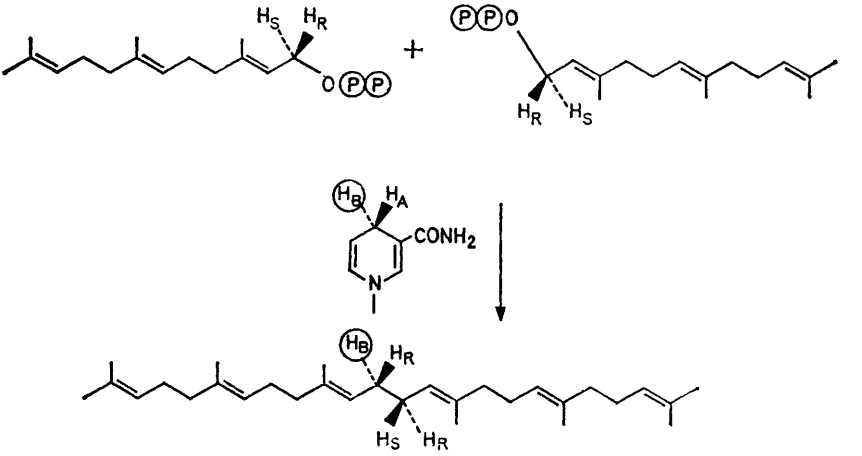
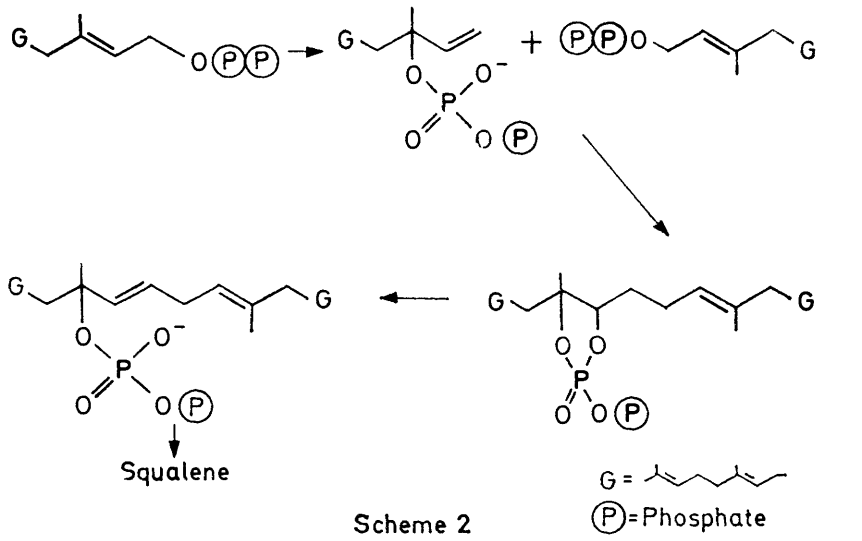
[1] R. B. Clayton, *Quart. Rev.*, 1965, **19**, 168, and references therein.
[2] (*a*) J. W. Cornforth and G. Popják, *Biochem. J.*, 1966, **101**, 553; (*b*) J. W. Cornforth, *Quart. Rev.*, 1969, **23**, 125.
[3] (*a*) I. D. Frantz and G. J. Schroepfer, jun., *Ann. Rev. Biochem.*, 1967, **36**, 691; (*b*) C. J. Sih and H. W. Whitlock, *Ann. Rev. Biochem.*, 1968, **37**, 661.
[4] L. J. Goad in 'Natural substances formed biologically from mevalonic acid', ed. T. W. Goodwin, Academic Press, London and New York, 1969, p. 45.

tail-to-tail linkage of farnesyl units by the enzymes of a rat-liver homogenate (Scheme 1). The process produces an overall inversion of stereochemistry of the hydrogen atoms attached to the prochiral† carbon atom C-1 of one farnesyl



Scheme 1

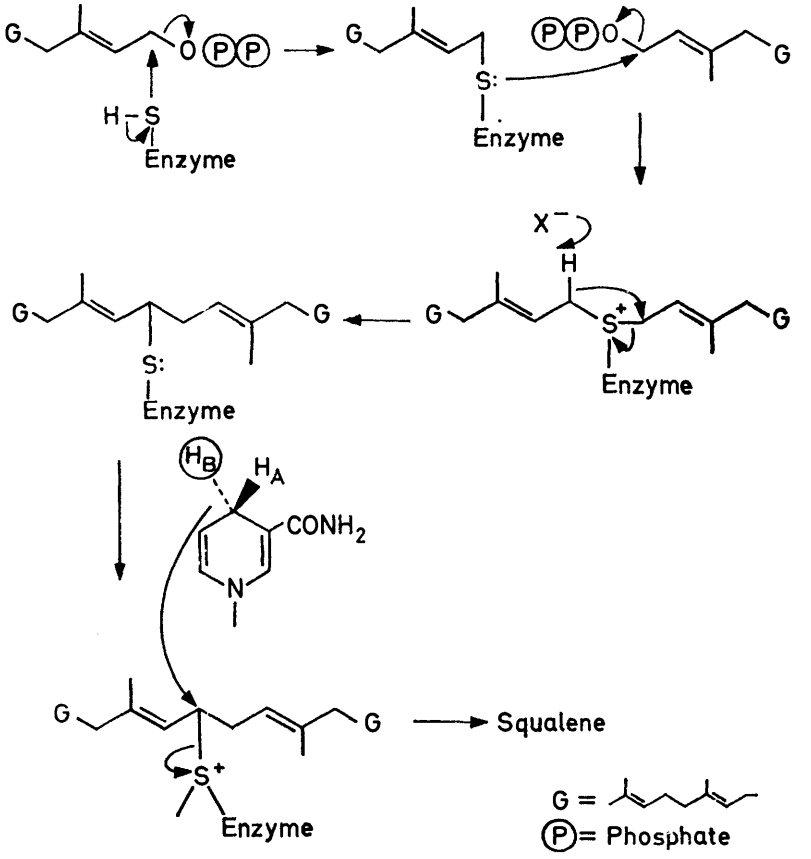


Scheme 2

† For general definition see K. R. Hanson, *J. Amer. Chem. Soc.*, 1966, **88**, 2731.

residue and retention of stereochemistry of H_R attached to C-1 of the second farnesyl group, while H_S is lost and stereospecifically replaced by a hydrogen atom (H_B) from the coenzyme NADPH. Thus, the symmetrical molecule squalene is formed by an asymmetric process.‡

Two main mechanisms were proposed to explain this coupling process in the light of the stereochemical features outlined above. The first (Scheme 2)[5a] involved isomerization of one farnesyl residue to a nerolidyl derivative, generating a terminal methylene group capable of nucleophilic addition to the second farnesyl moiety with inversion of stereochemistry. The pyrophosphate group (or an analogous sulphonium group[5a]) could then be removed by reductive



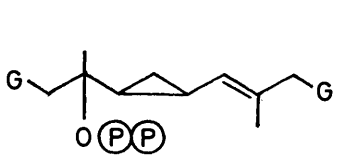
Scheme 3

‡ *trans*-Farnesyl pyrophosphate-squalene synthetase has now been isolated and purified 45-fold (I. Schechter and K. Bloch, *J. Biol. Chem.*, 1971, **246**, 7690).

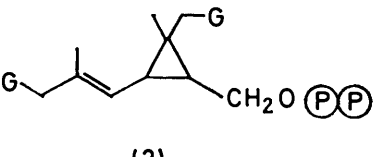
[5] (*a*) G. Popják, DeW. S. Goodman, J. W. Cornforth, R. H. Cornforth, and R. Ryhage, *J. Biol. Chem.*, 1961, **236**, 1934; (*b*) J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popják, *Proc. Roy. Soc.*, 1966, **B 163**, 492.

cleavage with hydride transfer from NADPH. This mechanism resembles that by which isopentenyl pyrophosphate reacts with dimethylallyl pyrophosphate and geranyl pyrophosphate to give farnesyl pyrophosphate. The second mechanism (Scheme 3) postulated displacement of the pyrophosphate group of one farnesyl unit by an enzymic sulphydryl group with inversion of stereochemistry.[5b] Addition of the second unit could then be followed by rearrangement to form the central carbon–carbon bond and reductive cleavage of the squalene–sulphur bond. The feasibility of the latter type of mechanism has been demonstrated by the chemical synthesis of squalene from farnesol by Stevens' rearrangement of a sulphonium ylide followed by reduction of the resulting sulphide.[6] However, subsequent work suggests that neither mechanism is operative in rat and yeast enzymes *in vitro*.

B. Presqualene Pyrophosphate.—It has been demonstrated[7] that free nerolidyl pyrophosphate is not an intermediate in the coupling of farnesyl units to give squalene in yeast, contrary to the predictions of Scheme 2 outlined above. In the absence of the cofactor NADPH a new intermediate is formed. This compound, 'presqualene pyrophosphate', was first isolated from a yeast microsomal preparation by Rilling,[8a] who showed the presence of two fifteen-carbon units and one pyrophosphate group. The intermediate retained only three of the four C-1 hydrogens of the initial farnesyl groups (as does squalene) and could be converted to squalene, in the same enzyme system, on addition of NADPH. The compound has since been isolated from a rat-liver microsomal preparation[8b]



(1)



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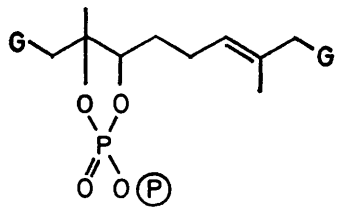

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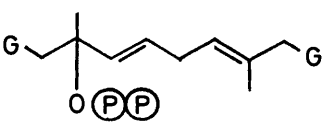


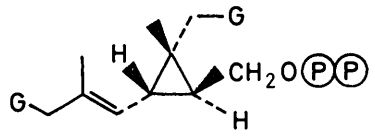
(4)

[6] (*a*) G. M. Blackburn, W. D. Ollis, C. Smith, and J. O. Sutherland, *Chem. Comm.*, 1969, 99; (*b*) J. E. Baldwin, R. E. Hackler, and D. P. Kelly, *J. Amer. Chem. Soc.*, 1968, **90**, 4758.
[7] S. S. Sofer and H. C. Rilling, *J. Lipid. Res.*, 1969, **10**, 183.
[8] (*a*) H. C. Rilling, *J. Biol. Chem.*, 1966, **241**, 3233; (*b*) H. C. Rilling, *J. Lipid. Res.*, 1970, **11**, 480.

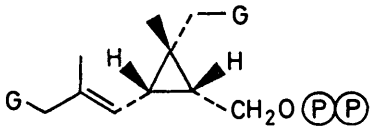
and is converted to squalene by either enzyme system in the presence of NADPH. The structure originally proposed[8a] for presqualene pyrophosphate (1) was shown to be incorrect by synthesis.[9] More detailed physical data were subsequently interpreted in terms of (2).[10]

A chromatographically identical compound was isolated in a similar way by Popják *et al.*,[11] who interpreted their data (in particular the reported retention of all four farnesyl C-1 hydrogens) in terms of structure (3). Subsequently, Wasner and Lynen[12] reported the isolation of an intermediate which retained only three farnesyl C-1 hydrogen atoms and which, when labelled with [1-^{14}C]-farnesyl pyrophosphate, gave [^{14}C]malondialdehyde on ozonolysis. The compound was formulated as (4). Both (3) and (4) had been proposed[5] as possible intermediates in squalene formation (Scheme 2).

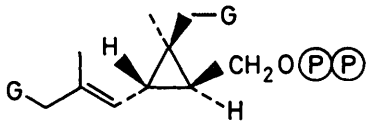
Recently, the structure (2) proposed by Rilling[10] for presqualene pyrophosphate has been confirmed by degradation and by unambiguous synthesis.[13]* Of the eight possible isomers [(5)—(8) and optical antipodes] only one is bio-



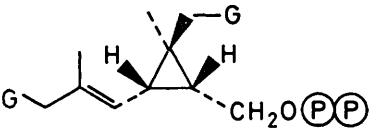
(5)



(6)



(7)



(8)

logically active. The absolute stereochemistry of this compound has been defined as (5). Although (5) has not been obtained in pure form by synthesis, incorporation of various mixtures of isomeric pyrophosphates into yeast homogenates containing NADPH results in efficient conversion of the active isomer

* The degradation results have recently been confirmed and extended (J. Edmond, G. Popják, S. Wong, and V. P. Williams, *J. Biol. Chem.*, 1971, **246**, 6254).

[9] E. J. Corey and P. R. Ortiz de Montellano, *Tetrahedron Letters*, 1968, 5113.

[10] W. W. Epstein and H. C. Rilling, *J. Biol. Chem.*, 1970, **245**, 4597.

