BIOSYNTHESIS OF STEROLS, STEROIDS, AND TERPENOIDS. PART I.
BIOGENESIS OF CHOLESTEROL AND THE FUNDAMENTAL STEPS IN TERPENOID BIOSYNTHESIS

By R. B. CLAYTON

(DEPARTMENT OF PSYCHIATRY, STANFORD UNIVERSITY SCHOOL OF MEDICINE, PALO ALTO, CALIFORNIA)

Introduction

THE principal aim of this and the following Review (Part II) will be to survey the work relating to the biosynthesis of sterols and to some major aspects of the conversion of cholesterol into steroid hormones and bile acids. Intensive studies of cholesterol biosynthesis, particularly those conducted in the 1950s in the laboratories of Bloch, Cornforth and Popják, and Lynen, have elucidated most of the essential features of the biogenesis not only of cholesterol and related sterols, but of terpenoid compounds in general. The resulting advances in knowledge have covered a front far too broad for detailed treatment in these Reviews and the salient results in several areas of terpene biochemistry will be dealt with only briefly in Part II. So far as possible detailed discussion will be reserved for more recent work, but for coherence it seems unavoidable to reiterate much older material although it has been covered extensively in several earlier Reviews. An excellent concise discussion of the chemical principles involved in most of the steps in cholesterol biosynthesis will be found in the Review by Cornforth.[1] Fieser and Fieser[2] present a valuable outline of the biosynthetic pathway with emphasis on the chemical techniques (degradative procedures, etc.) used in the biochemical studies up to 1958 and Crabbé[3] has reviewed cholesterol biosynthesis in the broader context of the biogenesis of terpenes. The most comprehensive recent Review of sterol biosynthesis, covering both enzymology and chemical mechanisms, is that of Popják and Cornforth[4] and earlier work has been discussed in detail by Bloch.[5,6] Other valuable surveys are those of Tchen[7] and Wright.[8] Many important contributions to the field were published in a CIBA Foundation Symposium in 1959.[9] A single volume contains excellent recent

[1] Cornforth, *J. Lipid Res.*, 1959, **1**, 3.
[2] Fieser and Fieser, "Steroids", Reinhold, New York, 1959, Ch. 13.
[3] Crabbé, *Rec. Chem. Progr.*, 1959, **20**, 189.
[4] Popják and Cornforth in "Advances in Enzymology", ed. Nord, Interscience, 1960, vol. 22, 281.
[5] Bloch, Harvey Lectures, 1952, **48**, 68.
[6] Bloch, "Vitamins and Hormones," eds. Harris, Marrian, and Thimann, Academic Press, New York, 1957, vol. 15, p. 119.
[7] Tchen, "Metabolic Pathways," ed. Greenberg, Academic Press, New York, 1960, p. 389.
[8] Wright, "Annual Review of Biochemistry," ed. Luck, Annual Reviews, Palo Alto, 1961, **30**, p. 525.
[9] CIBA Foundation Symposium: Biosynthesis of Terpenes and Sterols, ed. Wolstenholme and O'Connor, Little, Brown and Co., Boston, 1959.

reviews of both the chemistry[10] and biochemistry[11] of the adrenal steroids. The biochemistry of the steroid hormones has also been reviewed by Samuels[12] and by Engel and Langer,[13] and that of the bile acids by Bergström, Danielsson, and Samuelsson[14] and by Danielsson.[15]

The Major Features of the Pathway of Cholestrol Biosynthesis.—Isotope studies by Bloch and his co-workers revealed the origins of the individual carbon atoms of the side chain of cholesterol in either $C_{(1)}$ or $C_{(2)}$ of acetate, and in the light of suggestions made by Bonner and Arreguin[16] concerning the biogenesis of the isoprene units of rubber, in which a similar pattern of distribution of acetate carbons was postulated, these results led to a revival of a hypothesis originally due to Heilbron, Kamm, and Owens[17] and stated more specifically by Robinson,[18] that the triterpene hydrocarbon, squalene, was a biological precursor of cholesterol. This early work, including the first direct demonstration of the biosynthesis of squalene in mammalian tissue and its conversion into cholesterol[19] has been reviewed by Bloch.[5] A crucial contribution to proper understanding of the role of squalene in sterol biogenesis was the structural elucidation of lanosterol, a non-saponifiable component of wool wax, which was shown by Ruzicka and Jeger and their associates[20] to be 4,4′,14α-trimethylcholesta-8,24-dien-3β-ol. In consideration of this structure, Woodward and Bloch[21] suggested a pattern of cyclisation of squalene (Fig. 1, I) which could account for the biological derivation of both lanosterol (II) and cholesterol (III) from this hydrocarbon, the 4,4′-*gem*-dimethyl structure of lanosterol being derived from a terminal isopropylidene group of squalene. They pointed out that the departure from the strict "isoprenoid" arrangement of methyl groups at $C_{(13)}$ and $C_{(14)}$ of lanosterol would have to be accounted for in terms of a shift of methyl groups of squalene in the course of cyclisation. The squalene cyclisation scheme of Woodward and Bloch differed from that suggested earlier by Robinson[18] in which the central bond of squalene corresponded to that between $C_{(6)}$ and $C_{(7)}$ of cholesterol. Assuming that the distribution of acetate carbon atoms in the isoprene units throughout the squalene molecule conformed to the pattern found in the cholesterol side chain, the labelling pattern in

[10] Moore and Heftmann, "Handbuch Experimentellen Pharmakologie," ed. Deane, Springer, Berlin, 1962, vol. 14, pt. 1, p. 186.
[11] Dorfman, ref. 10, p. 511.
[12] Samuels, ref. 7, p. 471.
[13] Engel and Langer, ref. 8, p. 499.
[14] Bergström, Danielsson, and Samuelsson, "Lipid Metabolism," ed. Bloch, Wiley Sons, New York, 1960, p. 291.
[15] Danielsson, "Advances in Lipid Research," ed. Paoletti and Kritchevsky, Academic Press, 1963, vol. 1, p. 335.
[16] Bonner and Arreguin, *Arch. Biochem. Biophys.*, 1949, **21**, 109.
[17] Heilbron, Kamm, and Owens, *J.*, 1926, 1630.
[18] Robinson, *Chem. and Ind.*, 1934, **53**, 1062.
[19] Langdon and Bloch, *J. Biol. Chem.*, 1953, **200**, 129.
[20] Voser, Mijovic, Heusser, Jeger, and Ruzicka, *Helv. Chim. Acta*, 1952, **35**, 2414.
[21] Woodward and Bloch, *J. Amer. Chem. Soc.*, 1953, **75**, 2023.

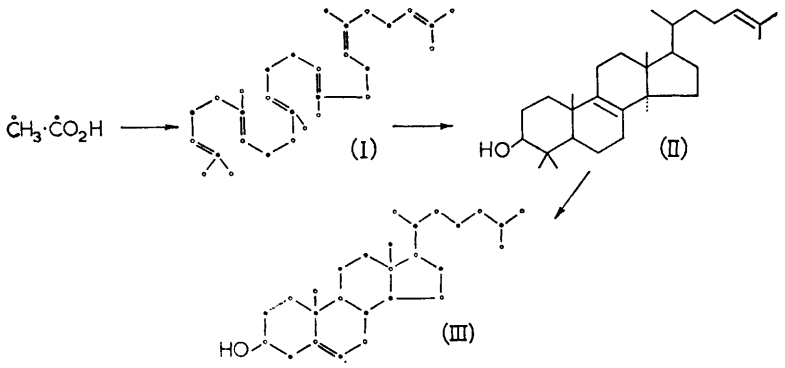


FIG. 1. Biogenetic relationship between squalene, lanosterol, and cholesterol.

the cholesterol nucleus must differ, depending upon which scheme was valid. In support of their hypothesis, Woodward and Bloch[21] gave evidence that the mixed $C_{(10)}$ and $C_{(13)}$ of cholesterol contained methyl carbon of acetate, and Bloch[22] later showed that $C_{(7)}$ was also derived from the methyl carbon of acetate. Further confirmation came from the demonstration that lanosterol was synthesised in rat tissue both *in vitro*[23] and *in vivo*[24] and in turn was efficiently metabolised to cholesterol. Finally the total degradation of squalene[25] and of cholesterol[26] biosynthesised from labelled acetate, with identification of the origin of each carbon atom of both structures from either $C_{(1)}$ or $C_{(2)}$ of acetate, gave results (I and III) in total agreement with the postulated biogenetic relationship.

Intermediate reactions between acetate and squalene

The incorporation of acetate into squalene can best be discussed as four sequential phases: (1) conversion of acetate into mevalonate; (2) conversion of mevalonate into isopentenyl pyrophosphate, which is probably the immediate precursor of the isoprene unit in all living systems; (3) the head-to-tail condensation of three isopentenyl pyrophosphate molecules yielding farnesyl pyrophosphate; and (4) the tail-to-tail union of two of these farnesyl residues to give the symmetrical structure of squalene. The essential biochemical mechanisms of phases (2) and (3) were clarified before 1960 and have been reviewed in detail.[1,4,7] Some new proposals concerning phase (1) have been put forward and the stereochemical aspects of phases (2)—(4) have recently been the subject of a penetrating analysis.

[22] Bloch, *Helv. Chim. Acta*, 1953, **36**, 1611.
[23] Clayton and Bloch, *J. Biol. Chem.*, 1956, **218**, 305, 319.
[24] Schneider, Clayton, and Bloch, *J. Biol. Chem.*, 1957, **224**, 175.
[25] Cornforth and Popják, *Biochem. J.*, 1954, **58**, 403.
[26] Cornforth, Hunter, and Popják, *Biochem. J.*, 1953, **54**, 590, 597; Cornforth, Gore, and Popják, *ibid.*, 1957, **65**, 94.

The Conversion of Acetate into Mevalonate.—In the early 1950s the search for the biological equivalent of the isoprene unit, conducted in several laboratories, followed along lines that were related to a mechanism suggested for the biogenesis of the isoprene units of rubber by Bonner and Arreguin.[16,27] The five-carbon branched chain acid radicals: isovalerate, β-hydroxyisovalerate, ββ-dimethylacrylate, as well as the six-carbon acid radical β-hydroxymethylglutarate, were all utilised as precursors of cholesterol but with extensive randomisation of their carbon atoms. β-Hydroxymethylglutarate was suggested as a precursor of sterols by Bloch[5] but although the synthesis of hydroxymethylglutaryl-CoA (VI) (CoA = coenzyme A) from acetyl-CoA (IV) and acetoacetyl-CoA (V) by enzyme extracts of both liver and yeast, was demonstrated[28,29] (Fig. 2,

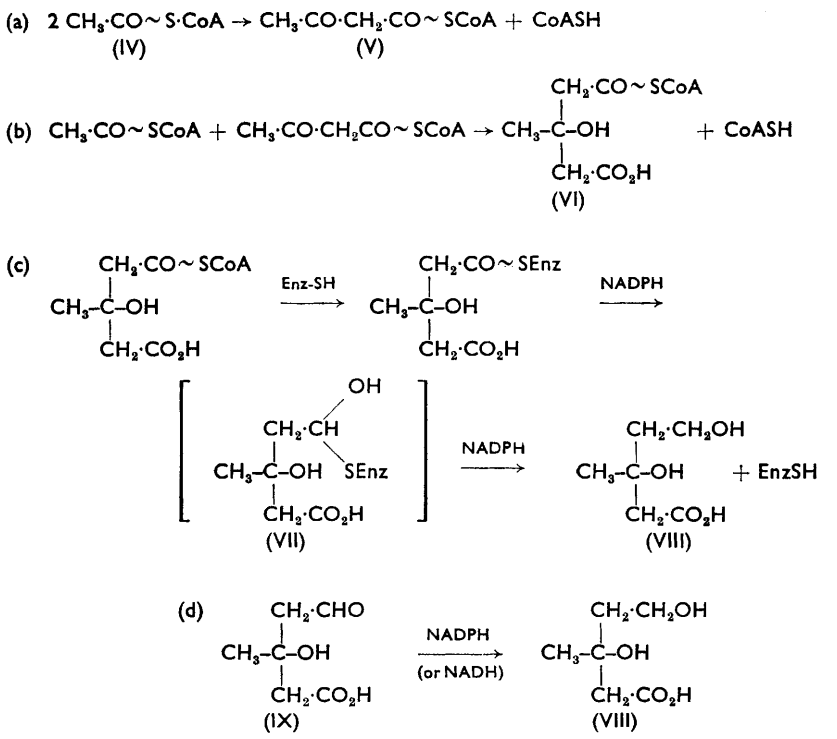


FIG. 2. Synthesis of mevalonic acid from acetate.

a, b), further insight into the pathway between acetate and the terpenoid compounds finally came from the fortuitous discovery of mevalonic acid

[27] Arreguin and Bonner, *Arch. Biochem. Biophys.*, 1950, **26**, 178.
[28] Rudney, ref. 9, p. 75.
[29] Lynen, Henning, Bublitz, Sörbo, and Kroplin-Rueff, *Biochem. Z.*, 1958, **330**, 269.