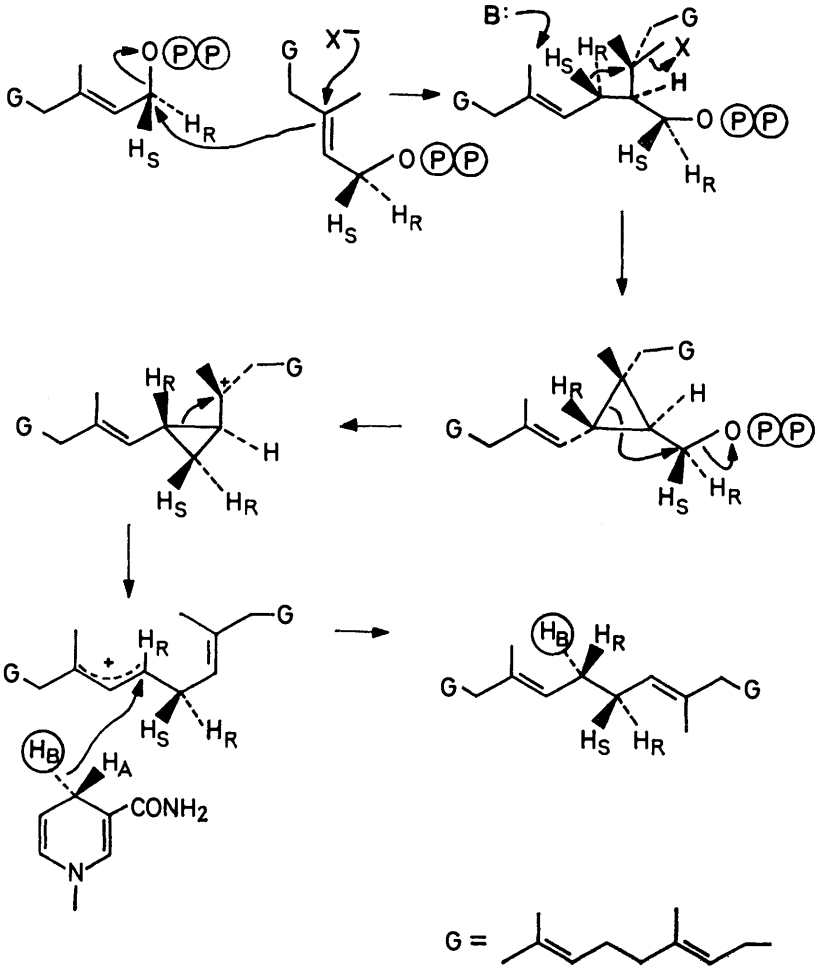
[11] G. Popják, J. Edmond, K. Clifford, and V. Williams, *J. Biol. Chem.*, 1969, **244**, 1897.

[12] H. Wasner and F. Lynen, *F.E.B.S. Letters*, 1970, **12**, 54.

[13] (*a*) R. V. M. Campbell, L. Crombie, and G. Pattenden, *Chem. Comm.*, 1971, 218; (*b*) L. J. Altman, R. C. Kowerski, and H. C. Rilling, *J. Amer. Chem. Soc.*, 1971, **93**, 1782; (*c*) H. C. Rilling, C. D. Poulter, W. W. Epstein, and B. Larsen, *ibid.*, p. 1783; (*d*) R. M. Coates and W. H. Robinson, *ibid.*, p. 1785.

to squalene. In the light of these results, Scheme 4 has been proposed to explain the conversion of farnesyl pyrophosphate, *via* (5), to squalene.[13c]

C. Investigation of Reaction Sequence.—As described above, the conversion of farnesyl pyrophosphate to the symmetrical molecule squalene is an asymmetric process in which a hydrogen atom is lost from one farnesyl residue and replaced



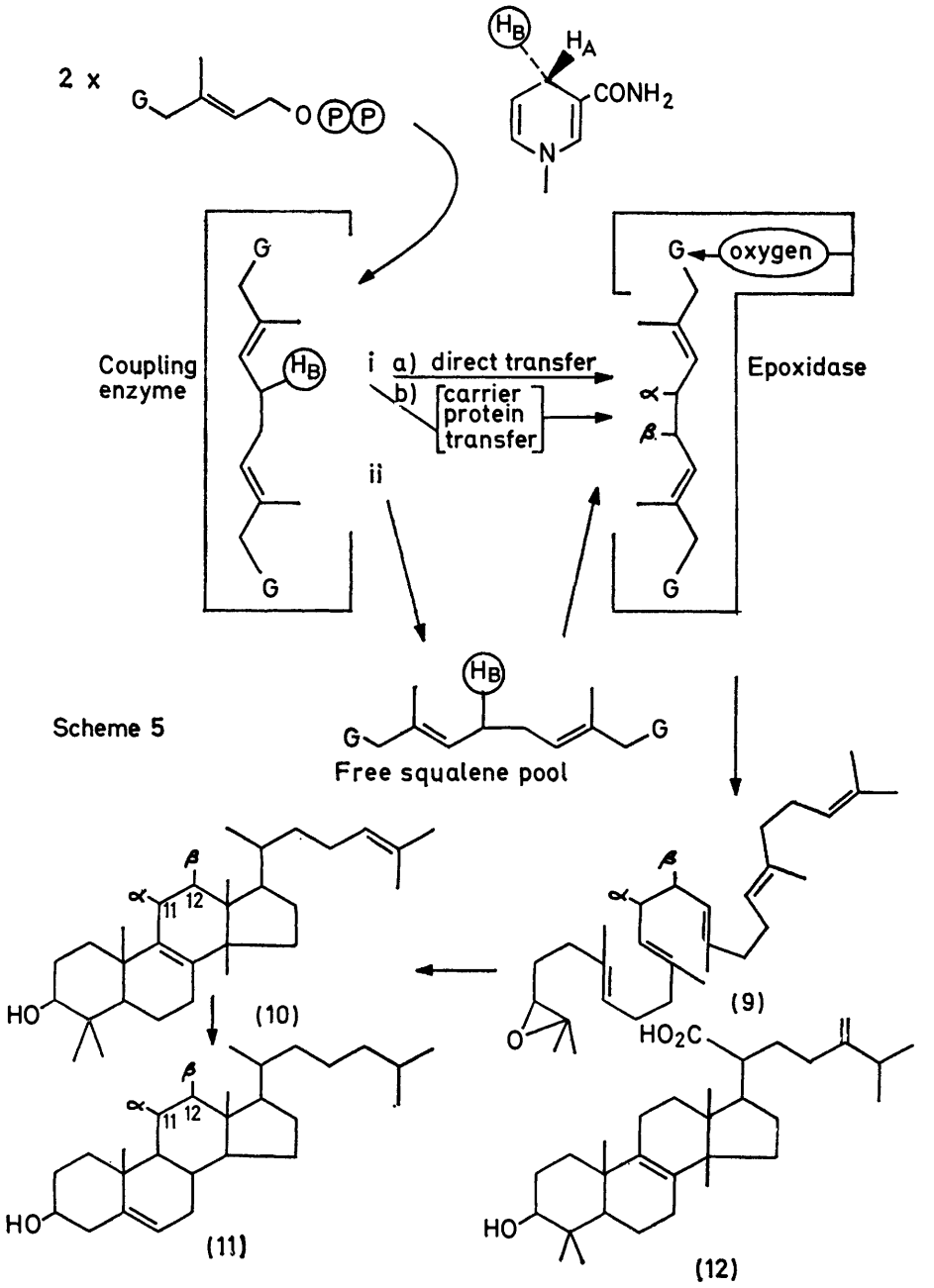
Scheme 4

by a hydrogen from NADPH. In many organisms the next step is the conversion of squalene to 2,3-epoxysqualene (see Section 3). Since in rat liver the microsomal fraction contains the enzymes responsible for both the coupling of farnesyl groups and the epoxidation of squalene, it has been suggested[2b,14] that an ordered arrangement of enzymes in the microsomes could result (Scheme 5) in a geometrically controlled transfer of the coupled squalene molecules [containing a hydrogen from NADPH (H_B) in one half of the molecule] to the epoxidase. This could occur either directly (path i*a*) or by means of a carrier protein (path i*b*) in such a way that H_B would be located specifically at either α or β during epoxidation. However, if squalene were first liberated into a metabolic pool (path ii), detachment from the enzymic environment would randomize the orientation of the molecules and subsequent transfer to the epoxidase would distribute H_B equally between α and β. Thus by using tritiated NADPH these two pathways should be distinguishable. The distribution of radioactivity at α and β can be measured by conversion of labelled 2,3-epoxysqualene (9) to lanosterol (10) or cholesterol (11) followed by specific oxidation at C-11 or C-12.

An experiment in which squalene was formed anaerobically from farnesyl pyrophosphate and [^{3}H]NADPH by a rat-liver microsome preparation and subsequently converted aerobically to cholesterol[15] showed equal distribution of radioactivity at C-11 and C-12. However, the possibility that anaerobic incubation promoted liberation of free squalene (path ii) by blocking the action of the epoxidase cast doubt on this result. A study using [^{3}H]NADPH in a continuous incubation of farnesyl pyrophosphate with pig liver homogenate[14a] gave lanosterol having a ratio of tritium activity at C-11 : C-12 of 1·28 : 1. This small difference, together with the presence of some radioactivity at unknown positions in the molecule, did not allow a decision to be made between the operation of paths i and ii. Also, the presence of free squalene in the homogenate suggested[2b] that the enzyme systems may have been disrupted and the microsomal order lost during preparation, since intact liver contains very little squalene, and what free squalene is found is not in equilibrium with newly biosynthesized squalene.[16] Studies on intact cells are more likely to provide a definite answer on this point.

A similar experiment on incorporation of [1-$^3H_2$]farnesyl pyrophosphate into eburicoic acid (12) in the fungus *Polyporus sulphureus*[17] showed equal distribution of activity at C-11 and C-12. It is noteworthy, however, that recent studies of the squalene epoxidase from yeast[18] have shown it to be a soluble enzyme, in contrast to the rat liver epoxidase which is microsomal. This variation of enzyme properties suggests the possibility that reactions which appear to be

[14] A. H. Etemadi, G. Popják, and J. W. Cornforth, *Biochem. J.*, 1969, **111**, 445.
[15] B. Samuelson and DeW. S. Goodman, *J. Biol. Chem.*, 1964, **239**, 98.
[16] (*a*) G. Popják, *Arch. Biochem. Biophys.*, 1945, **48**, 102; (*b*) A. V. Loud and N. R. L. Bucher, *J. Biol. Chem.*, 1958, **233**, 37.
[17] W. Lawrie, J. McLean, P. L. Pauson, and J. Watson, *Chem. Comm.*, 1965, 623.
[18] I. Schechter, F. W. Sweat, and K. Bloch, *Biochim. Biophys. Acta*, 1970, **220**, 463.


2 x
G
O
P
P
H_B
H_A
$CONH_2$
N
Coupling enzyme
i a) direct transfer
b) carrier protein transfer
ii
oxygen
Epoxidase
Free squalene pool
(9)
(10)
(11)
(12)
HO
HO_2C
O
11
12

Scheme 5

chemically identical in various organisms may occur by different processes at the enzymic level.

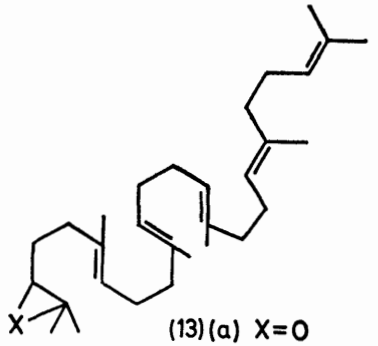
3 Conversion of Squalene to Triterpenes

A. Oxidative Route; 2,3-Epoxysqualene.—The central role of squalene in the biosynthesis of sterols and triterpenes[19] has been verified by extensive tracer studies. Tchen and Bloch [20] observed that cyclization in a rat liver homogenate containing D_2O or $H_2{}^{18}O$ gave lanosterol (10) devoid of deuterium or ^{18}O while incubation in the presence of $^{18}O_2$ resulted in incorporation of the isotope. The oxidation–cyclization process was shown to require NADPH and oxygen together with both microsomal and supernatant fractions of the homogenate.

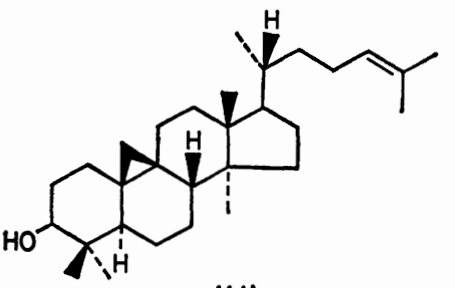
In 1966, Corey *et al.*[21] and van Tamelen *et al.*[22] showed that this conversion involves a new intermediate, 2,3-epoxysqualene (13a). This compound could be cyclized to lanosterol anaerobically and, as expected, [^{18}O]-2,3-epoxysqualene gave [^{18}O]lanosterol.[22b] Examination of the enzymes mediating this conversion, squalene epoxidase and epoxysqualene cyclase, showed that both are located in the microsomes of liver. The epoxidase,[23] which has not been obtained in soluble form (in contrast to the epoxidase in yeast[18]), requires NADPH and oxygen together with a supernatant fraction. The cyclase, which has been solubilized and purified, appears to require no cofactors.[24] It is inhibited by the analogue 2,3-iminosqualene (13b).[21b]

The widespread role of 2,3-epoxysqualene in the conversion of squalene to triterpenes has been rapidly established. In higher plants, cycloartenol (14) replaces lanosterol as the principal triterpene[4] and its formation from 2,3-epoxysqualene has been demonstrated in a cell-free preparation from bean leaves[25] and in the microsomal fraction of cultured tobacco cells.[26] A similar pathway is known to operate in *Euphorbia* latex[27] and in several other species.[4] Cycloartenol also appears to be the key triterpene in algae since the brown alga *Fucus spiralis*[28] and the phytoflagellate *Ochromonas malhamensis*[29] incorporate MVA and 2,3-epoxysqualene into this triterpene rather than into lanosterol.