(VIII) by Folkers and his associates.[30,31] The structural relationship of this compound to hydroxymethylglutarate prompted a study of its possible rôle as a cholesterol precursor,[32] which showed that one enantiomorph of racemic mevalonic acid was almost quantitatively converted into the isoprene unit with concomitant loss of the carboxyl group.[33]

The pattern of labelling in squalene[34,35] and cholesterol[36] biosynthesized from [2-^{14}C]mevalonate conformed to this mode of utilisation of mevalonate. Moreover, labelled mevalonic acid was isolated from a liver enzyme system by a trapping technique in which unlabelled mevalonic acid was added to the system during synthesis of squalene from [^{14}C]acetate.[37]

The pathway outlined in Fig. 2 (a)—(c) has become generally accepted as the route of biosynthesis of mevalonic acid in yeast and mammalian tissues.[28,38–42] It has been fully discussed by Popják and Cornforth[4] and set forth in more or less detail by numerous other reviewers (see, *e.g.*, ref. 43). The evidence points to a reduction of hydroxymethylglutarate to mevalonate which is only reversible with difficulty, thus accounting for the efficient utilisation of mevalonate for sterol synthesis, in contrast to the poor utilisation of hydroxymethylglutaryl-CoA which is subject to cleavage to acetyl-CoA and acetoacetate. Mevaldic acid (IX), although reduced by a specific reductase to mevalonic acid (Fig. 2d) and hence made available for sterol synthesis,[30,39,41,44–46] is probably not a normal intermediate.[38,40,42,45] An enzyme-bound intermediate (VII) of equivalent oxidation state has been postulated (Fig. 2c).[4]

A different view of the origin of mevalonic acid has recently been put forward by Brodie *et al.*[47] (Fig. 3) who have studied an avian liver enzyme preparation that was active in fatty acid synthesis, with a view to testing the intermediates in that process for their availability as precursors of mevalonic acid. Attention was directed[48–50] primarily to the utilisation of

[30] Folkers, Shunk, Linn, Robinson, Wittreich, Huff, Gilfillan, and Skeggs, ref. 9, p. 20.
[31] Wolf, Hoffman, Aldrich, Skeggs, Wright, and Folkers, *J. Amer. Chem. Soc.*, 1956, **78**, 4499; 1957, **79**, 1486.
[32] Tavormina, Gibbs, and Huff, *J. Amer. Chem. Soc.*, 1956, **78**, 4498.
[33] Tavormina and Gibbs, *J. Amer. Chem. Soc.*, 1956, **78**, 6210.
[34] Dituri, Gurin, and Rabinowitz, *J. Amer. Chem. Soc.*, 1957, **79**, 2650.
[35] Cornforth, Cornforth, Popják, and Gore, *Biochem. J.*, 1958, **69**, 146.
[36] Isler, Ruegg, Würsch, Gey, and Pletscher, *Helv. Chim. Acta*, 1957, **40**, 2369.
[37] Knauss, Porter, and Wasson, *J. Biol. Chem.*, 1959, **234**, 2835.
[38] Durr and Rudney, *J. Biol. Chem.*, 1960, **235**, 2572.
[39] Lynen, ref. 9, p. 95.
[40] Knappe, Ringelmann, and Lynen, *Biochem. Z.*, 1959, **332**, 195.
[41] Coon, Kupiecki, Dekker, Schlesinger, and del Campillo, ref. 9, p. 62.
[42] Brodie and Porter, *Biochem. Biophys. Res. Comm.*, 1960, **3**, 173.
[43] Bernfeld, "The Biogenesis of Natural Compounds," Pergamon Press, New York, 1963.
[44] Wright, Cleland, Dutta, and Norton, *J. Amer. Chem. Soc.*, 1957, **79**, 6572.
[45] Schlesinger and Coon, *J. Biol. Chem.*, 1961, **236**, 2421.
[46] Knauss, Brodie, and Porter, *J. Lipid Res.*, 1962, **3**, 197.
[47] Brodie, Wasson, and Porter, *J. Biol. Chem.*, 1963, **238**, 1294.
[48] Brady, *Proc. Nat. Acad. Sci., U.S.*, 1958, **44**, 993.
[49] Wakil and Ganguly, *J. Amer. Chem. Soc.*, 1959, **81**, 2597.
[50] Lynen, *J. Cell. Comp. Physiol.*, 1959, **54** (Suppl. 1), 33.

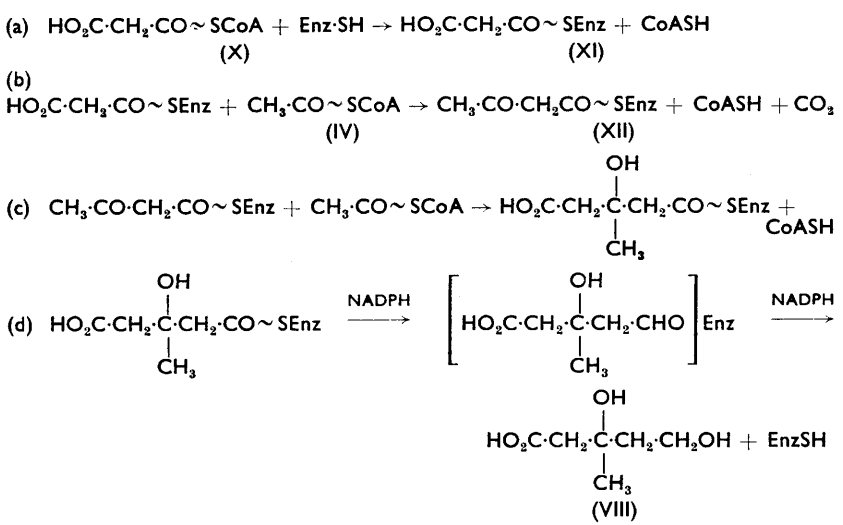


FIG. 3. Alternative scheme for mevalonic acid biosynthesis involving enzyme-bound intermediates.[47,51]

acetyl-CoA and malonyl-CoA (X), each of which was found to enhance the incorporation of the other into hydroxymethylglutarate in the absence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). However, the initial condensation–decarboxylation reaction between these two compounds apparently led to acetoacetyl units that were firmly bound to the enzyme (XII), since addition of an unlabelled pool of acetoacetyl-CoA had little effect on the efficiency of incorporation of labelled acetyl-CoA or malonyl-CoA into hydroxymethylglutaryl-CoA. In the presence of NADPH the product was mevalonic acid (VIII). The authors interpret their results to support the alternative reaction pathway (Fig. 3 a—d) in which malonyl-enzyme (XI) yields acetoacetyl-enzyme (XII) as a key intermediate common to both fatty acid and sterol synthesis. They suggest that acetoacetyl-enzyme may be a focal point for mechanisms controlling the relation between these two processes. According to this view the previously observed reactions (Fig. 2) are due to slow exchange of reactants between their coenzyme A and enzyme-bound forms. These results have so far not been confirmed by other workers but whereas Porter and his co-workers have extended them[51] and have recently claimed[52] to have obtained results with rat liver preparations that were consonant with their earlier findings with avian liver systems, one attempt to test their findings in a rat liver preparation was reported to be unsuccessful.[53]

[51] Brodie, Wasson, and Porter, *J. Biol. Chem.*, 1964, **239**, 1346.
[52] Porter, Guchhast, and Vadlamundi, 6th International Congress of Biochemistry, New York, 1964, Abstracts, p. 590.
[53] Fimognari and Rodwell, 6th International Congress of Biochemistry, New York, 1964, Abstracts, p. 573.

The Conversion of Mevalonate into Isopentenyl Pyrophosphate.—The first clue to the mechanism whereby mevalonic acid provides the isoprene unit for the biosynthesis of terpenoid compounds was the observation that a dialysed enzyme system from yeast catalysed the conversion of mevalonic acid into squalene only on addition of adenosinetriphosphate (ATP), Mg^{++}, and pyridine nucleotides.[54] The formation of a monophosphorylated derivative of mevalonic acid, which served as an efficient precursor of squalene, was shown by Tchen[55,56] to be catalysed by a mevalonic kinase of yeast. Similar enzymic activity was also observed in mammalian liver.[57] On the basis of its elementary analysis, stability to both acid and alkaline hydrolysis, and its failure to undergo lactone formation,[58] the new compound was suspected to be mevalonic acid 5-phosphate (XIII) and was identified as such by chemical synthesis.[30,39] Subsequent intensive work in the laboratories of Bloch,[59,60] Popják,[57,58] and Lynen,[39,61] showed that mevalonic acid monophosphate underwent a second ATP-dependent phosphorylation to the 5-pyrophosphate (XIV)[62,63] followed by a concerted dehydration and decarboxylation reaction which required a third molecule of ATP. The product of these reactions (Fig. 4a) was identified by Bloch and his co-workers[59] as isopentenyl pyrophosphate (XV). The transformation entailed the loss of the carboxyl carbon of mevalonic acid phosphate but retention of its original phosphate group and acquisition of a second phosphate from ATP. The diphosphate was readily hydrolysed in acid in keeping with its proposed pyrophosphate structure. The structure of the isopentenyl moiety was demonstrated by phosphatase hydrolysis followed by steam distillation in the presence of carrier isopentenol and the formation of isopentenyl 3,5-dinitrobenzoate. The structure of the compound has also been confirmed by organic synthesis.[61,64]

Several mechanisms for the dehydration–decarboxylation reaction which constitutes the last step in isopentenyl pyrophosphate formation have been considered by Bloch and his co-workers[62] who have purified the enzyme involved, 120-fold, from yeast autolysates. The strict stoicheiometry between ATP and mevalonic pyrophosphate consumed and isopentenyl pyrophosphate, ADP, and CO_2 produced, argue in favour of a concerted process involving a 3-phosphorylated intermediate (XVI) as shown (Fig. 4b). Such an intermediate has, however, never been isolated and may well be incapable of more than transient existence.

[54] Amdur, Rilling, and Bloch, *J. Amer. Chem. Soc.*, 1957, **79**, 2646.
[55] Tchen, *J. Amer. Chem. Soc.*, 1957, **79**, 6344.
[56] Tchen, *J. Biol. Chem.*, 1958, **233**, 1100.
[57] Popják, ref. 9, p. 148.
[58] DeWaard and Popják, *Biochem. J.*, 1959, **73**, 410.
[59] Chaykin, Law, Phillips, Tchen, and Bloch, *Proc. Nat. Acad. Sci., U.S.A.*, 1958, **44**, 998.
[60] DeWaard, Phillips, and Bloch, *J. Amer. Chem. Soc.*, 1959, **81**, 2913.
[61] Lynen, Eggerer, Henning, and Kessel, *Angew. Chem.*, 1958, **70**, 738.
[62] Bloch, Chaykin, Phillips, and DeWaard, *J. Biol. Chem.*, 1959, **234**, 2595.
[63] Henning, Moslein, and Lynen, *Arch. Biochem. Biophys.*, 1959, **83**, 259.
[64] Yuan and Bloch, *J. Biol. Chem.*, 1959, **234**, 2605.

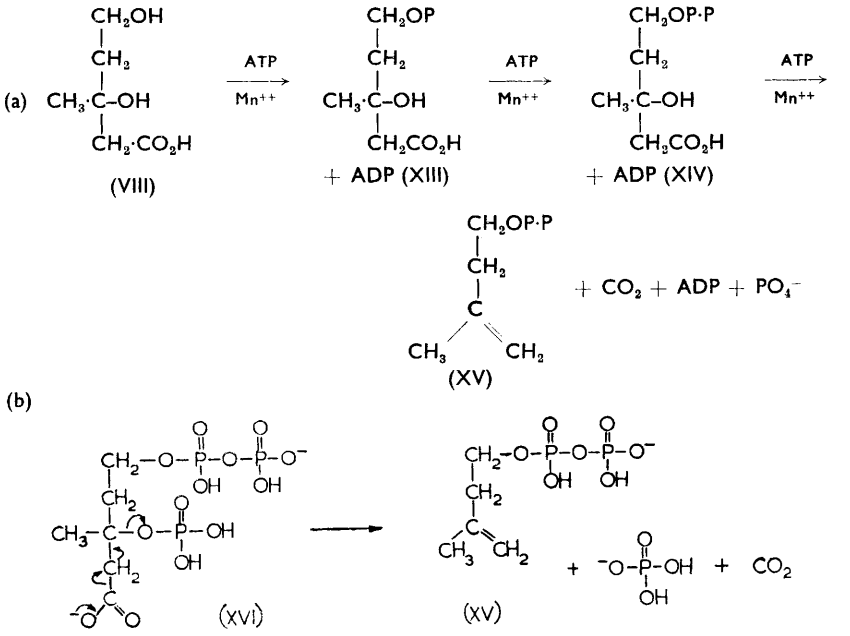


FIG. 4. Conversion of mevalonic acid into isopentenyl pyrophosphate.