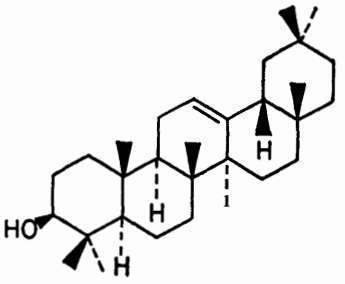
[19] (*a*) R. B. Woodward and K. Bloch, *J. Amer. Chem. Soc.*, 1953, **75**, 2023; (*b*) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, 1955, **38**, 1890.
[20] T. T. Tchen and K. Bloch, *J. Biol. Chem.*, 1957, **226**, 931.
[21] (*a*) E. J. Corey, W. E. Russey, and P. R. Ortiz de Montellano, *J. Amer. Chem. Soc.*, 1966, **88**, 4750; (*b*) E. J. Corey, P. R. Ortiz de Montellano, K. Lin, and P. D. G. Dean, *J. Amer. Chem. Soc.*, 1967, **89**, 2797.
[22] (*a*) E. E. van Tamelen, J. D. Willett, R. B. Clayton, and K. E. Lord, *J. Amer. Chem. Soc.*, 1966, **88**, 4752; (*b*) E. E. van Tamelen, J. D. Willett, and R. B. Clayton, *J. Amer. Chem. Soc.*, 1967, **89**, 3371.
[23] S. Yamamoto and K. Bloch, ref. 4, page 35.
[24] (*a*) P. D. G. Dean, P. R. Ortiz de Montellano, K. Bloch, and E. J. Corey, *J. Biol. Chem.*, 1967, **242**, 3014; (*b*) J. D. Willett, K. B. Sharpless, K. E. Lord, E. E. van Tamelen, and R. B. Clayton, *J. Biol. Chem.*, 1967, **242**, 4182.
[25] H. H. Rees, L. J. Goad, and T. W. Goodwin, *Tetrahedron Letters*, 1968, 723.
[26] (*a*) U. Eppenberger, L. Hirth, and G. Ourisson, *European J. Biochem.*, 1969, **8**, 180; (*b*) R. Heinz and P. Benveniste, *Phytochemistry*, 1970, 9, 1499.
[27] G. Ponsinet and G. Ourisson, *Phytochemistry*, 1968, **7**, 757.
[28] L. J. Goad and T. W. Goodwin, *European J. Biochem.*, 1969, **7**, 502.
[29] H. H. Rees, L. J. Goad, and T. W. Goodwin, *Biochim. Biophys. Acta*, 1969, **176**, 892.



(13) (a) X=O
(b) X=NH



(14)



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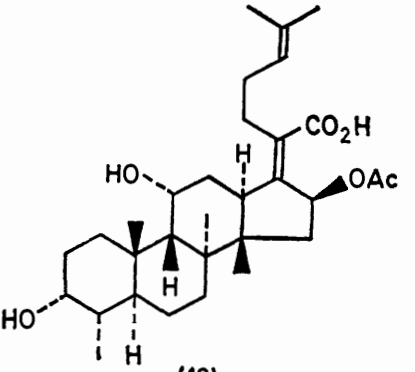


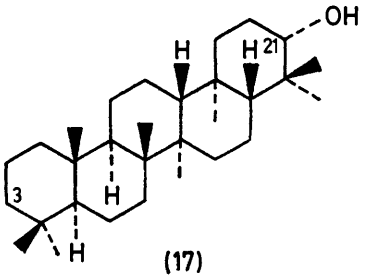
(16)

The cyclase enzyme of *O. malhamensis* appears to be partially soluble,[29] in contrast to that in rat liver, which is located in the microsomes.[24] A cell-free system from peas causes cyclization of 2,3-epoxysqualene to β-amyrin (15), a process which, as in the rat liver enzyme system, is inhibited by 2,3-iminosqualene.[30] In yeast the epoxide is efficiently cyclized to lanosterol[31] while the fungus *Fusidium coccineum*[32] converts it to the protosterol fusidic acid (16) and related triterpenes.

B. Non-oxidative Route of Squalene Cyclization.—The intermediacy of 2,3-epoxysqualene is not, however, universal. While the protozoan *Tetrahymena pyriformis* does not produce sterols, the pentacyclic triterpene tetrahymanol (17)

[30] E. J. Corey and P. R. Ortiz de Montellano, *J. Amer. Chem. Soc.*, 1967, **89**, 3362.
[31] D. H. R. Barton, A. F. Gosden, G. Mellows, and D. A. Widdowson, *Chem. Comm.*, 1968, 1067.
[32] W. O. Godtfredsen, H. Lorck, E. E. van Tamelen, J. D. Willett, and R. B. Clayton, *J. Amer. Chem. Soc.*, 1968, **90**, 208.

has been isolated.[33] Simultaneous feeding of [^{3}H]-2,3-epoxysqualene and [^{14}C]-squalene resulted in incorporation of only ^{14}C activity into tetrahymanol, using either whole cells or a soluble enzyme preparation.[34] Further, anaerobic cyclization of squalene in media containing D_2O or $H_2{}^{18}O$ gave tetrahymanol containing deuterium (probably at C-3) and ^{18}O (at C-21). These results are consistent with the theory that the triterpene is formed by proton-initiated cyclization of squalene,[34] terminating in acquisition of a hydroxide ion from the medium.



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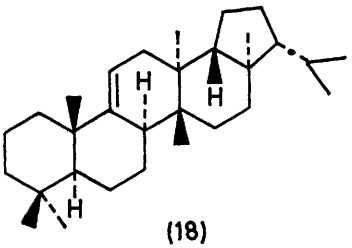
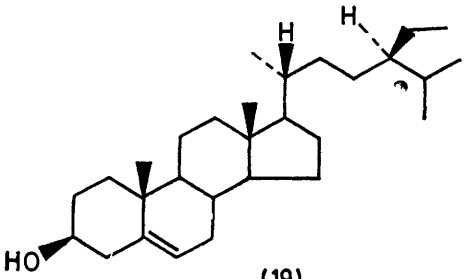


(18)



(19)

A similar non-oxidative pathway for squalene cyclization appears to be operative in the fern *Polypodium vulgare*[35] where squalene is converted to the triterpene fern-9-ene (18) while 2,3-epoxysqualene is not incorporated. It is significant that this organism does convert 2,3-epoxysqualene to β-sitosterol (19) and other sterols.[35] This is the only demonstration to date of the operation

[33] (*a*) F. B. Mallory, J. T. Gordon, and R. L. Conner, *J. Amer. Chem. Soc.*, 1963, **85**, 1362; (*b*) Y. Tsuda, A. Morimoto, T. Sano, Y. Inubushi, F. B. Mallory, and J. T. Gordon, *Tetrahedron Letters*, 1965, 1427.

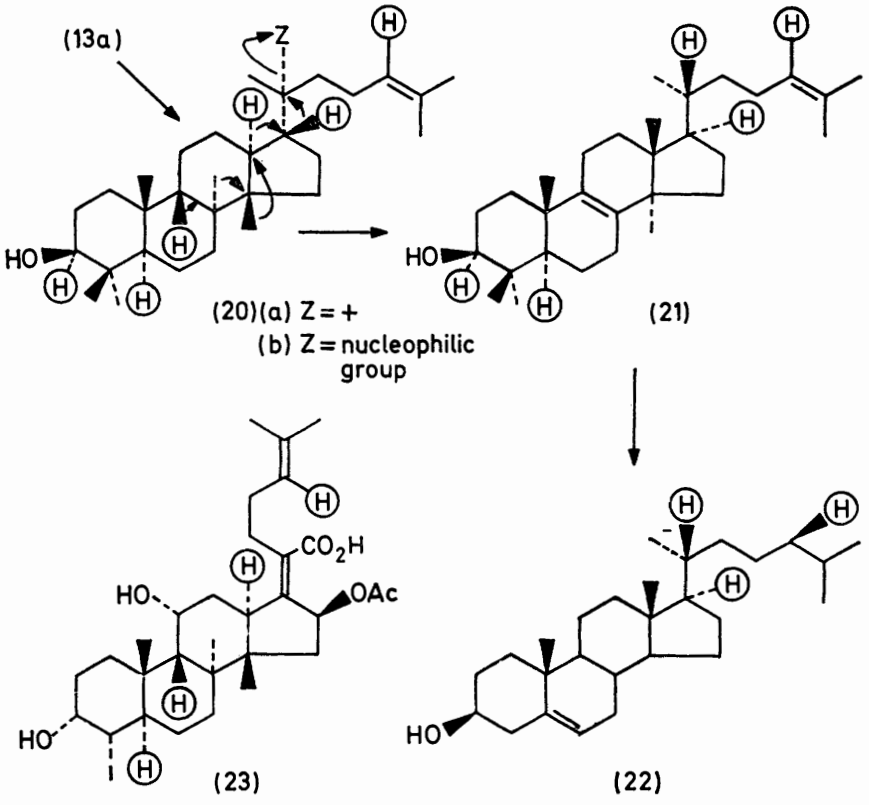
[34] (*a*) E. Caspi, J. M. Zander, J. B. Greig, F. B. Mallory, R. L. Conner, and J. R. Landrey, *J. Amer. Chem. Soc.*, 1968, **90**, 3563, 3564; (*b*) E. Caspi, J. B. Greig, J. M. Zander, and A. Mandelbaum, *Chem. Comm.*, 1969, 28; (*c*) J. M. Zander, J. B. Greig, and E. Caspi, *J. Biol. Chem.*, 1970, **245**, 1247.

[35] (*a*) D. H. R. Barton, A. F. Gosden, G. Mellows, and D. A. Widdowson, *Chem. Comm.*, 1969, 184; (*b*) D. H. R. Barton, G. Mellows, and D. A. Widdowson, *J. Chem. Soc.* (*C*), 1971, 110.

of both the oxidative and non-oxidative pathways of squalene cyclization in a single organism.

4 Mechanisms of Formation of Tetracyclic Triterpenes

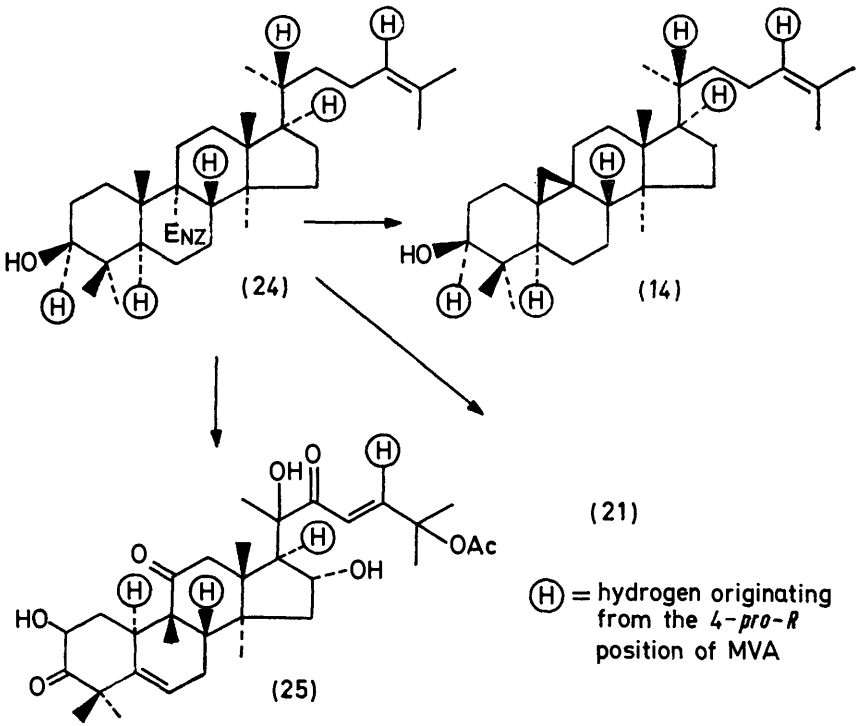
The processes involved in the cyclization of 2,3-epoxysqualene have been studied most intensively in the context of lanosterol formation by rat liver enzymes. It has been postulated that cyclization proceeds (Scheme 6) to give the protosterol carbonium ion (20a)[19b] or its stabilized equivalent (20b)[36] followed by a concerted series of hydrogen and methyl migrations terminating in the loss of the 9β hydrogen of (20) to give lanosterol (21). The demonstration that squalene containing six 4-*pro-R* protons of MVA gives lanosterol retaining only five of



Scheme 6

[36] J. W. Cornforth, *Angew. Chem. Internat. Edn.*, 1968, **7**, 903.

these hydrogens[37] supported this scheme. Also, the protons at C-17α and C-20β of lanosterol were shown to have originated from C-13 and C-17 of (20) by degradation of cholesterol (22).[37,38a] The process was also shown to involve two 1:2 migrations rather than a single 1 : 3 migration.[38b] This result has since been confirmed by direct degradation of lanosterol biosynthesized in a yeast enzyme system.[38c] Migration of the C-8 and C-14 methyl groups of (20) had previously been shown to involve two 1 : 2 migrations by the use of ^{13}C-labelled substrates.[1] Indirect support for the involvement of a protosterol intermediate has been obtained by studies on the fungal protosterol fusidic acid (23). The hydrogens at C-9β and C-13α were shown to be derived from the 4-*pro-R* position of MVA as predicted for the unrearranged protosterol skeleton.[39]*



Scheme 7

* A possible protosterol intermediate (20b; Z = OH) has recently been synthesized. However, on incubation with a rat liver homogenate no incorporation into lanosterol could be demonstrated (H. Immer and K. Huber, *Helv. Chim. Acta*, 1971, **54**, 1346).