The Conversion of Isopentenyl Pyrophosphate into Farnesyl Pyrophosphate—Lynen and his collaborators identified farnesyl pyrophosphate (Fig. 5, XIX) as a precursor of squalene[61] in yeast and it was later shown[65] that farnesyl pyrophosphate was preceded in the biosynthetic sequence by geranyl pyrophosphate (XVIII). The first step in the conversion of isopentenyl pyrophosphate into farnesyl pyrophosphate was the enzymic isomerisation of isopentenyl pyrophosphate (XV) to dimethylallyl pyrophosphate (XVII) Fig. 5a).[66,67] The mechanism of formation of the terminal isopropylidene groups of squalene from the isomeric isopentenyl structure was thus clarified, since each terminal isoprene unit of squalene arises from a molecule of dimethylallyl pyrophosphate. Dimethylallyl pyrophosphate is converted into geranyl pyrophosphate (XVIII) and the latter, in turn, to *trans-trans*-farnesyl pyrophosphate (XIX), by sequential reactions involving isopentenyl pyrophosphate (Fig. 5b, c). Thus, one half of the symmetrical carbon skeleton of squalene is assembled. This pathway has been shown to operate in mammalian liver by Popják and his coworkers[68,69] who have also established the *trans-trans*-structure for the

[65] Lynen, Agranoff, Eggerer, Henning, and Moslein, *Angew. Chem.*, 1959, **71**, 657.
[66] Agranoff, Eggerer, Henning, and Lynen, *J. Amer. Chem. Soc.*, 1959, **81**, 1254.
[67] Agranoff, Eggerer, Henning, and Lynen, *J. Biol. Chem.*, 1960, **235**, 326.
[68] Popják, *Tetrahedron Letters*, 1959, **19**, 19.
[69] Goodman and Popják, *J. Lipid Res.*, 1961, **1**, 286.

farnesyl pyrophosphate and demonstrated the biosynthesis of squalene from farnesyl pyrophosphate that had been prepared by organic synthesis.[70,71] Some important advances in knowledge of the stereochemistry of these reaction are discussed below.

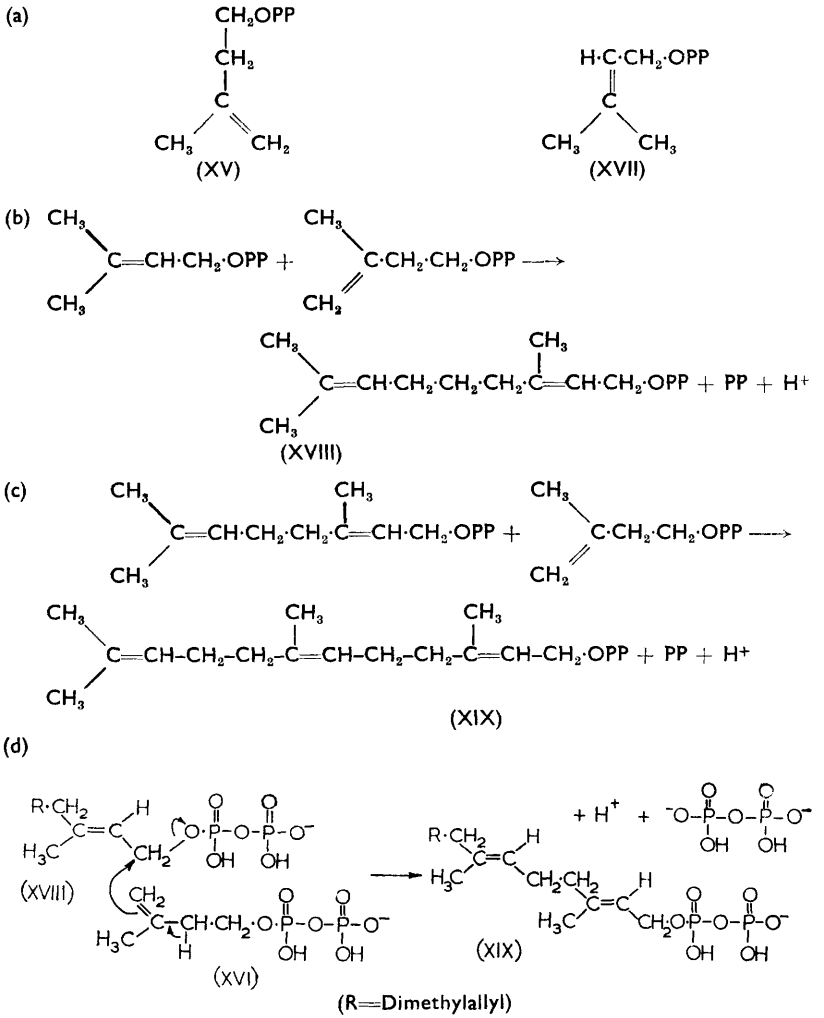


FIG. 5. Synthesis of farnesyl pyrophosphate and general mechanism of condensation of isopentenyl pyrophosphate with allyl pyrophosphates.

[70] Popják, Cornforth, Cornforth, Ryhage, and Goodman, *Biochem. Biophys. Res. Comm.*, 1961, **1**, 204.

[71] Popják, Cornforth, Cornforth, Ryhage, and Goodman, *J. Biol. Chem.*, 1962, **237**, 56.

In the process of the formation of the new carbon–carbon bond in each of these condensation reactions, an allylic pyrophosphate group is eliminated. While it seems most probable that the enzymic condensation takes place in concerted fashion as shown in Fig. 5d, it may formally be considered as a two-stage process of which the first stage consists of an elimination of the allylic pyrophosphate group as the inorganic anion with the formation of a cation that is subject to resonance stabilisation.[1] The situation becomes energetically favourable when a new nucleophilic group [*i.e.*, the methylene group of isopentenyl pyrophosphate (XVI)] is available for attack by the carbonium ion, and the resulting structure is stabilised by ejection of a proton with the formation of a new allyl pyrophosphate. The essential characteristics of this mechanism were discussed on a theoretical basis by Rilling and Bloch[72] and by Lynen *et al.*[61] before completely substantiating experimental data were available, and were elaborated by Cornforth and Popják[73] in an early attempt to account for the formation of squalene. There is now evidence from work with a variety of biological systems that the same mechanism applies generally to the head-to-tail linkage of isoprene units in terpenoid compounds. Although it does not appear to have been confirmed at an enzymic level, the same principle of attack upon a neighbouring double bond by a resonance-stabilised carbonium ion derived from an allylic pyrophosphate, is probably the basis of formation of a wide variety of cyclic terpenoid compounds. An interesting non-enzymic analogy with the postulated biochemical mechanism has recently been described[74] (Fig. 6), in which an ethereal

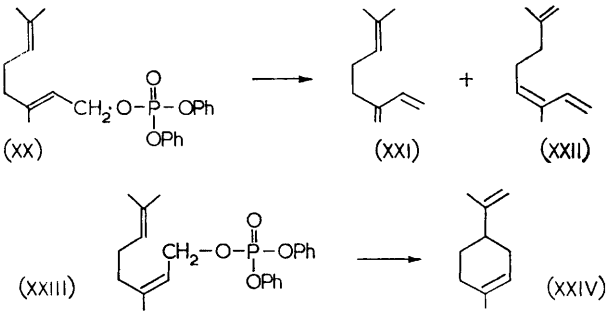


FIG. 6.

solution of geranyl diphenyl phosphate (XX) was partially transformed, on prolonged standing, into a mixture of myrcene (XXI) and ocimene (XXII), while neryl diphenyl phosphate (XXIII) with a *cis*-allylic double bond, gave a 45% yield of limonene (XXIV) under the same conditions.

[72] Rilling and Bloch, *J. Biol. Chem.*, 1959, **234**, 1424.
[73] Cornforth and Popják, *Tetrahedron Letters*, 1959, **19**, 29.
[74] Miller and Wood, *Angew. Chem., Internat. Edn.*, 1964, **3**, 310.

The Conversion of Farnesyl Pyrophosphate into Squalene and the Stereochemistry of Squalene Biosynthesis.—The mechanism of condensation of two molecules of farnesyl pyrophosphate with the formation of squalene remains to be fully elucidated, though many features of the transformation may now be described in extraordinary detail, primarily as a result of the brilliant contributions of Cornforth and Popják and their collaborators.

When squalene is synthesised from [5,5-$^2H_2$]mevalonate by a rat liver enzyme system, 11 of the theoretically possible 12 deuterium atoms are retained.[75] The deuterium atom which is lost is detached from one of the two centre carbon atoms of the squalene molecule and, in a reaction medium in which all other components are unlabelled, is replaced by a normal hydrogen atom. This conclusion was based on a detailed, mass-spectrometric examination of the succinic acid fragment, derived from the ozonolysis of squalene, representing the four central carbon atoms of the molecule. In the mass spectrometer the anhydride of this succinic acid gave peaks with mass/charge ratios of 31, corresponding to trideutero-ethylene ion; 44 and 43, corresponding to the ketene fragments CD_2CO and CHDCO; and, most significantly, a peak at mass/charge ratio 59 corresponding to ($CHDCD_2CO$) in high abundance. Confirmatory results were obtained in the mass spectrometric analysis of the succinate dimethyl ester which yielded peaks with mass/charge ratios of 90 ($CH_3O{\cdot}CO{\cdot}CHD{\cdot}CD_2$) and 118 ($CH_3O{\cdot}CO{\cdot}CHD{\cdot}CD_2{\cdot}CO$). The interpretation of these results was based on a careful comparison with the mass spectra of normal (unlabelled) succinate, tetradeuterosuccinate and symmetrical dideutero- and asymmetrical dideutero-succinates, all of which were prepared by organic synthesis.

The origin of the hydrogen which displaces a deuterium atom from one of the centre carbon atoms of squalene was examined by experiments in which [^{14}C]farnesyl pyrophosphate was incubated with microsomes in the absence of oxygen and in a medium containing either ^{3}HHO or NADP^{3}H. In the former case only 0·013—0·015 μg.-atom ^{3}H per μmole of squalene was incorporated whereas, in the latter, 0·55—0·82 μg.-atom ^{3}H per μmole was incorporated, leaving no doubt that NADPH is the source of the entering hydrogen atom. On the other hand, in a more complex enzyme system (microsomes + supernatant fraction) containing ^{3}HHO, and [^{14}C]mevalonate was shown to be incorporated into farnesol with an uptake of 0·38—0·49 μg.-atom ^{3}H per μmole and into squalene with an uptake of 1·48—1·16 μg.-atom ^{3}H per μmole. The enhanced incorporation of ^{3}H from ^{3}HHO which evidently accompanied the condensation of the farnesyl pyrophosphate molecules under these conditions was attributed to the possibility of labelling the NADPH from ^{3}HHO by concurrent side reactions in the less pure enzyme preparation and was offered as an explanation of the earlier findings of Rilling and Bloch.[72] These authors,

[75] Popják, Goodman, Cornforth, Cornforth, and Ryhage, *J. Biol. Chem.*, 1961, **236**, 1934.

in experiments similar to those of Popják *et al.* but using, instead of the rat liver enzyme system, a crude autolysate of yeast, had concluded that two atoms of hydrogen from the water of the incubation medium were introduced into the centre of squalene during its biosynthesis. In a re-examination of the problem with the use of purer enzyme preparations from yeast and employing mass spectrometric analysis of the 4-carbon centre fragment of squalene in the form of 1,4-propanediol rather than succinic acid, Childs and Bloch[76] obtained results that were in essential agreement with those of Popják *et al.* In a further detailed study of the formation of squalene from farnesyl pyrophosphate (Fig. 7) Popják *et al.*[71] were able

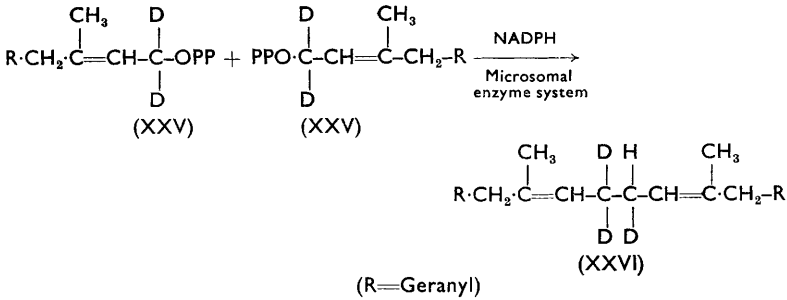


FIG. 7. Loss of deuterium from [1,1-$^2H_2$]farnesyl pyrophosphate in coupling to form squalene.

to show that synthetic all-*trans*-[2-^{14}C; 1,1-$^2H_2$]farnesyl pyrophosphate (XXV) yielded squalene in which the centre two carbon atoms retained only three of the four possible deuterium atoms (XXVI). Similarly, when the corresponding[2-^{14}C; 1,1-$^3H_2$]-labelled substrate was used the $^3H/^{14}C$ ratio in squalene was 75% of that in the farnesyl pyrophosphate.

The incorporation of [$^2H_2$]farnesyl pyrophosphate into squalene took place without any measurable isotope effect, suggesting that the enzymic process was stereospecific. This interpretation was confirmed unambiguously by experiments which involved (1) a demonstration that the hydrogen transferred from NADPH to one of the centre carbons of squalene was removed from the "B" face[77] of the pyridine ring of the nucleotide,[78] and (2) the isolation of the four centre carbons of squalene (Fig. 8a, XXVII), synthesised from [2-^{14}C; 5,5-$^2H_2$]mevalonate in the form of optically active succinate (XXVIII) containing three atoms of deuterium and one of normal hydrogen per molecule.[79] The structure of the succinic acid obtained in this experiment was deduced from its behaviour in the mass

[76] Childs and Bloch, *J. Biol. Chem.*, 1962, **237**, 62.
[77] Cornforth, Ryback, Popják, Donninger, and Schroepfer, *Biochem. Biophys. Res. Comm.*, 1962, **9**, 371.
[78] Popják, Schroepfer, and Cornforth, *Biochem. Biophys. Res. Comm.*, 1961/62, **6**, 438.
[79] Cornforth, Cornforth, Donninger, Popjak, Ryback, and Schroepfer, *Biochem. Biophys. Res. Comm.*, 1963, **11**, 129.

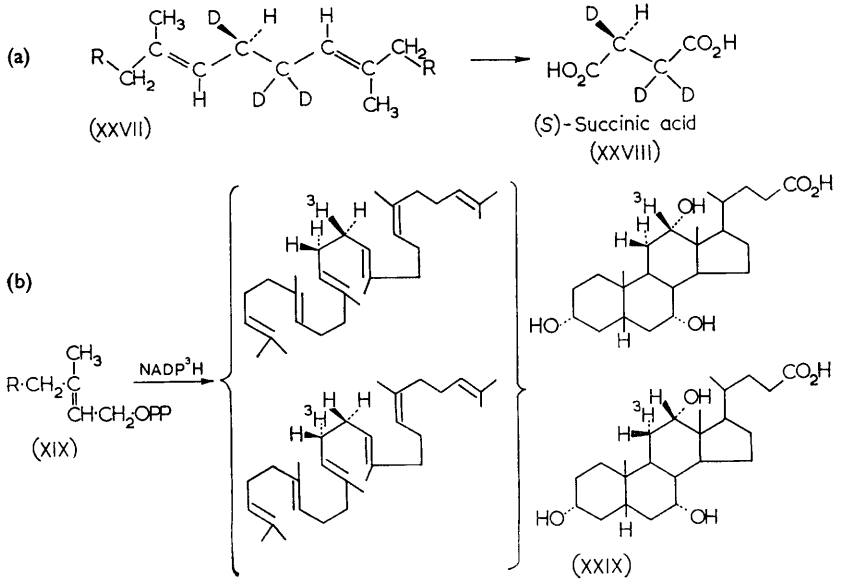


FIG. 8. (a) Absolute stereochemistry at the centre of squalene formed from [1,1-$^2H_2$] farnesyl pyrophosphate or [5,5-$^2H_2$]mevalonate.[79]

(b) The experiment of Samuelsson and Goodman.[81]

spectrometer, and from the finding that the optical rotatory dispersion curve for this material was the mirror image of that of a synthetic sample of [2-$^2H_1$]succinic acid of known absolute configuration, *R*,[80] it was concluded that the absolute configuration of the trideuterosuccinic acid (XXVIII), and hence of the asymmetric carbon at the centre of the squalene synthesised in this experiment, was s. It was pointed out by Cornforth *et al.*[78] that the foregoing observations permit the prediction that tritium introduced into squalene in the course of biosynthesis from farnesyl pyrophosphate in the presence of NADP^{3}H, should appear in either the 11α or 12β position in the steroid nucleus, depending upon the direction of cyclisation of the squalene molecule. This expectation was confirmed independently by Samuelsson and Goodman[81] (Fig. 8b) who prepared cholesterol biosynthetically labelled from [^{14}C]farnesyl pyrophosphate (XIX), and NADPH and injected it into a rat from which both cholic and chenodeoxycholic acids were recovered *via* a bile fistula with approximately equal ^{3}H/^{14}C ratios. The cholic acid (XXIX) was converted into the 3α,7α-diacetoxy-methyl ester and this in turn was oxidised under non-enolising conditions to yield the 12-ketone in which approximately

[80] Cahn, Ingold, and Prelog, *Experientia*, 1956, **12**, 81.

[81] Samuelsson and Goodman, *Biochem. Biophys. Res. Comm.*, 1963, **11**, 125; *J. Biol. Chem.*, 1963, **239**, 98.

one half of the tritium content of the cholic acid had been lost. On enolisation to labilise the hydrogen atom at $C_{(11)}$, all but 2·9% of the tritium was displaced. The results thus indicate approximately equal distribution of tritium between $C_{(11)}$ and $C_{(12)}$ with all of the label in the latter position in the β-configuration. In view of the accumulated evidence that hydroxylations in the steroid nucleus take place without inversion,[82,83] the configuration of tritium at $C_{(12)}$ in the precursor cholesterol must also be presumed to be 12β and while no evidence was available for that at $C_{(11)}$, it is reasonable to assume that it is in the α-position.

Information concerning the stereochemistry of hydrogen exchange at $C_{(1)}$ of farnesyl pyrophosphate during squalene biosynthesis was obtained[84] by the use of mevalonate (Fig. 9a, XXX) stereospecifically labelled by

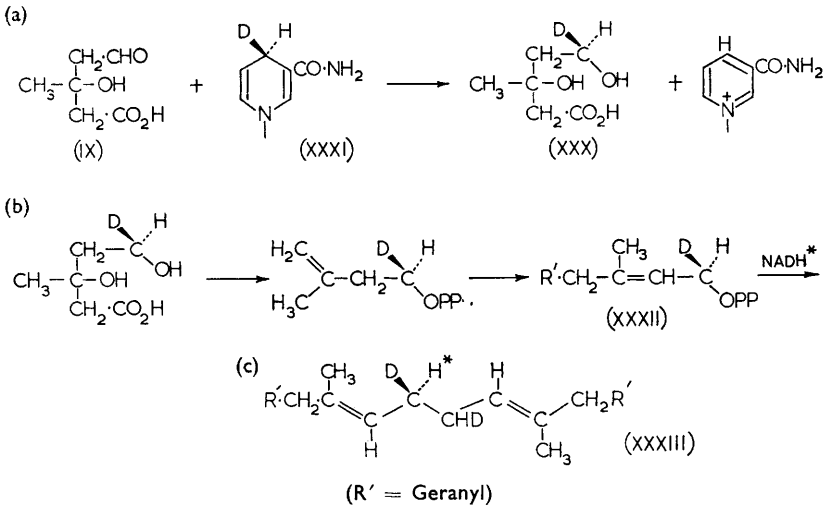


FIG. 9. (a) Preparation of 5-R-[5-^{2}H]mevalonic acid and its incorporation (b) into farnesyl pyrophosphate and (c) into squalene.
The hydrogen atom derived from NADH in the last step is designated H*

reduction of mevaldic acid (IX) with mevaldic reductase and 4-*R*-[4-$^2H_1$]-reduced nicotinamide adenine dinucleotide (NADH) or 4-*R*-[4-$^3H_1$] NADPH (XXXI). [5-$^3H_1$]-Mevalonate prepared in this way was mixed with [4-^{14}C]mevalonate so that loss or retention of tritium atoms in the course of enzymic conversions could be assessed in terms of $^3H/^{14}C$-ratios. Thus the $^3H/^{14}C$ ratio in farnesol biosynthesised from

[82] Bergström, Lindstedt, Samuelsson, Corey, and Gregoriou, *J. Amer. Chem. Soc.* 1958, **80**, 2337.
[83] Hayano, Gut, Dorfman, Sebek, and Peterson, *J. Amer. Chem. Soc.*, 1958, **80**, 2336.
[84] Popják, 6th International Congress of Biochemistry, New York, 1964, Abstract, p. 545.

this material was assumed to represent a value of 3 atoms ^{3}H/3 atoms ^{14}C. The absolute configuration of $C_{(1)}$ of farnesyl pyrophosphate (XXXII) was presumed to be unchanged from that of $C_{(5)}$ of the precursor [$^{3}H_1$]mevalonate (Fig. 8b). It was also assumed that this configuration was *R*, since liver alcohol dehydrogenase removed the labelled hydrogen atom from $C_{(1)}$ of farnesol biosynthesised from the enzymically labelled mevalonate.[85] (That this assumption was correct was supported[86] by the results of other experiments in which geraniol, stereo-specifically labelled by a non-enzymic method, was oxidised by liver alcohol dehydrogenase in the presence of NAD.) When 1-*R*-[1,5,9-$^{2}H_3$]-farnesyl pyrophosphate formed from 5-*R*-[5-$^{2}H_1$]mevalonate was converted enzymically into squalene (XXXIII) no loss of deuterium could be detected. The conclusion that both centre carbon atoms of squalene retained the labelled hydrogens present in farnesyl pyrophosphate was confirmed by ozonolysis of the squalene and gas-radiochromatographic analysis of the fragments. From these results it was concluded that the hydrogen atom removed from $C_{(1)}$ of farnesyl pyrophosphate in the course of condensation to squalene is epimeric to that removed from farnesol by alcohol dehydrogenase and that during the formation of squalene there is either no inversion at $C_{(1)}$ of the farnesyl pyrophosphate molecule involved in the hydrogen exchange reaction or there is an even number of inversions resulting in an apparent retention of configuration. There is no evidence yet available that distinguishes between these two possibilities.

These results define the stereochemical outcome of the changes taking place at $C_{(1)}$ of one farnesyl pyrophosphate molecule in the course of squalene formation, but they shed no light on the stereochemistry of the concomitant changes that occur at $C_{(1)}$ of the other. Further experiments[84] have given evidence on this point and also on the stereochemistry of the changes involving the $C_{(1)}$ carbon atoms of dimethylallyl pyrophosphate and geranyl pyrophosphate in the course of their respective condensations with isopentenyl pyrophosphate (Fig. 10). 5-*R*-[5-$^{2}H_1$]Mevalonate prepared from mevaldate and A-deutero NADH was converted into squalene (XXXIV) in a rat liver homogenate and the squalene isolated and degraded by ozonolysis to yield succinic (XXV) and levulinic (XXXVI) acids. The dideutero-succinic acid, representing the four middle carbon atoms of squalene, may be of two kinds, depending upon whether retention of configuration occurs at both, or only one, of the farnesyl pyrophosphate $C_{(1)}$ centres. The succinate should be optically active -*SS* in the former case and optically inactive (meso) -*RS* in the latter and, in fact, was found to be optically inactive. Thus, the overall process of the union of two farnesyl pyrophosphate molecules to yield squalene results in retention of configuration at $C_{(1)}$ of one molecule and inversion at the other. From the same experiment evidence was obtained for inversion of configuration at $C_{(1)}$ in

[85] Donninger and Popják, *Biochem. J.*, 1964, **91**, 10P.
[86] Donninger and Ryback, *Biochem. J.*, 1964, **91**, 11P.

both dimethylallyl pyrophosphate and geranyl pyrophosphate in their reactions with isopentenyl pyrophosphate. The two carbon atoms in question appear as $C_{(2)}$ of levulinic acid (XXXVI) and must be asymmetric since they are linked to one deuterium and one hydrogen atom. By hypoiodite oxidation the levulinic acid was converted into succinic acid (XXXVII) in which the asymmetric carbon atom retained its configuration. The optical rotation of the succinic acid indicated a configuration of *R*, which can only arise if inversion takes place at $C_{(1)}$ of the precursor pyrophosphates in the course of addition to the double bond of isopentenyl pyrophosphate. Such a result is consistent with a bimolecular nucleophilic substitution mechanism.

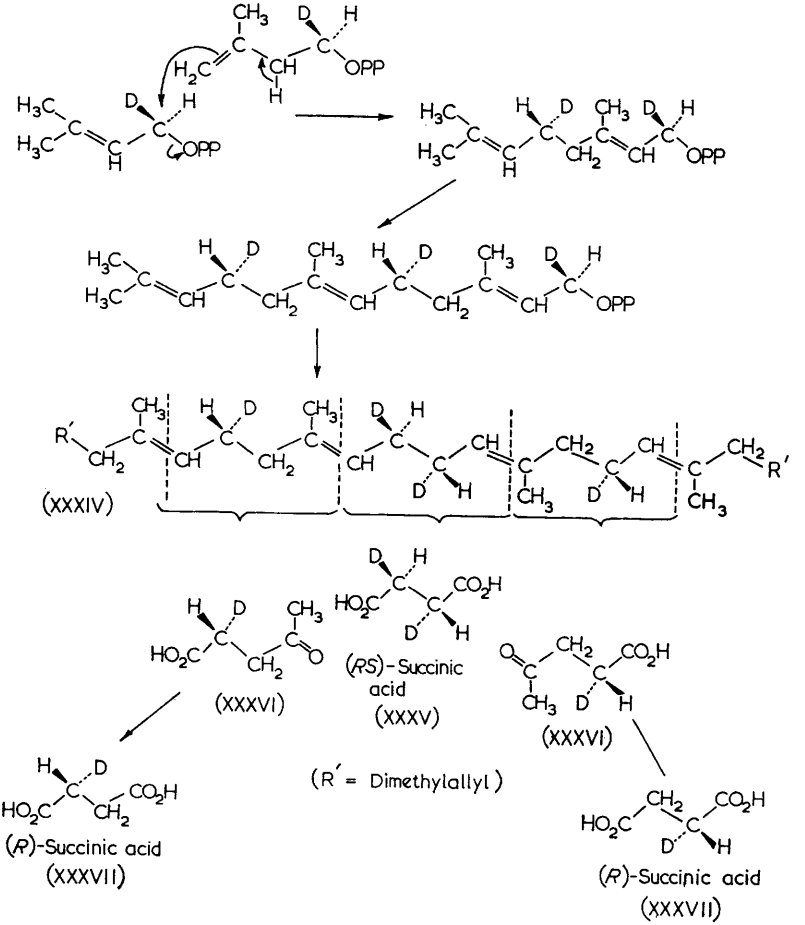


FIG. 10. Further analysis of stereochemistry of incorporation of 5-R-[5-$^2H_1$]mevalonic acid into geranyl and farnesyl pyrophosphates and into squalene.