[37] J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, Y. Shimizu, S. Ichii, E. Forchielli, and E. Caspi, *J. Amer. Chem. Soc.*, 1965, **87**, 3224.

[38] (*a*) E. Caspi and L. J. Mulheirn, *Chem. Comm.*, 1969, 1423; (*b*) M. Jayme, P. C. Schaefer, and J. H. Richards, *J. Amer. Chem. Soc.*, 1970, **92**, 2059; (*c*) D. H. R. Barton, G. Mellows, D. A. Widdowson, and J. J. Wright, *J. Chem. Soc.* (*C*), 1971, 1142.

[39] E. Caspi and L. J. Mulheirn, *J. Amer. Chem. Soc.*, 1970, **92**, 404.

While lanosterol appears to be the primary product of cyclization of 2,3-epoxysqualene in animals and fungi, the equivalent compound in higher plants and algae appears to be cycloartenol (14).[4] It has been postulated that this triterpene is formed *via* a protosterol intermediate similar to that envisaged for lanosterol.[40] Following a backbone rearrangement, the carbonium ion could be stabilized at C-9 by addition of an enzymic nucleophile to give (24) (Scheme 7). Elimination of this group would then allow formation of the cyclopropane ring.[41] This scheme is supported by the demonstration that all 4-*pro-R* protons of MVA are retained in cycloartenol.[41] The enzyme-stabilized moiety (24) may be invoked as a common intermediate in the formation of several triterpenes[4,41] since, in addition to cycloartenol formation in plants, lanosterol (21) could be derived (in animals and fungi) by loss of the C-8β hydrogen from (24). Also, the cucurbitacins [*e.g.* cucurbitacin B (25)] may be produced by further backbone rearrangement of (24), a route which is consistent with preliminary labelling studies.[42]†

5 The Action of 2,3-Epoxysqualene Cyclase on Synthetic Substrates

A. Factors Affecting Cyclization.—In two series of papers by Corey *et al.*[43–46] and van Tamelen *et al.*[47–50] a number of synthetic analogues of 2,3-epoxysqualene have been investigated as substrates for rat liver epoxysqualene cyclase and the structures of the enzymically generated products compared with those resulting from chemical cyclization of the epoxides. These investigations have provided information on (*a*) the basic structural features of 2,3-epoxysqualene which are essential for acceptance and cyclization by the enzyme, (*b*) the factors which enable the cyclase to control the conformation of the epoxide

† However, formation of lanosta-7,24-dien-3β-ol in rat skin has recently been shown to occur by a pathway which may not involve lanosterol since all six 4-*pro-R* protons of MVA are retained in this triterpene (G. M. Hornby and G. S. Boyd, *Biochem. J.*, 1971 **124**, 831).

[40] J. H. Richards and J. B. Hendrickson, 'Biosynthesis of Sterols, Terpenes and Acetogenins', Benjamin, New York, 1964, p. 274.

[41] H. H. Rees, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, 1968, **107**, 417.

[42] J. M. Zander and D. C. Wigfield, *Chem. Comm.*, 1970, 1599.

[43] (*a*) E. J. Corey and S. K. Gross, *J. Amer. Chem. Soc.*, 1967, **89**, 4561; (*b*) E. J. Corey and W. E. Russey, *ibid.*, 1966, **88**, 4751.

[44] E. J. Corey, K. Lin, and M. Jautelat, *J. Amer. Chem. Soc.*, 1968, **90**, 2724.

[45] (*a*) E. J. Corey, A. Krief, and H. Yamamoto, *J. Amer. Chem. Soc.*, 1971, **93**, 1493; (*b*) E. J. Corey, P. R. Ortiz de Montellano, and H. Yamamoto, *ibid.*, 1968, **90**, 6254; (*c*) E. J. Corey, K. Lin, and H. Yamamoto, *ibid.*, 1969, **91**, 2132.

[46] E. J. Corey and H. Yamamoto, *Tetrahedron Letters*, 1970, 2385.

[47] (*a*) R. J. Anderson, R. P. Hanzlik, K. B. Sharpless, E. E. van Tamelen, and R. B. Clayton, *Chem. Comm.*, 1969, 53, and refs. therein; (*b*) E. E. van Tamelen, K. B. Sharpless, R. Hanzlik, R. B. Clayton, A. L. Burlingame, and P. C. Wszolek, *J. Amer. Chem. Soc.*, 1967, **89**, 7150; (*c*) E. E. van Tamelen, J. D. Willett, M. Schwartz, and R. Nadeau, *J. Amer. Chem. Soc.*, 1966, **88**, 5937.

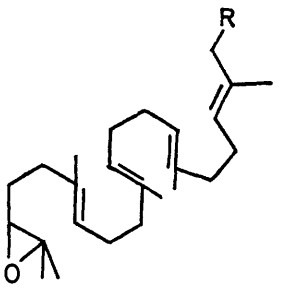
[48] (*a*) R. B. Clayton, E. E. van Tamelen, and R. G. Nadeau, *J. Amer. Chem. Soc.*, 1968, **90**, 820; (*b*) L. O. Crosby, E. E. van Tamelen, and R. B. Clayton, *Chem. Comm.*, 1969, 532.

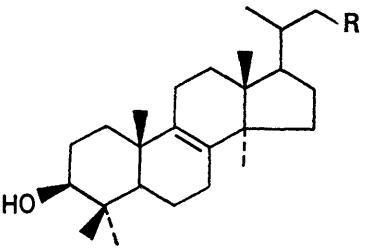
[49] (*a*) E. E. van Tamelen, R. P. Hanzlik, R. B. Clayton, and A. L. Burlingame, *J. Amer. Chem. Soc.*, 1970, **92**, 2137; (*b*) E. E. van Tamelen, R. P. Hanzlik, K. B. Sharpless, R. B. Clayton, W. J. Richter, and A. L. Burlingame, *ibid.*, 1968, **90**, 3284.

[50] E. E. van Tamelen and J. H. Freed, *J. Amer. Chem. Soc.*, 1970, **92**, 7206, and preceding papers.

and hence the configuration of the putative protosterol intermediate, and (*c*) the enzymic and thermodynamic factors involved in the conversion of the protosterol to lanosterol.

The Δ^{22} double bond and the terminal isopentenyl unit of the epoxide do not play an essential part in cyclization, since the analogues (26a—c) are converted efficiently to the corresponding lanosterol derivatives (27a—c).[43,47a] 18,19-Dihydro-2,3-epoxysqualene (28) is accepted by the enzyme but is cyclized to give the tricyclic compound (29), which is structurally related to that obtained by chemical cyclization, differing only in stereochemistry at one or more undefined positions[47b] (a dehydro-analogue was obtained on chemical cyclization of 2,3-epoxysqualene itself[47c]). 10,11-Dihydrosqualene is epoxidized randomly at either end but is not cyclized.[43b]





(26)(a) R = H

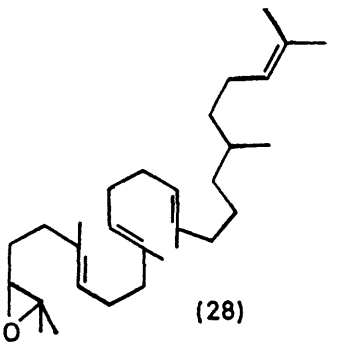
(b) R =

(c) R =

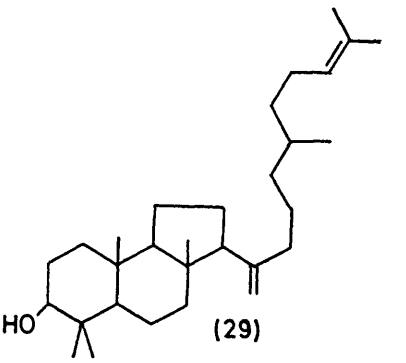
(27)(a) R = H

(b) R =

(c) R =

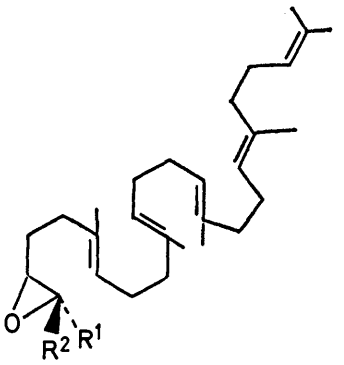


(28)

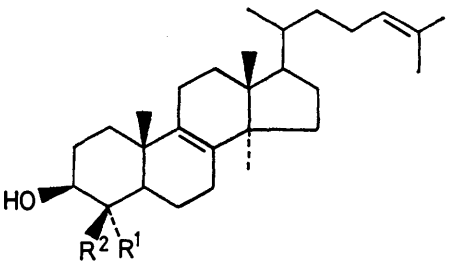


(29)

The role of the terminal methyl groups adjacent to the oxiran ring of 2,3-epoxysqualene has been shown to be important for the acceptance and orientation of the substrate by the cyclase.[44,48] When either methyl group is removed the efficiency of cyclization is greatly reduced. The epoxide (30a) is converted in 1—1.5% yield (6% of 2,3-epoxysqualene efficiency) to 4α,14α-dimethylcholesta-8,24-dienol (31a), while the isomeric epoxide (30b) and the bis-demethyl epoxide (30c) are not cyclized.[48a] In these cases the major products (15% and 95% respectively) are the corresponding glycols formed by enzymic hydration.[44,48a] The homologue (30d) is cyclized (45% of 2,3-epoxysqualene efficiency) to 4α-ethyl-4β,14α-dimethylcholesta-8,24-dienol (31b).[48b]

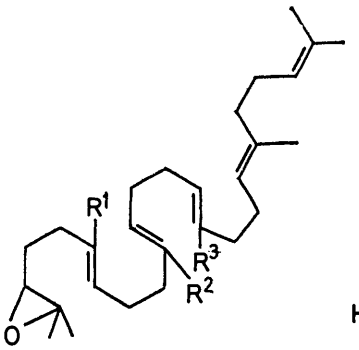


(30) (a) $R^1 = CH_3$; $R^2 = H$
(b) $R^1 = H$; $R^2 = CH_3$
(c) $R^1 = R^2 = H$
(d) $R^1 = C_2H_5$; $R^2 = CH_3$

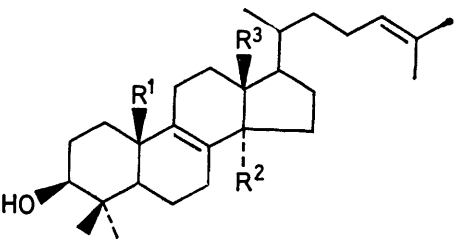


(31) (a) $R^1 = CH_3$; $R^2 = H$
(b) $R^1 = C_2H_5$; $R^2 = CH_3$

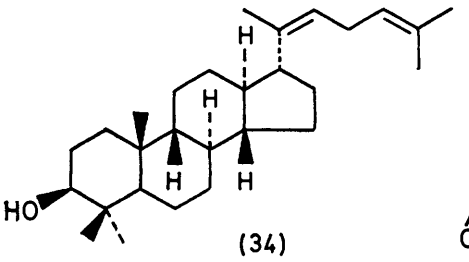
B. Factors Affecting Rearrangement of Protosterol Intermediates.—The importance of the central methyl groups at C-6, C-10, and C-15 of 2,3-epoxysqualene has been investigated by synthesis of demethyl analogues and their cyclization by rat liver homogenates.[45,49] The 6-, 10-, and 15-mono-demethyl compounds (32a—c) are efficiently cyclized and rearranged to the lanosterol derivatives (33a) (61%), (33b) (18—24%), and (33c) (40—50%) respectively.[45a,49] However, the 10,15-bis-demethyl epoxide (32d), which is accepted by the cyclase (38% conversion), gave a compound having an unrearranged skeleton[45b] and formulated as (34). Inhibition of this process by 2,3-iminosqualene indicated that the product was formed by the normal cyclase system. Similarly, 20-dehydro-2,3-epoxysqualene (35) is converted[45c] into a compound thought to be the protosterol (36). Again the normal enzyme system was implicated by the inhibitory effect of



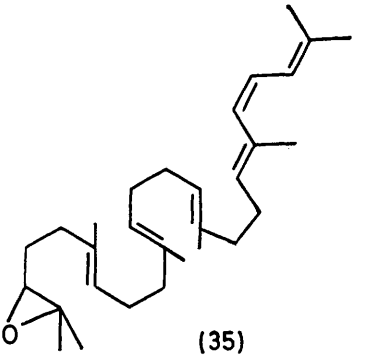
(32)(a) R^1=H; R^2=R^3=CH_3
(b) R^2=H; R^1=R^3=CH_3
(c) R^3=H; R^1=R^2=CH_3
(d) R^1=CH_3; R^2=R^3=H



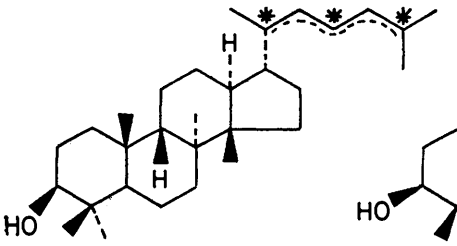
(33)(a) R^1=H; R^2=R^3=CH_3
(b) R^2=H; R^1=R^3=CH_3
(c) R^3=H; R^1=R^2=CH_3