In more recent studies by Cornforth and Popják and their co-workers[84] the loss of the hydrogen atom from $C_{(2)}$ of isopentenyl pyrophosphate in the course of its condensation to form *trans-trans*-farnesyl pyrophosphate has been examined with the use of mevalonic acid stereospecifically labelled at $C_{(4)}$ with deuterium in either the *S* or *R* configuration. $C_{(4)}$ of mevalonate becomes $C_{(2)}$ of isopentenyl pyrophosphate and hence the formation of the *trans*-double bonds of farnesol involves the loss of hydrogen atoms originally present at $C_{(4)}$ of mevalonate. No label was retained in farnesyl pyrophosphate when the precursor was 4-*S*-[4-$^2H_1$]mevalonate, and 4-*S*-[4-^{3}H;2-^{14}C]mevalonate yielded squalene that contained only ^{14}C, whereas squalene formed from the 4-*R*-isomer (Fig. 11, XXXVIII) retained the $^3H/^{14}C$ ratio of the starting material. The stereochemistry of labelling of farnesyl pyrophosphate (XXXIX) from 4-*R*-[4-^{3}H; 2-^{14}C]mevalonate (XXXVIII) must therefore be as shown (Fig. 11).

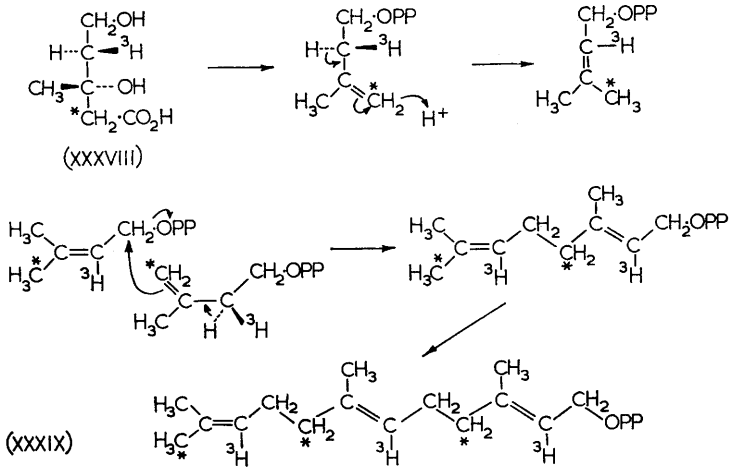
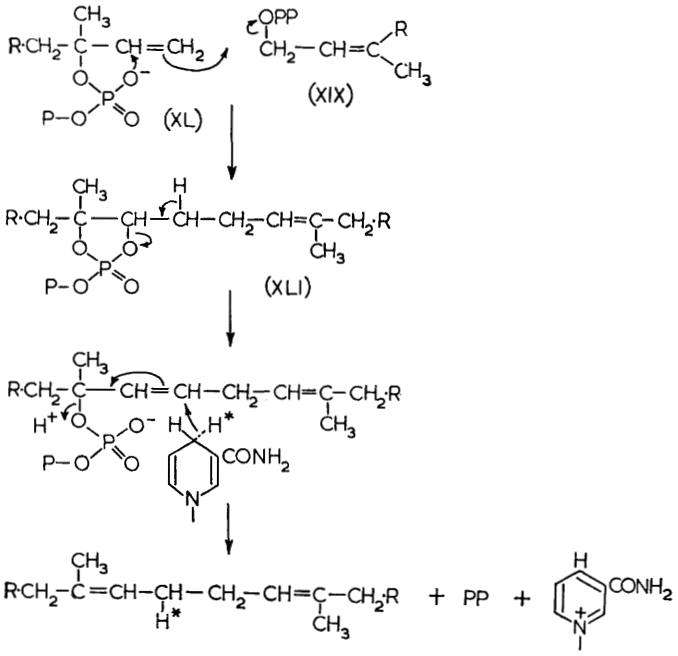


FIG. 11. Absolute stereochemistry of proton eliminations at C-2 of isopentenyl pyrophosphate during biosynthesis of farnesyl pyrophosphate.

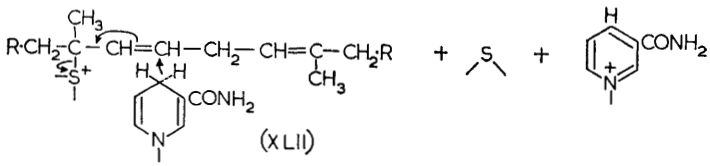
The unprecedented detail which these studies have revealed with respect to the stereochemistry of squalene biosynthesis still leaves unanswered the question of exactly how the two halves of the molecule become united, and clearly the answer must ultimately come from studies of the enzyme system involved.

Two general mechanisms have been suggested which are consistent with all of the experimental data. Two variants of the first type of mechanism[75] are shown in Fig. 12. The assumption in each case is that one of the reacting molecules of farnesyl pyrophosphate undergoes prior isomerisation to nerolidyl pyrophosphate (XL)[68,74] thus making $C_{(1)}$ of this molecule a methylene carbon capable of entering into an S_N2 type of interaction

with $C_{(1)}$ of the second farnesyl pyrophosphate molecule (XIX) in a manner analogous to the interaction between isopentenyl pyrophosphate and dimethylallyl pyrophosphate. This second farnesyl residue might be expected to undergo inversion at $C_{(1)}$ again by analogy with what is known of the reactions involving isopentenyl pyrophosphate. The positive charge imparted to the carbon chain by the leaving phosphate group may be neutralised either in the formation of a cyclic phosphate (XLI) (Scheme 1) or in the formation of a sulphonium compound (XLII) through reaction with a methionine residue (Scheme 2). In either case a final reductive cleavage is envisaged which results in the liberation of squalene and entails



SCHEME 1.



SCHEME 2.

(R = Geranyl)

FIG. 12. Hypothetical schemes for squalene biosynthesis involving nerolidol pyrophosphate.

the insertion of a new hydrogen atom at the carbon (originally $C_{(1)}$ of nerolidyl pyrophosphate) from which one hydrogen has been removed as a proton. On the basis of the evidence that has been cited, this exchange involves no change in configuration.

The second type of mechanism,[87] which may also be subject to modification of detail, is outlined in Fig. 13. The initial step is the displacement of pyrophosphate from one farnesyl pyrophosphate molecule by a sulphhydryl group of the enzyme (Fig. 13a). A reaction of the farnesyl–*S*-enzyme complex (XLIII) with a second farnesyl pyrophosphate could then yield a difarnesyl–enzyme complex (XLIV) which may undergo a Stevens rearrangement leading to the formation of the central carbon–carbon bond

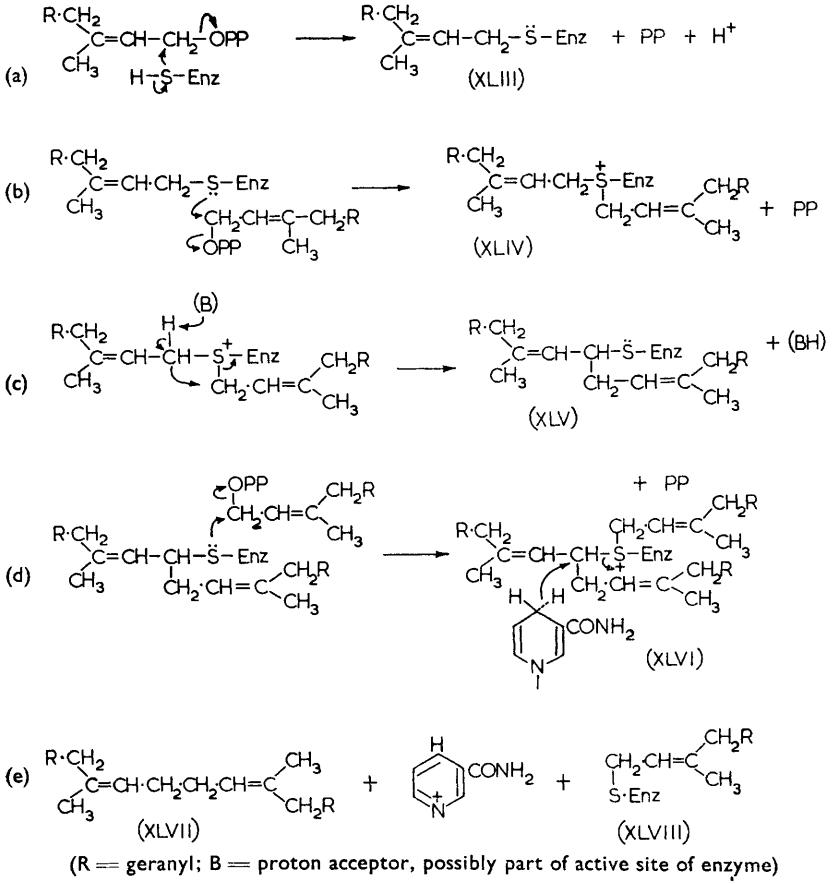


FIG. 13. Hypothetical mechanism for coupling of farnesyl pyrophosphate involving Stevens rearrangement.

[87] Popják, NATO-sponsored symposium; Metabolism and Physiological Significance of Lipids, ed. Dawson and Rhodes, Wiley and Son, London, 1964, p. 45.

of squalene (Fig. 13c). The product at this stage is a squalyl–*S*-enzyme complex (XLV). From the known characteristics of the Stevens rearrangement[88] it seems likely that it would be assisted by an appropriately situated proton-accepting group (B) at the active site. According to a suggestion put forward by Woodward (cited in ref. 87) the reductive liberation of squalene from the enzyme complex might be facilitated by prior reaction (Fig. 13d) with a third farnesyl pyrophosphate molecule to yield a new sulphonium complex (XLVI). The products of reductive cleavage (Fig. 13e) would now be squalene (XLVII) and farnesyl–*S*-enzyme (XLVIII). As pointed out by Popják, Woodward's suggestion should be amenable to experimental testing, since it implies that the enzyme, in its resting state, should always accommodate a farnesyl residue.

There have as yet been few detailed reports of experiments specifically designed to isolate and study the possible intermediates in the conversion of farnesyl pyrophosphate into squalene, but those that have appeared suggest a complex sequence of events and do not exclude either type of mechanism outlined above. Thus Gosselin[89] reported that when labelled farnesyl pyrophosphate was incubated with a microsomal preparation in the absence of NADPH which is an essential requirement for squalene formation, it was in part converted into a material bound to microsomal protein from which a labelled hydrocarbon could be liberated by acid hydrolysis. The hydrocarbon behaved similarly to squalene on chromatography, but failed to form a hexahydrochloride that co-crystallised with that of squalene. These observations have been confirmed and extended by Krishna *et al.*[90] in experiments performed with a "solublised" squalene synthetase preparation from beef liver microsomes.[91] These workers showed that the protein-bound hydrocarbon, after liberation by acid hydrolysis, behaved on gas–liquid chromatography like a C_{30} hydrocarbon somewhat more polar than squalene. They also presented evidence for the transformation of this unidentified material into squalene when it was incubated further (in the protein-bound state) in the presence of NADPH.

Gosselin and Lynen[92] have recently described experiments in which a rat liver preparation was incubated with labelled mevalonic acid under conditions which should carry the transformation of mevalonic acid as far as farnesyl pyrophosphate, followed by the addition of the microsomal enzyme components normally responsible for coupling the two farnesyl groups to produce squalene, but without addition of NADPH. This procedure resulted in the formation of a labelled substance having the characteristics of a phospholipid from which acid hydrolysis liberated a hydrocarbon similar to, but not identical with, squalene. The phospholipid-like material did not behave as a precursor of squalene but no datum was

[88] Thompson and Stevens, *J.*, 1932, **55**, 69.
[89] Gosselin, *Arch. Internat. Physiol. Biochem.*, 1962, **70**, 89.
[90] Krishna, Feldbruegge, and Porter, *Biochem. Biophys. Res. Comm.*, 1964, **14**, 363.
[91] Anderson, Rice, and Porter, *Biochem. Biophys. Res. Comm.*, 1960, **3**, 591.
[92] Gosselin and Lynen, *Biochem. Z.*, 1964, **340**, 186.

presented with reference to either the structure or the metabolic potential of the hydrocarbon released from it by acid hydrolysis. The relationship between this hydrocarbon and that described in earlier brief reports[89,90] therefore remains unclear. It is possible that these observations foreshadow further important advances in the understanding of squalene formation. However there is considerable evidence (discussed by Popják and Cornforth[4]) for alternative pathways of metabolism of farnesyl pyrophosphate and some of these may be accentuated when the normal pathway to squalene is obstructed as in the work described above.

The Biological Conversion of Squalene into Lanosterol.—The scheme of cyclisation of squalene put forward by Woodward and Bloch[21] implied that lanosterol or some closely related compound should be an intermediate in the biosynthetic pathway between squalene and cholesterol. After this intermediate rôle of lanosterol had been clearly established[23–26], a major aspect of subsequent investigations became the elucidation of the mechanism of conversion of squalene into lanosterol. Experimental work in this area was preceded by the extensive development of the theoretical concepts of Ruzicka, Eschenmoser, and their collaborators,[93,94] according to which squalene was visualised as a common biological precursor not only of lanosterol and related tetracyclic triterpenes, but also of the pentacyclic triterpenes such as the amyrins and lupeol. In this theoretical treatment, the cyclisation of squalene was considered as a steroelectronically controlled concerted process, initiated by electrophilic attack of a cationic species such as H^+ or OH^+ and involving a series of transient carbonium ion intermediates. Stabilisation of the final cyclisation product was presumed to take place either by loss of a proton or neutralisation of the positive charge by OH^-; some examples of the suggested biogenetic relationship are shown in Fig. 14. The biogenesis of the different classes of cyclic triterpene was rationalised in terms of several alternative conformations of the squalene chain and various carbonium ion rearrangements to which they should lead. Thus, an essential rôle of the enzyme catalysing such a cyclisation must be to impose the appropriate conformation upon the squalene chain. The conformation of squalene and the sequence of rearrangements suggested for its conversion into lanosterol by a concerted cyclisation initiated by OH^+, are depicted in Fig. 14a. This scheme accounts for the presence of the 14α and 13β methyl groups in lanosterol in terms of two 1,2 shifts of methyl groups of squalene. Eschenmoser *et al.*[94] pointed out that with ring B initially present in the boat conformation, this mechanism was more consistent with a non-stop cyclisation process than the 1,3 shift of a methyl group from $C_{(8)}$ to $C_{(13)}$.

The hypothesis of the central rôle of squalene as a biological precursor of polycyclic triterpenes in both animals and plants is now supported by various experiments, some of which will be discussed below. The bio-

[93] Ruzicka, *Experientia*, 1953, **9**, 357, 362.
[94] Eschenmoser, Ruzicka, Jeger, and Arigoni, *Helv. Chim. Acta.*, 1955, **38**, 1890.

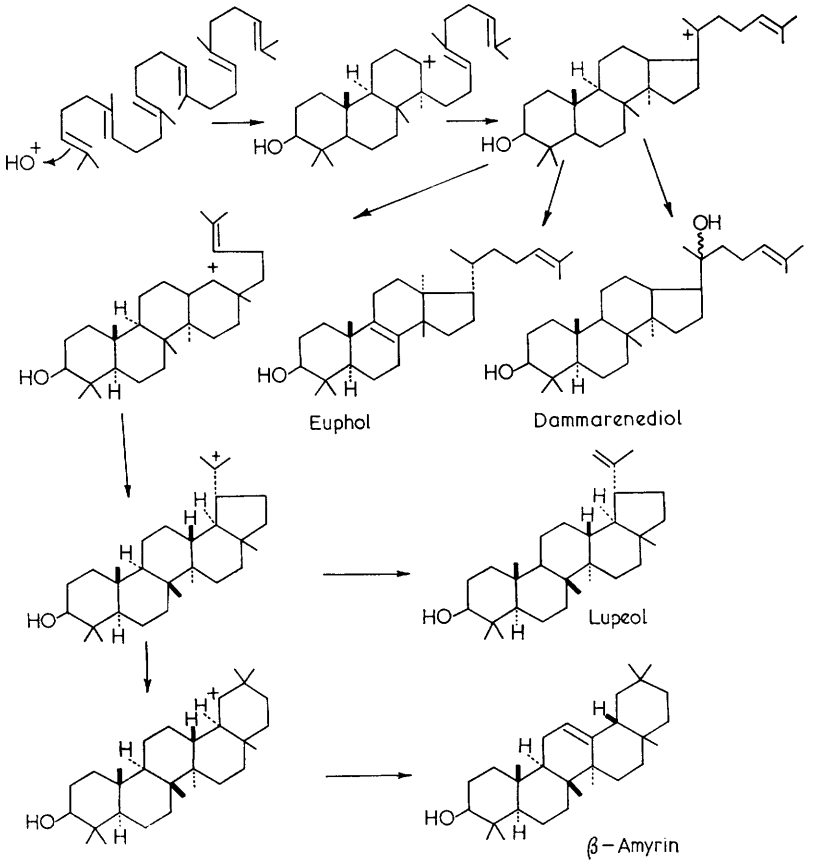


FIG. 14. Derivation of some tetracyclic and pentacyclic triterpenes from squalene.

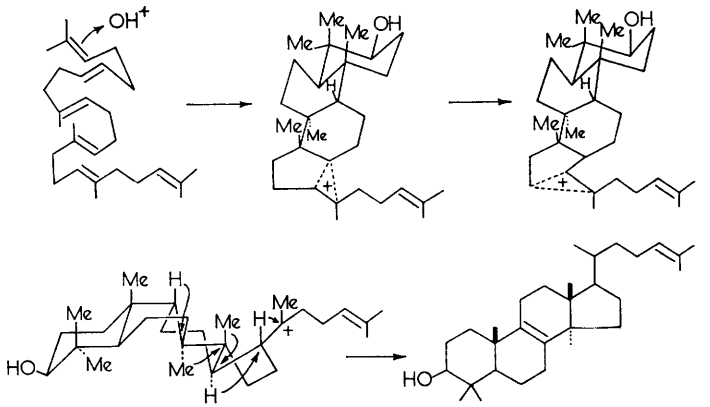


FIG. 14a. Stereochemical course of cyclisation of squalene to form lanosterol.

synthesis of squalene from labelled mevalonate and its active turnover have recently been demonstrated to take place in higher plant tissues[95] with incorporation of the label according to the same pattern as is found in squalene of animal tissues.[96] Moreover, a pentacyclic triterpene of animal origin has recently been isolated.[97]

The first experiments designed to test the postulated mechanism of squalene cyclisation were those of Tchen and Bloch,[98,99] who showed that the enzymic cyclisation of squalene in the presence of D_2O yielded lanosterol into which no measurable amount of deuterium had been incorporated, a result that is consistent with a concerted process without stable intermediates. Moreover, ^{18}O was incorporated into lanosterol when the incubation was carried out in an atmosphere enriched in $^{18}O_2$ but not when the medium contained $H_2{}^{18}O$. These and other observations strongly support the concept that the cationic species which initiates the cyclisation is either enzymically "activated" oxygen or OH^+ derived from it. The cyclisation reaction requires NADPH and enzymic factors from both the microsomal and soluble fractions of a liver homogenate.[100] Its characteristics are therefore those of a "mixed function oxidase" reaction.[101] Many such reactions are now known in the oxidative metabolism of the steroids and other examples will be discussed in a later section.

The possibility that squalene cyclisation may be initiated by the attack of OH^+ has prompted attempts to inhibit cholesterol formation by the use of catalase which would be expected to reduce the OH^+ concentration of tissues or tissue homogenates, and results have been reported[102] which seem to support this concept. However, a closer study of the effect shows that it is due not to the enzyme (hepatocatalase) *per se*, but to an impurity of low molecular weight which inhibits the conversion of mevalonate into squalene.[103]

The rearrangement of the methyl groups at the centre of the squalene molecule, leading to the 13β,14α-dimethyl structure of lanosterol, has been shown by two differently designed experiments to take place by 1,2-shifts of the methyl groups as postulated by Eschenmoser *et al.*[94] Maudgal, Tchen, and Bloch[104] synthesised ^{13}C-labelled all-*trans*-squalene by the method of Dicker and Whiting[105] using as an intermediate a mixture of geranylacetones, one of which contained ^{13}C in the carbonyl carbon and

[95] Nicholas, *J. Biol. Chem.*, 1962, **237**, 1485.
[96] Nes and Rosin, 6th International Congress of Biochemistry, New York, 1964, p. 588.
[97] Mallory, Gordon and Conner, *J. Amer. Chem. Soc.*, 1963, **85**, 1362.
[98] Tchen and Bloch, *J. Biol. Chem.*, 1957, **226**, 931.
[99] Tchen and Bloch, *J. Amer. Chem. Soc.*, 1956, **78**, 1516.
[100] Tchen and Bloch, *J. Biol. Chem.*, 1957, **226**, 921.
[101] Mason, *Adv. Enzymol.*, 1957, **19**, 79.
[102] Puig-Muset, Martin, and Fernande, International Symposium. Drugs Affecting Lipid Metabolism, Milan, 1960.
[103] Caravaca, May, and Dimond, *Biochem. Biophys. Res. Comm.*, 1963, **10**, 189.
[104] Maudgal, Tchen, and Bloch, *J. Amer. Chem. Soc.*, 1958, **80**, 2589.
[105] Dicker and Whiting, *J.* 1958, 1994.

the other contained ^{13}C in the methyl group attached to the carbonyl carbon. A mixture of three types of squalene as shown (Fig. 15, I, II, III) was obtained and subjected to enzymic cyclisation to lanosterol. The species III would be expected to yield two patterns of labelling in lanosterol depending upon which end of the molecule became involved in the initiation of the cyclisation process. Moreover, the pattern of lanosterol labelling would be expected to be different depending upon whether species (III) were to cyclise with a 1,2- or 1,3-rearrangement of methyl groups. Squalene species (III), when cyclised in one of the two possible directions with 1,2-rearrangement of methyl groups, will yield one molecule of acetic acid (that from $C_{14} + C_{30}$) as indicated in Fig. 15 which is labelled in both carbons with ^{13}C. Under no circumstances would such a doubly labelled acetic acid be obtained by a 1,3 shift of a methyl group. The lanosterol derived from the mixed synthetic squalenes was oxidised by a modified Kuhn–Roth procedure to acetic acid which represented the various methyl groups of the lanosterol molecule together with the carbons to which they were attached. The acetic acid obtained from the oxidation was converted into ethylene and analysed by mass spectrometry with the identification of a peak corresponding to the doubly labelled molecules in approximately the expected abundance. The 1,2-shift mechanism was thus supported. Further evidence for this mechanism was provided by the experiment of Cornforth and his co-workers[106] similar in principal to that of Maudgal *et al.*, but utilising mevalonolactone labelled with ^{13}C. In the mevalonolactone (I) used for the experiment, some molecules were labelled in the methyl group and in $C_{(4)}$, others were labelled in only one or the other of these positions and others were unlabelled. Squalene biosynthesised from this mixture would consist of many differently labelled species, of which two (Fig. 16, II, III) would yield cholesterols with different labelling distributions in $C_{(13)}$ and $C_{(18)}$,

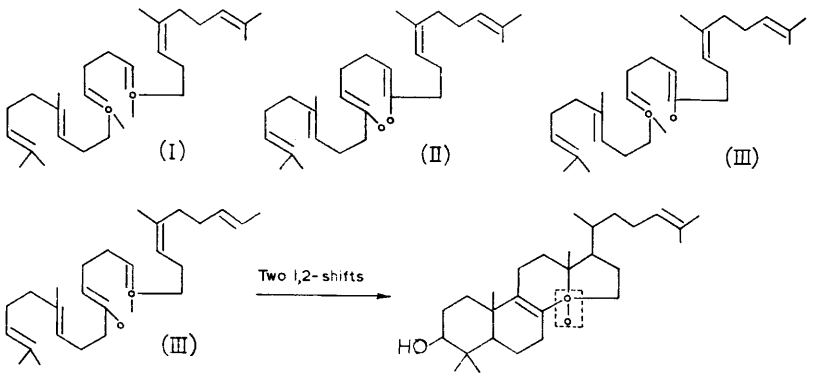


FIG. 15. Different ^{13}C- labelled squalenes used by Maudgal, Tchen, and Bloch to demonstrate the 1,2-shift of methyl groups in cyclisation to lanosterol.

[106] Cornforth, Cornforth, Pelter, Horning, and Popják, *Tetrahedron*, 1959, **5**, 311.

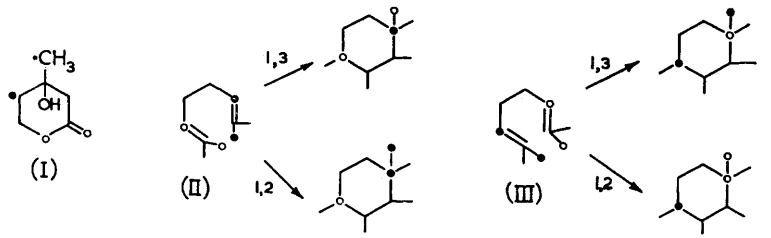


FIG. 16. Distribution of label (●) at centre of squalene and in cholesterol derived from [3′,4-$^{13}C_2$]mevalonolactone.

depending upon whether the $C_{(18)}$ methyl group became attached to $C_{(13)}$ by a 1,2 or 1,3 rearrangement. Cholesterol biosynthesised from this mixture was oxidised to acetic acid which was analysed by mass spectrometry to determine the proportion of doubly labelled molecules. These appeared with an abundance that could only be accounted for if a 1,2-methyl group migration were to occur during cyclisation.

Proof that the methyl carbons in the terminal isopropylidene groups of squalene retain their individual identity in the course of cyclisation has been presented by Arigoni.[107] Soya-bean seedlings were supplied with [2-^{14}C]mevalonate and the labelled soya-sapogenols subsequently isolated. The 1,3-glycol structure in ring A (Fig. 17, IL) was oxidised to give the

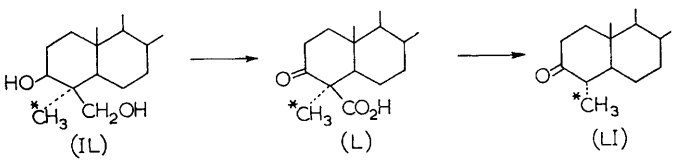
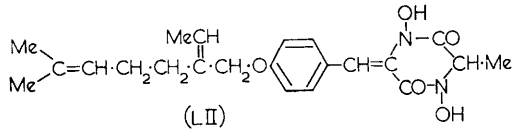


FIG. 17. Labelling of 4α-methyl group of soyasapogenol from [2-^{14}C]mevalonate.