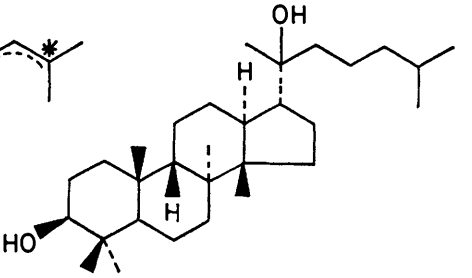
(34)



(35)



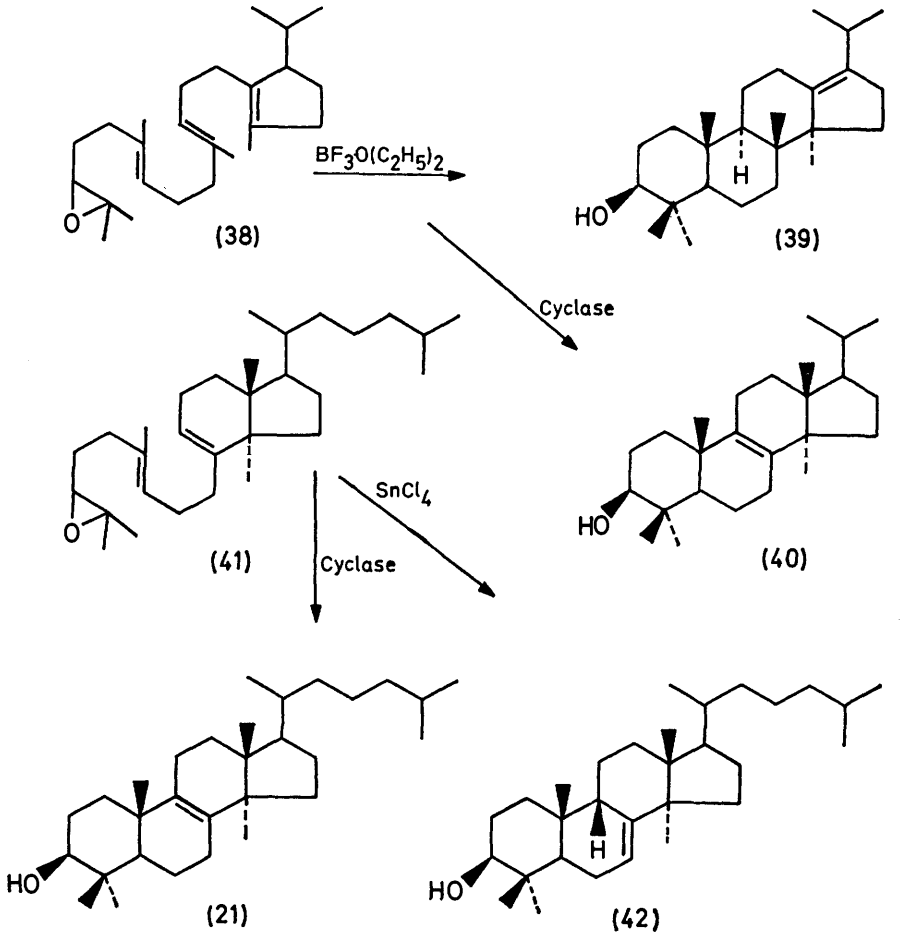
(36) OH at one position *



(37)

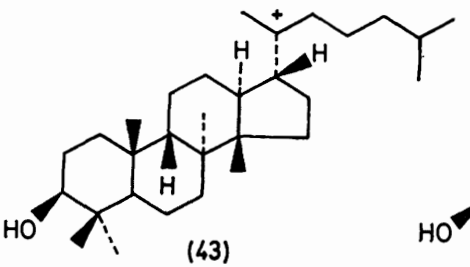
2,3-iminosqualene and by suppression of conversion of 2,3-epoxysqualene to lanosterol in the presence of the synthetic epoxide.

The failure of these two compounds to rearrange suggests that the repulsive forces generated by the presence of the two central methyl groups in the natural protosterol intermediate greatly facilitate the rearrangement process. Removal of only one methyl group does not reduce the strain sufficiently to prevent rearrangement. However, even when all methyl groups are present, rearrangement can be prevented and the protosterol skeleton stabilized by conjugation of the C-20 electron-deficient centre with double bonds in the side-chain. This thermodynamic interpretation of the results is supported by the observed conversion of the protosterol (37) to dihydrolanosterol by acidic treatment.[46] However, the

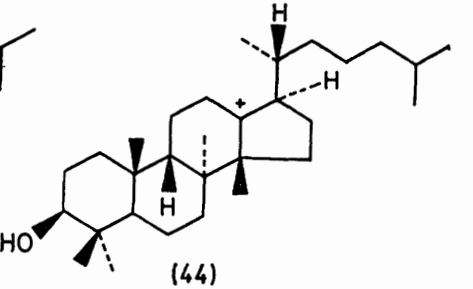


failure of these unnatural substrates to rearrange could be due to conformational changes which they may introduce within the enzyme. It has also been suggested that the cyclization and rearrangement process may occur on two different enzyme sites,[36] the initial cyclization product being stabilized by an enzymic or other nucleophile followed by transfer to the second site and rearrangement. The failure of the analogues (34) and (36) to rearrange could then result from their rejection by the second enzyme.

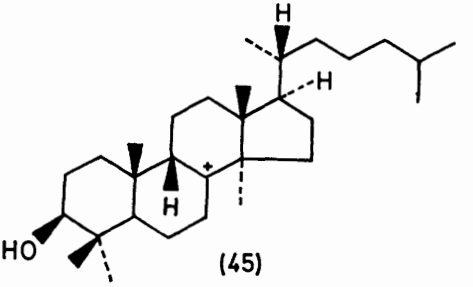
The role of the enzyme in termination of the protosterol rearrangement by introduction of the Δ^8 double bond of lanosterol has been studied by comparison of the products of enzymic and chemical reaction of certain synthetic substrates.[50] The epoxide (38) was cyclized chemically to give, among other products, the iso-euphenol analogue (39), resulting from cyclization in the all-chair conformation. However, in the presence of a liver cyclase system, this substrate was converted, in low yield, to the lanosterol analogue (40). Similarly, the epoxide (41) was cyclized chemically to 9β-Δ^7-lanostenol but (42) enzymically to



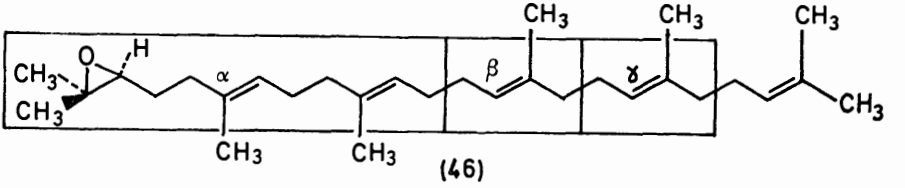
(43)



(44)



(45)



(46)

lanosterol (21). It was suggested that the enzymic cyclizations generate carbonium ions (44) and (45) which are intermediates in conversion of the protosterol carbonium ion (43) to lanosterol. The results demonstrate the role of the enzyme in inducing loss of the 9β-hydrogen of (43) to give the Δ^8 double bond rather than the thermodynamically more stable Δ^7 isomer, and are compatible with the proposed mechanism of lanosterol formation described in Section 4.

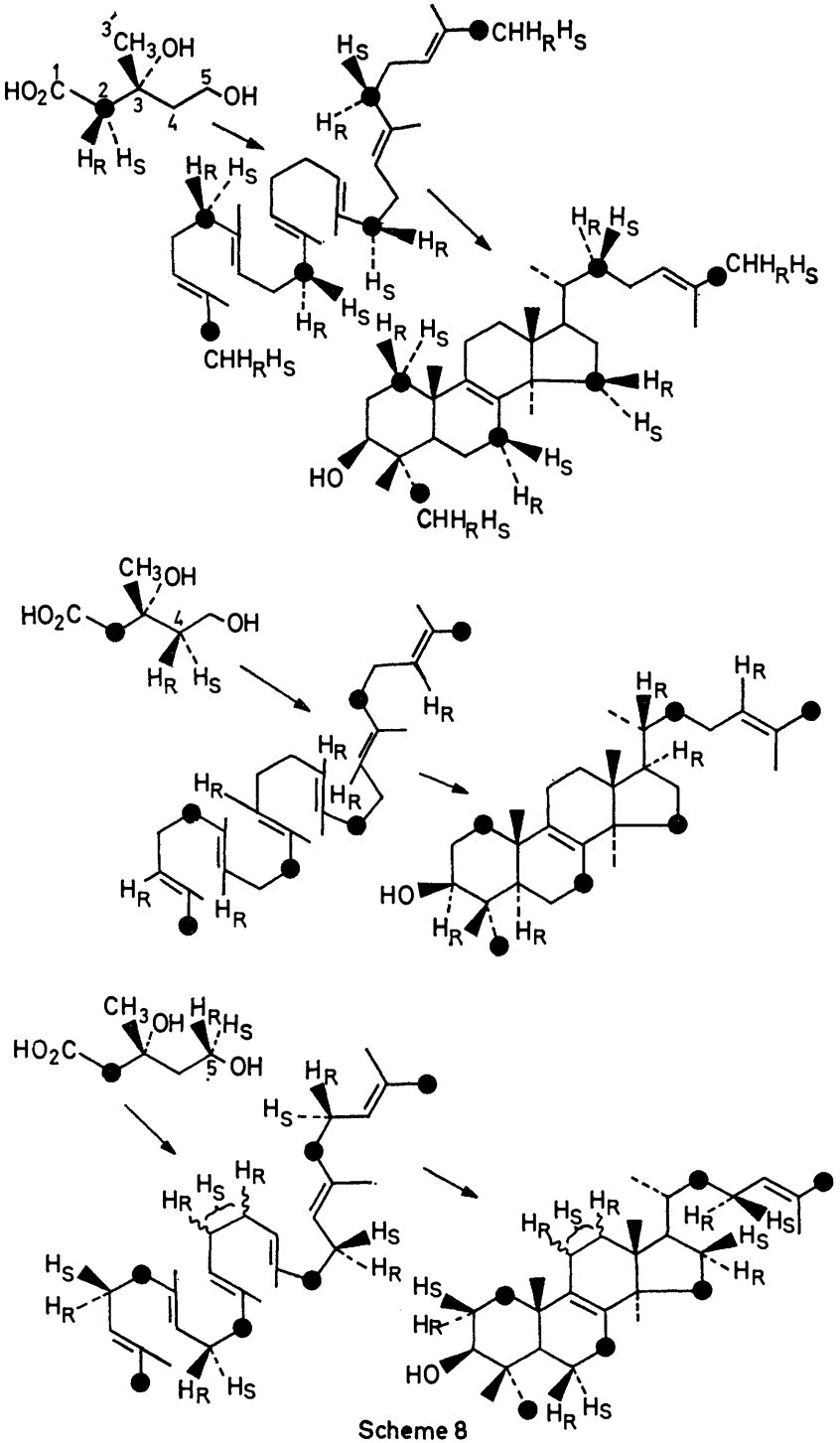
The information obtained with modified substrates has been collated in terms of the apparent role of the various parts of 2,3-epoxysqualene (46) during conversion to lanosterol.[50] The portion (α)—(γ) is the basic structure required for lanosterol formation, although (α) constitutes the minimum requirement for cyclase action. (β) appears to be involved in the enzyme control necessary to form a six-membered ring C rather than the chemically preferred five-membered ring. Finally, the methyl groups at C-10 and C-15 are implicated in rearrangement of the protosterol, while further enzymic participation may be required to stabilize the protosterol intermediate and to direct the termination of backbone rearrangement to give lanosterol.[50]

6 Uses of MVA in Studies on Later Stages of Sterol Biosynthesis

The three methylene groups at C-2, C-4, and C-5 of MVA each carry two prochiral hydrogen atoms. Replacement of individual hydrogens by an isotope gives six isomeric compounds. Five of the mono-tritiated isomers were synthesized by Cornforth and Popják using chemical and enzymatic methods, and these compounds were used to elucidate the stereochemical changes involved in conversion of MVA to squalene.[2] The remaining 5*S*-isomer has recently been prepared.[51] Incorporation of these isomers into squalene proceeds in a stereochemically defined manner. Therefore, following folding and cyclization of 2,3-epoxysqualene, the position and stereochemistry of the hydrogens which were labelled in MVA can be predicted in lanosterol (Scheme 8). Although little direct proof has been obtained by degradation of lanosterol itself, studies on cholesterol and other sterols fully support this scheme. Similar predictions on the mode of incorporation of hydrogens of MVA into other triterpenes, such as cycloartenol (14), tetrahymanol (17), fusidic acid (16), and others, have also received experimental verification.

The use of tritiated species of MVA in studies of triterpene and sterol formation is facilitated by the technique of 'double labelling'. Feeding a mixture of ^{14}C and ^{3}H species having a known ratio of specific activities enables the number of tritium atoms in the produced sterol to be determined by measuring changes in this ^{3}H:^{14}C ratio. The ^{14}C activity acts as an internal standard provided that allowance is made for the loss of any carbon atoms in the biosynthetic sequence. The position of incorporation of tritium can then be defined by specific degradations of the sterol and measurement of the resulting changes in ^{3}H:^{14}C ratio. In addition, any unexpected changes in tritium content occurring during the

[51] (*a*) P. Blattmann and J. Retey, *Chem. Comm.*, 1970, 1394; (*b*) J. W. Cornforth and F. P. Ross, *ibid.*, p. 1395; (*c*) A. I. Scott, G. T. Phillips, P. B. Reichardt, and J. G. Sweeney, *ibid.*, p. 1396.