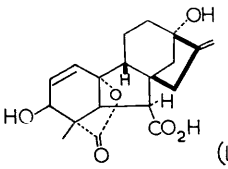
unstable 3-oxo-24-carboxylic acid (L) which was readily decarboxylated to the ketone (LI), giving CO_2 that contained no ^{14}C. Hence in the formation of this pentacyclic triterpene the axially oriented hydroxymethyl group at $C_{(4)}$ was derived from the methyl carbon of mevalonic acid. There is also retention of individual identity by the corresponding *gem.*-disubstituted carbon atoms in gibberellic acid (LIII)[108] and rosenonolactone (LIV),[107,108] and by the *gem.*-dimethyl groups of the isopropylidene structure of the terpenoid chain of mycelianamide (LII).[109] The evidence in the latter case is considered in more detail in a later section. The stereospecific fate of the two apparently identical methyl carbons of the isopropylidene group of squalene is entirely consistent with the stereospecificity, noted above, for the loss of hydrogen from $C_{(2)}$ of isopentenyl

[107] Arigoni, ref. 9, p. 231.
[108] Birch and Smith, ref. 9, p. 245.
[109] Birch, Kocor, Sheppard, and Winter, *J.*, 1962, 1502.

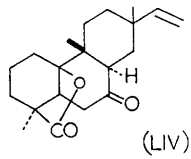
pyrophosphate during establishment of the allylic double bond. It is also clearly a further example of the now well established phenomenon of dissymmetric reactivity of a symmetrical substrate in association with an enzymic site.



(LII)



(LIII)



(LIV)

The Conversion of Lanosterol into Cholesterol.—The conversion of lanosterol into cholesterol entails the following changes: (1) Replacement of the methyl groups at $C_{(4)}$ and $C_{(14)}$ with hydrogen atoms; (2) shift of the Δ^8 bond to Δ^5; (3) saturation of the side chain. While much information has been obtained concerning the biochemical conditions necessary for these various changes individually, it has proved much more difficult to determine whether, in a given tissue, these reactions must always take place in a definite sequence and, if so, what that sequence is. In particular the stage (or stages) at which the saturation of the side chain takes place remains inadequately defined. Some of the studies bearing on this point are considered in further detail below but for the moment it is sufficient to note that ample evidence has been obtained for the conversion of various Δ^{24}-derivatives[24,110–113] and 24,25-dihydro-derivatives[24,110,111,113–117] into cholesterol.

Evidence for the sequence in which the three methyl groups attached to $C_{(4)}$ and $C_{(14)}$ are removed from lanosterol (II) (Fig. 18) has been presented by Bloch and his co-workers. A material slightly more polar than lanosterol, was isolated from the tissues in trace amounts but with high specific activity, at short time intervals after the injection of ^{14}C-acetate into rats. This material was further converted into cholesterol by a liver homogenate.[118] By experiments with trace amounts of the labelled compound, its individual structural features were characterised and it was tentatively

[110] Johnston and Bloch, *J. Amer. Chem. Soc.*, 1957, **79,** 1145.
[111] Lindberg, Gautschi, and Bloch, *J. Biol. Chem.*, 1963, **238,** 1661.
[112] Steinberg and Avigan, *J. Biol. Chem.*, 1960, **235,** 3127.
[113] Schroepfer, *J. Biol. Chem.*, 1961, **236,** 1668.
[114] Frantz, Davidson, Dulit, and Mobberley, *J. Biol. Chem.*, 1959, **234,** 2290.
[115] Schroepfer and Frantz, *J. Biol. Chem.*, 1961, **236,** 3137.
[116] Kandutsch and Russell, *J. Biol. Chem.*, 1960, **235,** 2256.
[117] Wells and Lorah, *J. Biol. Chem.*, 1960, **235,** 978.
[118] Gautschi and Bloch, *J. Amer. Chem. Soc.*, 1957, **79,** 684.

identified as 4,4′-dimethylcholesta-8,24-dien-3β-ol (LVIII) with some doubt remaining as to the exact position of the nuclear double bond. This sterol and its Δ^{7}- and $\Delta^{8(14)}$-isomers were subsequently prepared by partial synthesis.[119] The radioactive material easily separated from the $\Delta^{8(14)}$-compound on crystallisation but was inseparable from the $\Delta^{8(9)}$-isomer even

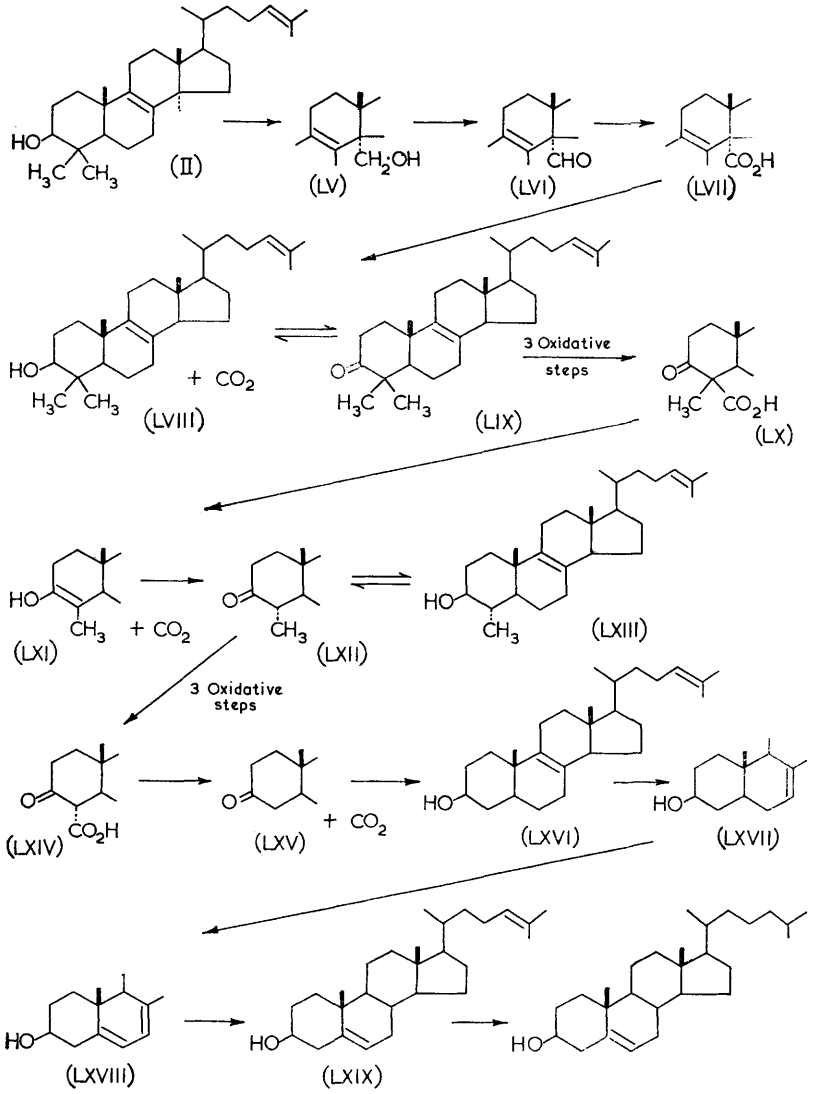
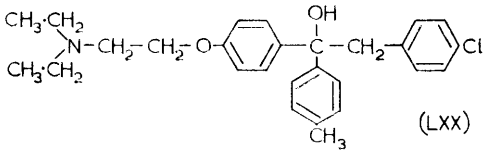


FIG. 18. Conversion of lanosterol into cholesterol *via* Δ^{24}-intermediates.

[119] Gautschi and Bloch, *J. Biol. Chem.*, 1958, **233**, 1343.

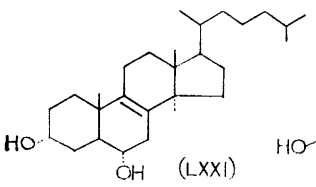
during reduction to the 24,25-dihydro-derivative and oxidative fission of the $\Delta^{8,9}$-bond. The derivative of the Δ^{7}-isomer was separated from the radioactive material by this procedure. With this confirmation of the structure it was concluded that the $C_{(14)}$-methyl group was the first to be removed from lanosterol. When incubated with rat liver homogenates, 4,4′-dimethylcholesta-8,24-dien-3β-ol[118] or lanosterol (II),[120] that had been labelled biologically with [2-^{14}C]acetate, yielded $^{14}CO_2$ equivalent to the



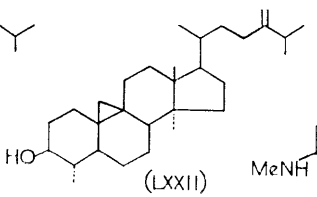
(LXX)

methyl groups removed. No labelled formaldehyde could be detected. The demethylating enzyme system was present in the microsomal fraction and required both oxygen and NADPH. Semicarbazide, added to the incubation medium, inhibited the conversion of lanosterol into both cholesterol and CO_2 and caused the accumulation of unidentified polar metabolites which could be converted in turn into cholesterol. These results were interpreted[120] to suggest that each methyl group at $C_{(4)}$ and $C_{(14)}$ of lanosterol was oxidised through the sequence $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow CO_2H$, (*e.g.*, LV—LVII) to be eliminated finally as CO_2. Up to the present time, however, none of the hypothetical intermediates with partially oxidised methyl groups has been identified and no separation of the enzymes involved in these oxidative steps has been reported.

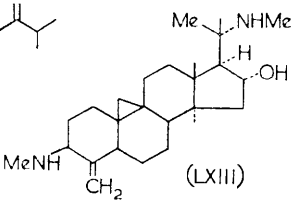
While the prior removal of the $C_{(14)}$-methyl group seems to be reasonably well established for the mammalian pathways of sterol synthesis, the fact that this may not hold in plants is suggested by the structure of macdougallin (LXXI) isolated[121] from the cactus *Peniocereus macdougalli*. This compound presumably arises from lanosterol by a route involving removal of the 4,4′-methyl groups without attack on that at $C_{(14)}$.



(LXXI)



(LXXII)



(LXIII)

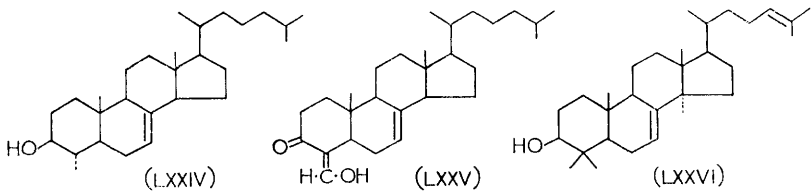
The presence of one methyl group at $C_{(4)}$ together with the one in the 14α-position is found[122] in cycloeucalenol (LXXII) and in the closely

[120] Olson, Lindberg, and Bloch, *J. Biol. Chem.*, 1957, **226**, 94.
[121] Djerassi, Knight, and Wilkinson, *J. Amer. Chem. Soc.*, 1963, **85**, 835.
[122] Cox, King, and King, *J.*, 1959, 514.

related alkaloid cyclobuxine (LXXIII).[123] Both of these structures imply removal of a 4-substituted methyl group before attack on the $C_{(14)}$-methyl group in the cyclopropano-steroid series. Indeed, the $C_{(14)}$-methyl group is unattacked in all of the cyclopropano-steroids so far isolated, suggesting that this structural feature is for some reason (possibly the absence of a Δ^8-bond) incompatible with oxidative attack on the methyl group at $C_{(14)}$.

The removal of the two methyl groups at $C_{(4)}$ of 4,4'-dimethylcholesta-8,24-dien-3β-ol (LVIII) probably takes place in stepwise fashion, with oxidation of the 3β-hydroxyl group to the ketone (LIX and LXII).[111] Thus, the 3α-^{3}H label is lost from lanosterol, 4,4,-dimethyl-Δ^8-cholestenol, 4α-methyl-Δ^7-cholestenol, and 4α-methyl-Δ^8-cholestenol when these compounds are metabolised to cholesterol, and the metabolism of lanosta-8,24-diene-3-one and 4,4'-dimethylcholest-8-enone to cholesterol has been demonstrated. On the other hand, zymosterol (LXVI), previously shown by Alexander and Schwenk[124] and by Johnston and Bloch[110] to be converted into cholesterol by mammalian enzymes, retained the 3α-^{3}H in the process. It has been pointed out by Bloch[125] that decarboxylation at $C_{(4)}$ of the intermediates LX and LXIV, would be facilitated by the $C_{(3)}$-keto-group and the extreme lability of a 3-oxo-4-carboxylic acid has been noted by Britt, Scheuerbrandt, and Bloch (unpublished observation quoted in ref. 111). Facilitation of the decarboxylation at $C_{(14)}$ by the Δ^8-bond (LVII) has also been suggested by Bloch.