Scheme 8

biosynthesis can be detected. These may be caused by equilibration (*e.g.* the loss of tritium from 2*R*-[2-^{3}H]MVA due to the reversibility of the isopentenyl pyrophosphate–dimethylallyl pyrophosphate isomerism in some organisms[4]) or from isotope effects causing preferential removal of the lighter hydrogen isotope. It is also important to note that, in most experiments, the synthetic MVA used is a mixture of 3*R* and 3*S* isomeric species; thus '2*R*'-tritiated MVA is actually a mixture of (2*R*, 3*R*)-[2-^{3}H] and (2*S*, 3*S*)-[2-^{3}H] isomers. However, in the few cases examined it has been shown that the 3*S*-isomer is not phosphorylated[52] and that hence only the 3*R*-isomer is metabolized, a result which is now assumed to be general.

The technique of ratio counting has been used extensively in studies on the conversion of variously labelled species of lanosterol to cholesterol in rat liver enzyme systems. Such investigations have given detailed information of the processes involved in the loss of the three methyl groups of lanosterol, the migration of the Δ^8 double bond to Δ^5, and the saturation of the Δ^{24} double bond. The following sections include a summary of these results and an outline of the progress which has been made recently in the application of this technique to studies of sterol biosynthesis in other organisms.

7 Demethylations of Lanosterol in Cholesterol Biosynthesis

A. Loss of the C-4 Methyl Groups.——Bloch and co-workers[53] demonstrated that the demethylations of lanosterol at C-4 and C-14 in rat liver result in the release of stoicheiometric amounts of CO_2. The sequence of reactions was thought to involve hydroxylation followed by oxidation to the corresponding aldehyde and acid with subsequent decarboxylation. Recent studies have provided support for this route and have established that, contrary to an earlier report,[54] the 4α-methyl is eliminated before the 4β-methyl.

A rat liver preparation rapidly converts 4α-hydroxymethyl-4β-methyl cholestanol (47a) and 4α-hydroxymethylcholestanol (47b) to cholestanol,[55] while the 4β-hydroxymethyl analogue (47c) is not metabolized. The initial hydroxylation of the methyl groups was shown to require molecular oxygen and NADPH, although the nature of the hydroxylating enzyme is still uncertain.[56] Oxidation of the alcohols occurs anaerobically and the microsomal enzyme involved could be replaced adequately by crystalline liver alcohol dehydrogenase.[57] The final stages of oxidation and decarboxylation have recently been

[52] (*a*) F. Lynen and M. Grassl, *Z. physiol. Chem.*, 1958, **313**, 291; (*b*) R. H. Cornforth, J. W. Cornforth, and G. Popják, *Tetrahedron*, 1962, **18**, 1351; (*c*) D. Arigoni, *Pure Appl. Chem.*, 1968, **17**, 331.

[53] (*a*) J. A. Olson, M. Lindberg, and K. Bloch, *J. Biol. Chem.*, 1957, **226**, 941; (*b*) F. Gautschi and K. Bloch, *J. Amer. Chem. Soc.*, 1957, **79**, 684; *J. Biol. Chem.*, 1958, **233**, 1343; (*c*) K. Bloch, *Ciba Symposium on The Biosynthesis of Terpenes and Sterols*, ed. G. E. W. Wolstenholme and M. O'Connor, 1959.

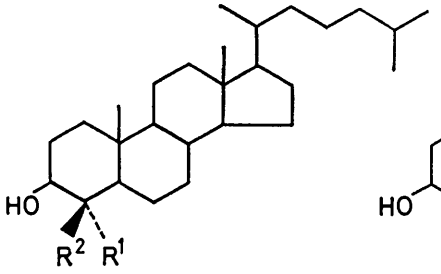
[54] J. L. Gaylor and C. V. Delwiche, *Steroids*, 1964, **4**, 207.

[55] K. B. Sharpless, T. E. Snyder, T. A. Spencer, K. K. Maheshwari, G. Guhn, and R. B. Clayton, *J. Amer. Chem. Soc.*, 1968, **90**, 6874.

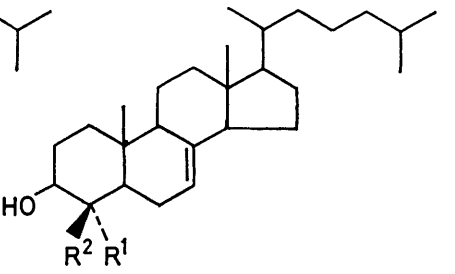
[56] (*a*) J. L. Gaylor and H. S. Mason, *J. Biol. Chem.*, 1968, **243**, 4966; (*b*) A. C. Swindell and J. L. Gaylor, *ibid.*, p. 5546.

[57] N. J. Moir, W. L. Miller, and J. L. Gaylor, *Biochem. Biophys. Res. Comm.*, 1968, **33**, 916.

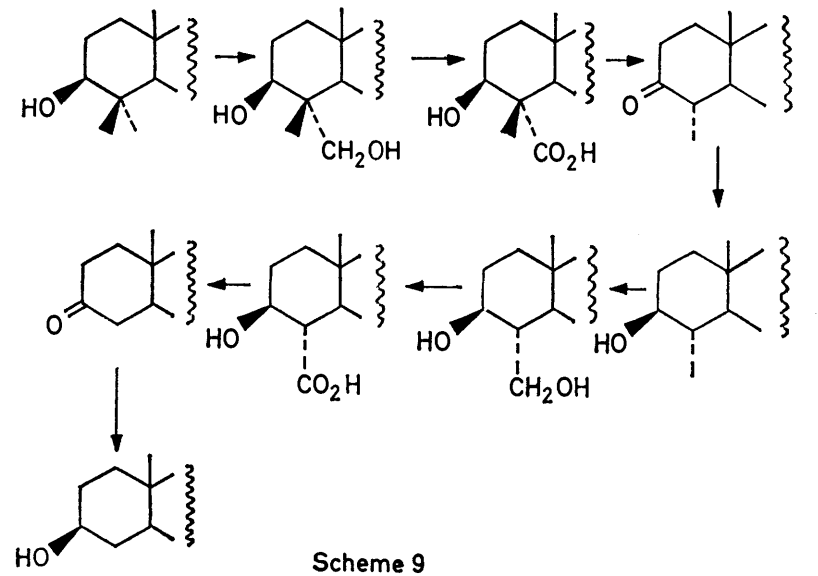
shown to require the cofactor NAD^+, and, by using rat liver microsomes deficient in this cofactor, 4,4-dimethylcholest-7-en-3β-ol (48a) and 4α-methylcholest-7-en-3β-ol (48b) were converted to the 4α-carboxylic acids (48c) and (48d) respectively.[58] Decarboxylation was completed on reincubation in the presence of NAD^+, indicating that the cofactor is required to oxidize the 3β-hydroxy-group



(47) (a) $R^1 = CH_2OH$; $R^2 = CH_3$
(b) $R^1 = CH_2OH$; $R^2 = H$
(c) $R^1 = CH_3$; $R^2 = CH_2OH$



(48) (a) $R^1 = R^2 = CH_3$
(b) $R^1 = CH_3$; $R^2 = H$
(c) $R^1 = CO_2H$; $R^2 = CH_3$
(d) $R^1 = CO_2H$; $R^2 = H$



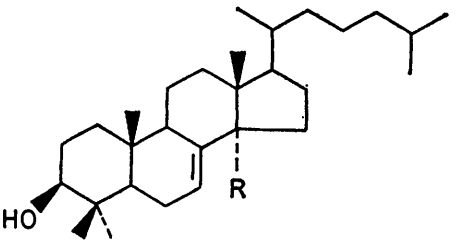
Scheme 9

[58] (*a*) W. L. Miller and J. L. Gaylor, *J. Biol. Chem.*, 1970, **245**, 5369, 5375; (*b*) G. M. Hornby and G. S. Boyd, *Biochem. Biophys. Res. Comm.*, 1970, **40**, 1452.

to a ketone either prior to, or concomitant with, decarboxylation. The cofactor NADPH has also been implicated in reduction of the ketone to a 3β-alcohol following decarboxylation.[56b] The results of several studies indicate that, following the loss of the 4α-methyl group, the remaining 4β-methyl equilibrates to the 4α-position (Scheme 9) prior to oxidation and elimination, probably by the same enzyme system.[58,59]

The sequence of reactions summarized in Scheme 9 is supported by studies on the biosynthetic origin of the 4α- and 4β-methyl groups. It has been demonstrated, both by n.m.r. spectroscopy[60] and by chemical degradation,[61] that 3*S*-2,3-epoxysqualene is the precursor of lanosterol and that the 4α-methyl group of lanosterol is derived exclusively from C-2 of MVA, while the 4β-methyl originates from C-3′. With this information at hand, [2-^{14}C]MVA was incubated with a rat liver enzyme preparation[62] and several precursors of cholesterol were isolated. While lanosterol and related compounds contained six ^{14}C atoms, 4α-methylcholest-7-en-3β-ol retained only five labelled atoms, confirming the loss of the original 4α-methyl group and epimerization of the 4β-methyl to the 4α-position. This scheme is also supported indirectly by the isolation of 4β-methylcholesta-8,24-dien-3β-ol from rat skin.[63]

B. Loss of the 14α-Methyl Group of Lanosterol.—The initial experiments of Bloch and co-workers[53] suggested that C-14 demethylation involves oxidation to the carboxylic acid followed by loss of CO_2 and that this process precedes those at C-4. The oxidative sequence has been supported by the demonstration that the 14α-hydroxymethyl and 14α-formyl compounds (49a) and (49b) are



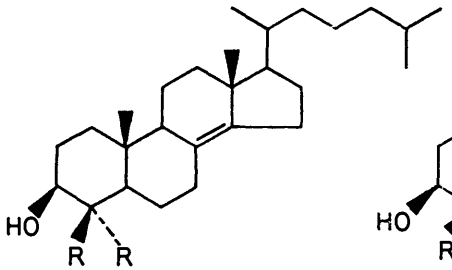
(49) (a) R = CH_2OH
(b) R = CHO

[59] K. B. Sharpless, T. E. Snyder, T. A. Spencer, K. K. Maheshwari, J. A. Nelson, and R. B. Clayton, *J. Amer. Chem. Soc.*, 1969, **91**, 3394.
[60] K. J. Stone, W. R. Roeske, R. B. Clayton, and E. E. van Tamelen, *Chem. Comm.*, 1969, 530.
[61] G. P. Moss and S. A. Nicolaides, *Chem. Comm.*, 1969, 1072, 1077.
[62] R. Rahman, K. B. Sharpless, T. A. Spencer, and R. B. Clayton, *J. Biol. Chem.*, 1970, **245**, 2667.
[63] (*a*) A. Sanghvi, D. Balasubramunian, and A. Moskowitz, *Biochemistry*, 1967, **6**, 869; (*b*) A. Sanghvi, *J. Lipid. Res.*, 1970, **11**, 124.

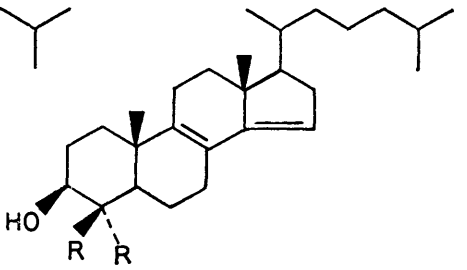
efficiently converted to cholesterol by liver microsomes.[64]† However, these compounds are probably not true intermediates since demethylation is thought to occur before migration of the nuclear double bond to the Δ^7 position (see below). It was also postulated that decarboxylation is accompanied by migration of the Δ^8 double bond to the $\Delta^{8(14)}$ position (Scheme 10, path *a*).[40,53c]

8 Migration of the Δ^8 Double Bond to Δ^5 in Cholesterol Formation

A. Double-bond Migrations Associated with Loss of the 14α-Methyl Group.— The observation that neither 4,4,14-trimethylcholestanol[55] nor 4,4,14-trimethylcholest-7-en-3β-ol[65] is metabolized by rat liver homogenates suggested the participation of the Δ^8 double bond of lanosterol in the loss of the 14α-methyl group. In addition, the conversion of cholest-8(14)-en-3β-ol (50a) and its 4,4-dimethyl analogue (50b) to cholesterol was demonstrated.[64,66] This process was



(50) (a) R = H
(b) R = CH_3



(51) (a) R = H
(b) R = CH_3

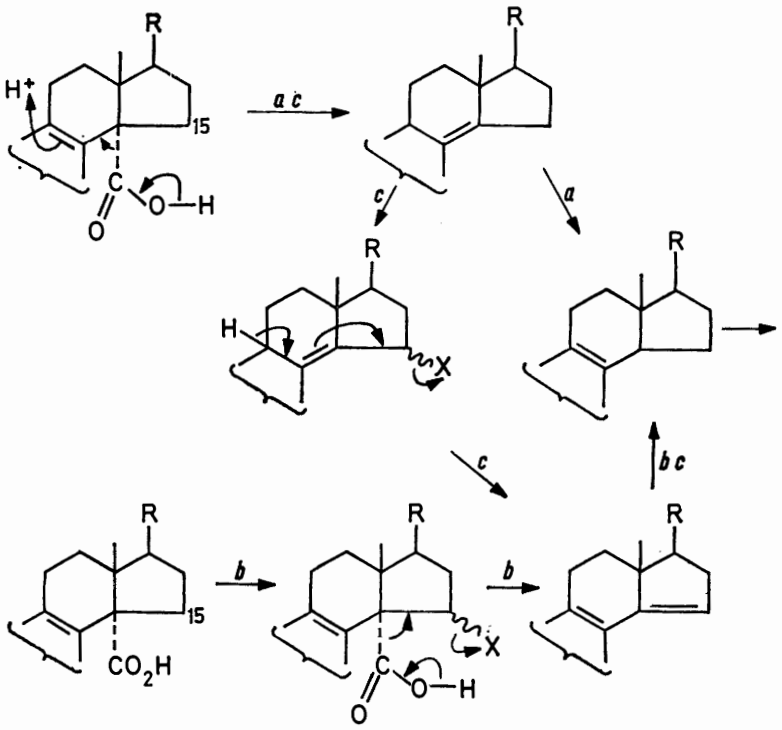
shown to require molecular oxygen since no conversion was obtained anaerobically. This suggested a decarboxylation mechanism as outlined in Scheme 10 (path *a*).

The use of lanosterol derived from 2*R*- and 2*S*-tritiated MVA provided more information on the reactions associated with C-14 demethylation.[67,68] Conversion of lanosterol to cholesterol was shown to proceed with loss of the 2-*pro-S* hydrogen of MVA located at the C-15α-position of lanosterol, while the 15β-hydrogen is retained. This result implicated the 15α-hydrogen in the C-14 demethylation sequence, and two mechanisms have been proposed to account for this observation.

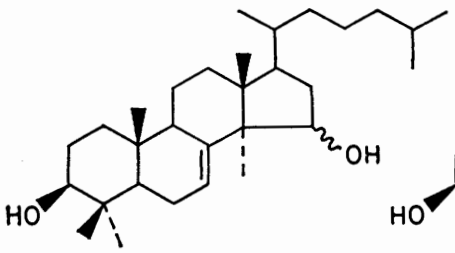
† Evidence has recently been obtained from studies with a microsomal enzyme system that C-14 demethylation occurs not by decarboxylation but by release of formic acid at the aldehyde level of oxidation (K. Alexander, M. Akhtar, R. B. Boar, J. F. McGhie, and D. H. R. Barton, *Chem. Comm.*, 1972, 383).

[64] J. Fried, A. Dudowitz, and J. W. Brown, *Biochem. Biophys. Res. Comm.*, 1968, **32**, 568.
[65] J. L. Gaylor, C. V. Delwiche, and A. C. Swindell, *Steroids*, 1966, **8**, 353.
[66] W. H. Lee and G. J. Schroepfer, *Biochem. Biophys. Res. Comm.*, 1968, **32**, 635.
[67] L. Canonica, A. Fiecchi, M. G. Kienle, A. Scala, G. Galli, E. G. Paoletti, and R. Paoletti, *J. Amer. Chem. Soc.*, 1968, **90**, 3597; *Steroids*, 1968, **12**, 445.
[68] G. F. Gibbons, L. J. Goad, and T. W. Goodwin, *Chem. Comm.*, 1968. 1458.

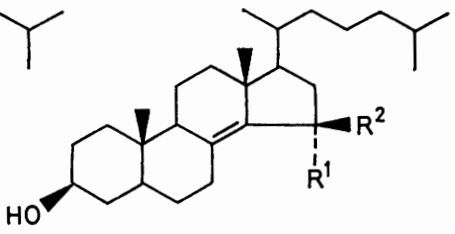
The involvement of a $\Delta^{8,14}$ diene intermediate formed as in Scheme 10 (path *b*) was supported by the fact that cholesta-8,14-dienol (51a) and its 4,4-dimethyl



Scheme 10



(52)



(53) (a) $R^1 = H$, $R^2 = OH$
(b) $R^1 = OH$, $R^2 = H$

analogue (51b) are metabolized in liver enzyme preparations[69–71] and that the latter compound became labelled when non-radioactive material was added to an incubation in which tritiated dihydrolanosterol was being metabolized to cholesterol.[71b] The group X may represent an enzymic or nucleophilic moiety or simply a hydrogen atom removed enzymatically as a hydride ion. The enzymic conversion of one of the epimeric diols (52) to cholesterol[72] is consistent with path *b*, although the stereochemistry of the active isomer is, as yet, unknown.

An alternative mechanism consistent with the stereospecific loss of the 15α-hydrogen atom is represented by Scheme 10 (path *c*). In this case the $\Delta^{8(14)}$ intermediate, formed as in path *a*, is subsequently dehydrogenated to cholesta-8,14-dienol. Reduction of the Δ^{14} bond would then give the Δ^{8} intermediate. Such an indirect process might be necessary to achieve the thermodynamically unfavourable $\Delta^{8(14)} \rightarrow \Delta^{8}$ migration. Here again several types of X group can be envisaged. In this connection, the diols (53a) and (53b) have been converted to cholesterol enzymically in high yield.[73]*

Reduction of the $\Delta^{8,14}$ diene to the Δ^{8} intermediate has been shown to involve addition of hydrogen from NADPH at C-14α and a proton from the medium at C-15.[74] The 15β-hydrogen of lanosterol, which is retained during conversion to the $\Delta^{8,14}$ diene, occupies the 15α-position in cholesterol,[75] indicating that reduction of the Δ^{14} bond involves addition of a proton at the 15β-position, giving an overall *trans* reduction.

B. Transposition of the Δ^{8} Double Bond to Δ^{5}.—The isomerization of the Δ^{8} double bond to Δ^{7} involves stereospecific loss of the 7β-hydrogen atom,[76] since lanosterol derived from (2*S*)-[2-^{3}H]MVA (and thus containing tritium at the 7β-position) gives cholesterol devoid of tritium at C-7, while the 7α-hydrogen of lanosterol is retained. An intramolecular migration of hydrogen from C-7

* However, a recent detailed investigation by means of incorporation and trapping experiments provides strong evidence that a $\Delta^{8(14)}$ intermediate is not involved and that the biosynthetic sequence is, in fact, as outlined in Scheme 10 path *b* (K. T. W. Alexander, M. Akhtar, R. B. Boar, J. F. McGhie, and D. H. R. Barton, *Chem. Comm.*, 1971, 1479).

[69] (*a*) B. N. Lutsky and G. J. Schroepfer, *Biochem. Biophys. Res. Comm.*, 1968, **33**, 492; (*b*) M. Akhtar, A. D. Rahimtula, I. A. Watkinson, D. C. Wilton, and K. A. Munday, *Chem. Comm.*, 1968, 1406.

[70] L. Canonica, A. Fiecchi, M. G. Kienle, A. Scala, G. Galli, E. G. Paoletti, and R. Paoletti, *J. Amer. Chem. Soc.*, 1968, **90**, 6532.

[71] (*a*) M. Akhtar, I. A. Watkinson, A. D. Rahimtula, D. C. Wilton, and K. A. Munday, *Biochem. J.*, 1969, **111**, 757; (*b*) I. A. Watkinson and M. Akhtar, *Chem. Comm.*, 1969, 206.

[72] J. A. Martin, S. Huntoon, and G. J. Schroepfer, *Biochem. Biophys. Res. Comm.*, 1970, **39**, 1170.

[73] S. Huntoon and G. J. Schroepfer, *Biochem. Biophys. Res. Comm.*, 1970, **40**, 476.

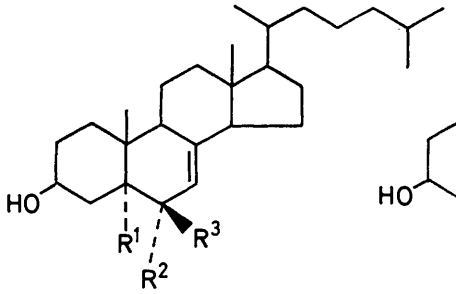
[74] M. Akhtar, A. D. Rahimtula, I. A. Watkinson, D. C. Wilton, and K. A. Munday, *Chem. Comm.*, 1969, 149.

[75] (*a*) E. Caspi, P. J. Ramm, and R. E. Gain, *J. Amer. Chem. Soc.*, 1969, **91**, 4012; (*b*) P. J. Ramm and E. Caspi, *J. Biol. Chem.*, 1969, **244**, 6064.

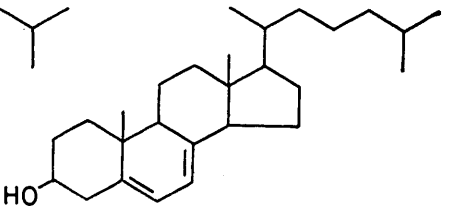
[76] (*a*) L. Canonica, A. Fiecchi, M. G. Kienle, A. Scala, G. Galli, E. G. Paoletti, and R. Paoletti, *Steroids*, 1968, **11**, 749; (*b*) E. Caspi, J. B. Greig, P. J. Ramm, and K. R. Varma, *Tetrahedron Letters*, 1968, 3829; (*c*) G. F. Gibbons, L. J. Goad, and T. W. Goodwin, *Chem. Comm.*, 1968, 1212.

to C-9 is not involved since the 9α-hydrogen originates from the medium.[77] The reversibility of this isomerization has recently been demonstrated by incorporation of radioactivity at C-9 of cholest-7-en-3β-ol re-isolated after incubation with liver microsomes in tritiated water.[78]

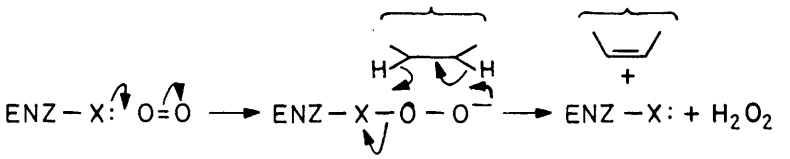
The conversion of cholest-7-en-3β-ol (54a) to cholesta-5,7-dien-3β-ol (55) results in stereospecific removal of the 5α- and 6α-hydrogen atoms.[79] The dehydrogenation process requires molecular oxygen[80] and a requirement for the cofactor $NADP^+$ has been detected.[81] Attempts to demonstrate a hydroxylation–dehydration mechanism for this step have been unsuccessful. Such a route should involve an aerobic hydroxylation followed by an anaerobic dehydration of the intermediate alcohol. While both 6α- and 6β-hydroxycholest-7-en-3β-ol



(54)(a) $R^1 = R^2 = R^3 = H$
(b) $R^1 = R^3 = H$; $R^2 = OH$
(c) $R^1 = R^2 = H$; $R^3 = OH$
(d) $R^1 = OH$; $R^2 = R^3 = H$



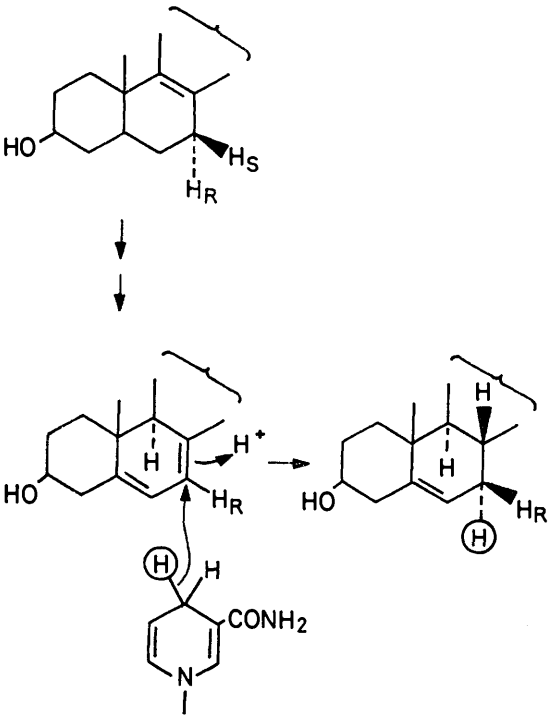
(55)



Scheme 11

[77] (*a*) M. Akhtar and A. D. Rahimtula, *Chem. Comm.*, 1968, 259; (*b*) L. Canonica, A. Fiecchi, M. G. Kienle, A. Scala, G. Galli, E. G. Paoletti, and R. Paoletti, *Steroids*, 1968, **11**, 287; (*c*) W. H. Lee, R. Kammereck, B. N. Lutsky, J. A. McCloskey, and G. J. Schroepfer, *J. Biol. Chem.*, 1969, **244**, 2033.
[78] D. C. Wilton, A. D. Rahimtula, and M. Akhtar, *Biochem. J.*, 1969, **114**, 71.
[79] (*a*) A. M. Paliokas and G. J. Schroepfer, jun., *J. Biol. Chem.*, 1968, **243**, 453; (*b*) M. Akhtar and S. Marsh, *Biochem. J.*, 1967, **102**, 462.
[80] M. E. Dempsey, *J. Biol. Chem.*, 1965, **240**, 4176.
[81] T. J. Scallen and M. W. Schuster, *Steroids*, 1968, **12**, 683.