That the oxidation and elimination of one methyl substituent at $C_{(4)}$ must be completed before the other is attacked has not been unambiguously established but it seems probable, since 4α-methyl-Δ^7-cholestenol (LXXIV) (methostenol or lophenol) has been identified in both animal[126–128]



and plant material[129] and its Δ^8-isomer has also been isolated from animal tissues.[130] As noted above[111] these compounds are metabolised to cholesterol and a putative partially oxidised intermediate (LXXV) in the con-

[123] Brown and Kupchan, *J. Amer. Chem. Soc.*, 1962, **84,** 4590, 4592.
[124] Alexander and Schwenk, *Arch. Biochem. Biophys.*, 1957, **66,** 381.
[125] Bloch, ref. 9, p. 4.
[126] Frantz, Davidson, and Dulit, *Fed. Proc.*, 1956, **15,** 255.
[127] Wells and Neiderhiser, *J. Amer. Chem. Soc.*, 1957, **79,** 6569.
[128] Neiderhiser and Wells, *Arch. Biochem. Biophys.*, 1959, **81,** 300.
[129] Djerassi, Krakower, Lemin, Liu, Mills, and Villotti, *J. Amer. Chem. Soc.*, 1958, **89,** 6284.
[130] Kandutsch and Russell, *J. Biol. Chem.*, 1960, **235,** 2253.

version of lophenol into cholesterol is also metabolised to cholesterol.[131] It has been pointed out by Popják and Cornforth[4] that the α-orientation of the remaining methyl group at $C_{(4)}$ in lophenol cannot be taken as evidence that this is the original orientation of the last methyl group of lanosterol to be removed, since an enol form of the ketone (LXI) would probably be involved in elimination of the first carboxyl group from $C_{(4)}$ and on reversion to the keto-form (LXII) the remaining $C_{(4)}$-methyl group would assume the less hindered α-configuration. Gaylor and Delwiche[132] used 4α-methyl-Δ^8-cholestenol as a trapping agent during enzymic demethylation of 24,25-dihydrolanosterol that had been labelled from [2-^{14}C]mevalonate, and concluded from their results that the stereospecific identity of the 4,4′-methyl groups in lanosterol was the same as that demonstrated in soya-sapogenols (IL) by Arigoni.[107] The several arguments used by these authors to support their conclusion do not, however, circumvent the difficulty of interpretation alluded to by Popják and Cornforth.[4]

The Final Steps in Cholesterol Biosynthesis.—The C_{27}-sterol resulting from demethylation of lanosterol, if no reduction occurred in the side chain, would be zymosterol (cholesta-8,24-dienol) (LXVI). In the studies by Johnston and Bloch[110] of the *in vitro* conversion of this sterol into cholesterol, liver homogenates prepared under the gentle conditions of Bucher[133] converted both zymosterol (LXVI) and Δ^8-cholestenol (24-dihydro-zymosterol) into cholesterol, but a homogenate prepared by the more disruptive Waring Blendor procedure metabolised only the first of these two compounds. On the basis of these observations and the report by Stokes *et al.*[134] of the isolation of desmosterol ($\Delta^{5,24}$-cholestadienol, LXIX) from chick embryos, with evidence for its rôle as an intermediate in cholesterol synthesis,[134,135] it was argued[110,112] that, as indicated in Fig. 18, the reduction of the Δ^{24}-bond probably occurred as the final reaction in cholesterol biosynthesis, and that the conversion of various 24-25-dihydro-sterols into cholesterol represented deviations from the normal pathway. (For full discussion see Bloch.[6]) However, it is now evident that all of the nuclear transformations between lanosterol and cholesterol can take place equally well in the 24,25-dihydro-series as in the Δ^{24}-series and since they are more accessible than their Δ^{24}-analogues, many 24-25-dihydro-compounds have been used in studies of the changes taking place in the nucleus.

Johnston and Bloch[110] found that the conversion of zymosterol into cholesterol required oxygen and assumed that the oxidative step was involved in the shift of the nuclear double bond. It was suggested that the $\Delta^{5,6}$-bond might arise by hydroxylation followed by dehydration in ring B with the possible formation of a 5,7-diene structure such as (LXVIII)

[131] Pudles and Bloch, *J. Biol. Chem.*, 1960, **235**, 3417.
[132] Gaylor and Delwiche, *Steroids*, 1964, **4**, 207.
[133] Bucher, *J. Amer. Chem. Soc.*, 1953, **75**, 498.
[134] Stokes, Fish, and Hickey, *J. Biol. Chem.*, 1956, **220**, 415.
[135] Stokes, Hickey, and Fish, *J. Biol. Chem.*, 1958, **232**, 347.

as an intermediate.[6] It has subsequently been shown that the conversion of Δ^7-cholestenol into cholesterol requires oxygen[114] but that the conversion of 7-dehydrocholesterol into cholesterol does not.[115,136] 7-Dehydrocholesterol is formed during conversion of Δ^7-cholestenol into cholesterol[137] and the sequence $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ is therefore supported. Moreover, convincing evidence for the irreversibility of the last two of these steps in mammalian tissues has been presented by Frantz *et al.*[138] in contrast to the finding of Glover and his co-workers[139,140] whose data suggest that they may to some extent be reversible. Evidence such as that of Fagerlund and Idler[141] for the conversion of cholesterol into Δ^7-cholestenol in starfish probably has little relevance to the mammalian biosynthetic pathway, since there is evidence that the invertebrates have special mechanisms for the modification of ring B in the preformed sterol nucleus, as for example in the conversion of cholestanol into Δ^7-cholestenol in insects.[142]

Though the intermediate rôle of Δ^7-cholestenol and 7-dehydrocholesterol in the conversion of Δ^8-cholestenol into cholesterol seems to be reasonably well established, the biochemical mechanisms involved require further clarification. Nothing is known of the enzymic mechanism of the shift of the double bond from the 8(9) position, though some results relevant to this problem are discussed in the following section. While the overall conversion of Δ^7-cholestenol into cholesterol requires both oxygen and NADPH, the latter appears to be necessary only for the reduction of the double bond of 7-dehydrocholesterol but not for the oxygen-requiring step from Δ^7-cholestenol to 7-dehydrocholesterol.[137] If the $\Delta^7 \rightarrow \Delta^{5,7}$-transformation involved a hydroxylated intermediate such as a 6-hydroxy-Δ^7-cholestenol, it would be expected that this hydroxylation, in common with other steroid hydroxylations, would require both oxygen and NADPH. In this connection it may be noted that Harvey and Bloch[143] failed to demonstrate a conversion of Δ^7-3β-6β-dihydroxycholestenol into cholesterol under anærobic conditions.

Reduction of the Δ^{24}-bond and the effects of triparanol

The question of the stage in the biosynthetic sequence at which reduction of the Δ^{24}-bond takes place has given rise to much discussion, usually on the basis of inadequate evidence, and it is only recently that serious attempts have been made to approach this difficult problem by means of appropriate experiments.

[136] Kandutsch, *J. Biol. Chem.*, 1962, **237**, 358.
[137] Dempsey, Seaton, Schroepfer, and Trockman, *J. Biol. Chem.*, 1964, **239**, 1381.
[138] Frantz, Sanghvi, and Schroepfer, *J. Biol. Chem.*, 1964, **239**, 1007.
[139] Mercer and Glover, *Biochem. J.*, 1961, **80**, 552.
[140] Glover and Stainer, *Biochem. J.*, 1959, **72**, 79.
[141] Fagerlund and Idler, *Canad. J. Biochem. Physiol.*, 1960, **38**, 997.
[142] Clayton and Edwards, *J. Biol. Chem.*, 1963, **238**, 1966.
[143] Harvey and Bloch, *Chem. and Ind.*, 1961, 595.

The observation[144–146] that triparanol {MER-29, 1-[4(diethylaminoethoxy)phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl)ethanol (LXX)}, an inhibitor of cholesterol synthesis, caused accumulation of desmosterol (LXIX) *in vivo* and *in vitro* at first seemed to strengthen the view that the reduction of the Δ^{24}-bond occurred principally, if not exclusively, as the last step. However, it is now clear that triparanol does not inhibit the conversion of desmosterol into cholesterol specifically. In various systems it can also prevent reduction of the Δ^{24}-bond of zymosterol (LXVI),[113] $\Delta^{7,24}$-cholestadienol (LXVII),[147] $\Delta^{5,7,24}$-cholestatrienol (LXVIII),[148] and lanosterol (II),[149] and probably in many other compounds.[150,151] Desmosterol probably accumulates as the major sterol of liver during triparanol treatment, because of the high efficiency of the liver enzymes responsible for the various nuclear transformations. Under normal conditions the liver contains the intermediates between squalene and cholesterol only in fractions of a percent of the cholesterol concentration. It was pointed out by Bloch[6] that this situation does not hold for mammalian skin and there is also evidence that it does not hold for intestinal tissues. Rodent skin normally contains measurable amounts of several 3β-monohydroxysterols representative of intermediate stages in cholesterol biosynthesis. It has been possible to achieve a complete resolution of this mixture of sterols which, with the exception of lanosterol, were found to occur in the normal animal almost exclusively as the 24,25-dihydro-derivatives. The major effect of triparanol was to cause their accumulation as the Δ^{24}-analogues[150] and all possible 3-monohydroxy-sterol intermediates of both the Δ^{24}- and 24,25-dihydro-series, between squalene and cholesterol, were chromatographically identified, several of them for the first time.

The enzymes responsible for reduction of the Δ^{24}-bond in both desmosterol[152] and lanosterol[149] are similar with respect to their intracellular distribution (in the microsomal fraction), co-factor requirements (NADPH but not NADH), and their response to various inhibitors, and Avigan *et al.*[149] suggest that the same enzyme could carry out the reduction of the side chain independently of the structure of the nucleus. This would certainly be consistent with the various findings, discussed above, but as the authors point out, it cannot be regarded as proven on the basis of the limited data available.

Goodman *et al.*[153] studied the time-course of incorporation of [^{14}C]-mevalonate *in vivo* into rat liver sterols which were separated into fractions

[144] Blohm and Mackenzie, *Arch. Biochem. Biophys.*, 1959, **85**, 245.
[145] Blohm, Kariya, and Laughlin, *Arch. Biochem. Biophys.*, 1959, **85**, 250.
[146] Avigan, Steinberg, Vroman, Thompson, and Mosettig, *J. Biol. Chem.*, 1960, **235**, 3123.
[147] Frantz and Mobberley, *Fed. Proc.*, 1961, **20**, 285.
[148] Frantz, Sanghvi, and Clayton, *J. Biol. Chem.*, 1962, **237**, 3381.
[149] Avigan, Goodman, and Steinberg, *J. Biol. Chem.*, 1963, **238**, 1283.
[150] Clayton, Nelson, and Frantz, *J. Lipid Res.*, 1963, **4**, 166.
[151] Horlick and Avigan, *J. Lipid Res.*, 1963, **4**, 160.
[152] Avigan and Steinberg, *J. Biol. Chem.*, 1961, **236**, 2898.
[153] Goodman, Avigan, and Steinberg, *J. Biol. Chem.*, 1963, **238**, 1287.

differing with respect to the degree of substitution at $C_{(4)}$, and the presence or absence of the Δ^{24}-bond. The results suggested that neither dihydrolanosterol nor desmosterol was on the main pathway of cholesterol synthesis but precise identification of the stage of reduction of the side chain was not made. This result agrees with the earlier observation[23] that lanosterol, but not dihydrolanosterol, acquires the label from [^{14}C]acetate in preparation from rat liver *in vitro*. It is also consistent with the absence of dihydrolanosterol from rat skin,[150] though not with its reported presence in a preputial gland tumour of mice.[154]

The detailed study of the sterols of rat skin[150] further revealed that in this tissue all components beyond lanosterol occur as mixtures of Δ^{7}- and Δ^{8}-isomers. The question therefore arises of whether one or several enzymes are responsible for this type of isomerisation. Triparanol not only inhibited the 24-reduction but also effected the ratio of Δ^{8} to Δ^{7}-isomers. In the compounds following lanosterol in the biosynthetic sequence, triparanol shifted this ratio in favour of the Δ^{8}-components, but lanosterol itself was apparently almost entirely replaced by its Δ^{7}-analogue (LXXVI). The significance of these observations is uncertain. Apart from its well established inhibition of the Δ^{24}- reductase, triparanol also inhibits the reduction of the Δ^{7}-bond in $\Delta^{5,7}$-intermediates.[155] If it also inhibits rearrangements between Δ^{8} and Δ^{7}, these observations suggest that $\Delta^{7,24}$-lanostadienol may be an alternative to lanosterol as an initial product of cyclisation of squalene in the skin. This possibility, though speculative, is also suggested by the work of Gaylor,[156] who has isolated $\Delta^{7,24}$-lanostadienol (LXXVI) from rat skin and has presented data consistent with its possible formation independently of lanosterol. It also recalls the unexplained observation[23] that pure lanosterol was less effective than crude "iso-cholesterol" in trapping radioactivity from [^{14}C]acetate in a liver homogenate. Isocholesterol contains lanosterol and agnosterol ($\Delta^{7,9,24}$-lanostatrienol) as well as both of their 24,25-dihydro-derivatives. Its effect as a trapping agent was approached, though not equalled, by a mixture of lanosterol with agnosterol of about 80% purity. It is possible, in the light of these more recent observations, that the synergistic effect of agnosterol could be due to its structural resemblance to $\Delta^{7,24}$-lanostadienol, or even to the presence of this compound as a previously unidentified contaminant of agnosterol.

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[154] Kandutsch and Russell, *J. Biol. Chem.*, 1959, **234**, 2037.
[155] Dempsey, 6th International Congress of Biochemistry, New York, 1964, Abstracts, p. 570.
[156] Gaylor, *J. Biol. Chem.*, 1963, **238**, 1643, 1649.