(54b and c) are converted to cholesterol by rat liver enzymes in the presence of oxygen, anaerobic incubation of these compounds results in conversion to cholest-7-en-3β-ol (54a) rather than cholesterol, indicating that they are not true intermediates in the dehydrogenation process.[82] Similar investigations on the metabolism of 5α-hydroxycholest-7-en-3β-ol (54d) have also led to the conclusion that this diol is not an intermediate in cholesterol biosynthesis. The failure to detect hydroxylated intermediates in the C-5 dehydrogenation step has led to the suggestion[83,84] that this and other *cis* dehydrogenations may be accomplished by an enzyme–oxygen complex (Scheme 11), similar to that postulated for analogous reactions in fatty acid biosynthesis.[85]*



Scheme 12

* It has now been shown that the 6α-hydrogen is not transferred to the cofactor, NAD^+ and is, therefore, probably removed as a proton (D. J. Aberhart and E. Caspi, *J. Biol. Chem.*, 1971, **246**, 1387).

[82] M. Slaytor and K. Bloch, *J. Biol. Chem.*, 1965, **240**, 4598.

[83] S. M. Dewhurst and M. Akhtar, *Biochem. J.*, 1967, **105**, 1187.

[84] (*a*) D. C. Wilton and M. Akhtar, *Biochem. J.*, 1970, **116**, 337; (*b*) J. M. Zander and E. Caspi, *J. Biol. Chem.*, 1970, **245**, 1682.

[85] (*a*) G. J. Schroepfer and K. Bloch, *J. Biol. Chem.*, 1965, **240**, 54; (*b*) W. Stoffel and H. G. Schiefer, *Z. physiol. Chem.*, 1966, **345**, 41.

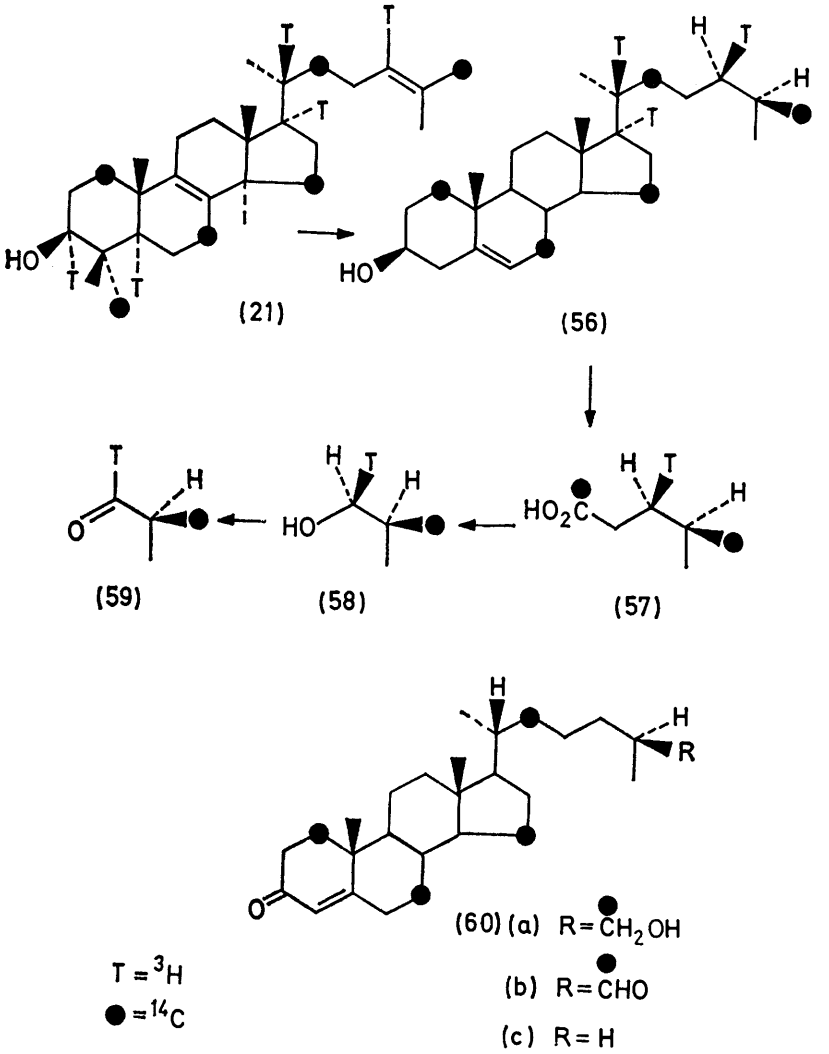
The final reaction of this sequence is the saturation of the Δ^7 double bond of the 5,7-diene intermediate. This involves the addition of a proton from the medium at C-8β and a hydride from NADPH at C-7α (Scheme 12).[86] The assignment of orientation at C-7 follows from the observation that the 7α-hydrogen of lanosterol, which is retained in the 5,7-diene intermediate, occupies the 7β-position in cholesterol.[76b,c] Again, reduction of the double bond involves a *trans* addition of hydrogens.

9 Stereochemistry of Hydrogenation of Δ^{24} of Lanosterol

Although it is still uncertain at which point saturation of the Δ^{24} double bond of lanosterol occurs in the overall process of conversion to cholesterol, the stereochemistry of the process has been elucidated. The addition of hydrogen at C-24 was studied by the use of cholesterol (56) biosynthesized from (4*R*)-[2-^{14}C, 4-^{3}H]MVA.[87] The side-chain was cleaved by a bovine adrenal enzyme preparation, giving 4-methylpentanoic acid (57) which was subsequently degraded to 2-methylpropanol (58) containing one tritium atom as predicted.[87] Oxidation to 2-methylpropionic acid resulted in loss of tritium activity and thus located the tritium at C-24 of cholesterol. Oxidation of 2-methylpropanol with yeast alcohol dehydrogenase, which removes only the 1-*pro-R* proton of aliphatic primary alcohols, gave 2-methylpropanal (59) with retention of tritium. This established the tritium atom at the 24-*pro-R* position of cholesterol (56) and indicated that reduction of Δ^{24} of lanosterol involved addition of a hydrogen at the 24-*pro-S* position.[87]

The stereochemistry of hydrogen addition at C-25 followed from two pieces of evidence. Firstly, it was shown by *X*-ray diffraction that cholesterol is converted by the organism *Mycobacterium smegmatis* to (25*S*)-26-hydroxycholestenone (60a)[88a] [rather than the (25*R*)-isomer as previously reported[88b]]. Secondly, when cholesterol biosynthesized from [2-^{14}C]MVA in a rat liver enzyme system is oxidized by this organism, the ^{14}C-labelled terminal methyl group is hydroxylated. This was shown by oxidation of (60a) to the aldehyde (60b) followed by decarbonylation to (60c). Since it is known that in the Δ^{24}-double bond of lanosterol (21) the labelled carbon atom is *cis* to the C-24 hydrogen, it follows that the double bond is saturated by *cis* addition of two hydrogens to C-24 and C-25. The cofactor NADPH is involved in the reduction and the process has been shown to result in addition of a proton from the medium at C-24 and a hydride ion at C-25.[89] This *cis* reduction involving NADPH contrasts with the *trans* reductions of the Δ^{14} and Δ^7 double bonds of other intermediates in cholesterol biosynthesis which have been described above.

[86] D. C. Wilton, K. A. Munday, S. J. M. Skinner, and M. Akhtar, *Biochem. J.*, 1968, **106**, 803.
[87] (*a*) E. Caspi, K. R. Varma, and J. B. Greig, *Chem. Comm.*, 1969, 45; (*b*) J. B. Greig, K. R. Varma, and E. Caspi, *J. Amer. Chem. Soc.*, 1971, **93**, 760.
[88] (*a*) D. J. Duchamp, C. G. Chidester, J. A. F. Wickramasinghe, E. Caspi, and B. Yagen, *J. Amer. Chem. Soc.*, 1971, **93**, 6283; (*b*) E. Caspi, M. G. Kienle, K. R. Varma, and L. J. Mulheirn, *J. Amer. Chem. Soc.*, 1970, **92**, 2161.
[89] M. Akhtar, K. A. Munday, A. D. Rahimtula, I. A. Watkinson, and D. C. Wilton, *Chem. Comm.*, 1969, 1287.



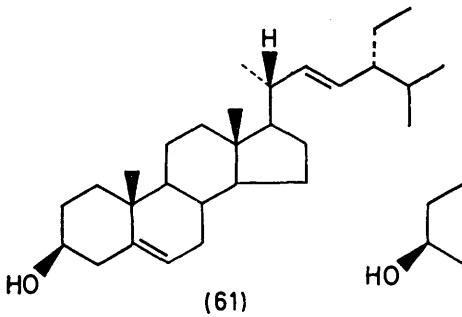
10 Sterol Formation in Other Organisms

The investigations of cholesterol biosynthesis reported above have stimulated similar investigations in other species. The structures of the sterols of plants, fungi, and algae suggest a biosynthetic route parallel to that of cholesterol. However, while the overall process appears to be common to all organisms, some unexpected variations of sequence and mechanism have been detected.

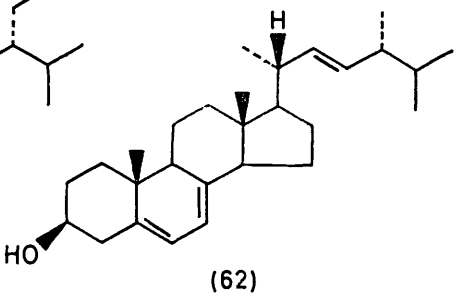
As mentioned previously, the triterpene cycloartenol (14) is known to replace lanosterol as the product of cyclization of 2,3-epoxysqualene in higher plants

and some algae.[4,25–29] However, lanosterol, although it is not a true intermediate, can be metabolized to phytosterols in some plants.[90] This result has been attributed to a lack of specificity of certain enzymes for the natural substrate. Incorporation of [2-^{14}C]MVA into the triterpenes and sterols of various plants indicates that, as in lanosterol demethylation in animals, loss of the 4α-methyl group is followed by epimerization of the 4β-methyl to the 4α-position before removal.[91] The isolation of many plant triterpenes lacking a methyl group at C-4 indicates that the overall demethylation sequence in plants may be C-4α → C-14α → C-4β (after epimerization),[4] rather than that believed to be operating in rat-liver homogenates; C-14α → C-4α → C-4β (after epimerization). In contrast, the fungal triterpene fusidic acid (16) retains all six labelled atoms when biosynthesized in the presence of [2-^{14}C]MVA, indicating that this compound has resulted from direct elimination of the 4β-methyl group of the original triterpene and retention of the 4α-methyl group.[92]

Few investigations have yet been reported on the nuclear double-bond migrations associated with C-14 demethylation of the triterpenes of lower organisms. In the formation of poriferasterol (61) by the phytoflagellate *Ochromonas malhamensis* the 7β-hydrogen is lost during Δ^8–Δ^7 isomerization,[93] while in both this organism and the leaves of the larch, *Larix decidua*, the 6α-hydrogen atom is lost on introduction of Δ^5 unsaturation.[94] Both processes are identical to those observed in cholesterol biosynthesis. In contrast, the Δ^8–Δ^7 isomerization occurring during the biosynthesis of ergosterol (62) and related sterols in



(61)



(62)

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[94] (*a*) A. R. H. Smith, L. J. Goad, and T. W. Goodwin, *Chem. Comm.*, 1968, 1259; (*b*) L. J. Goad and T. W. Goodwin, *European J. Biochem.*, 1969, **7**, 502.

yeast results in elimination of the 7α-hydrogen atom,[95] although introduction of the Δ^5 double bond again proceeds by loss of the 5α- and 6α-hydrogen atoms.[96]

The presence of a *trans* Δ^{22}-double bond is a feature of many sterols produced by plants, fungi, and algae. Here again, variations have been observed in the stereochemical course of dehydrogenation. The fungus *Aspergillus fumigatus* produces ergosterol (62) by elimination of the 22-*pro-S* and 23-*pro-S* protons,[96b] while the alga *Ochromonas malhamensis* produces poriferasterol (61) by loss of the 22-*pro-R* and 23-*pro-R* protons.[93,94a] Similar results in related species[4,96c] suggest that all fungi and algae may show this divergence of hydrogen elimination at C-22 and C-23. Finally, the alkylation at C-24 of phytosterols, which has been shown to involve transfer of methyl groups from *S*-adenosylmethionine, also appears to occur by a variety of mechanisms. The principal routes have been reviewed elsewhere.[97,98] However, more detailed investigations will be needed before the extent and significance of these variations can be assessed.

11 Conclusion

The identification of the component reactions of sterol biosynthesis, described in this review, has been achieved by the application of three main lines of investigation. Detailed knowledge of the biosynthesis of squalene from MVA has been utilized to study individual demethylation, migration, and elimination processes occurring at later stages of the sequence. The chemical synthesis of putative intermediates has allowed the feasibility of various hypotheses to be examined, while investigation of the enzymes and cofactors involved in individual reactions is proving to be a powerful tool for the analysis of reaction sequences in cell homogenates. The results obtained from these studies give a detailed picture of the biosynthesis of cholesterol from MVA in rat liver homogenates. Parallel investigations are now being conducted with other organisms. However, the principal remaining question concerns the absolute sequence of these reactions, if indeed there is one. Such investigations will probably involve the application of current techniques to intact cells and organisms. The reported identification of proteins capable of solubilizing squalene and other sterol precursors, and which catalyse several stages of sterol biosynthesis in rat liver enzyme systems,[99] has added a further dimension for future investigation.

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