THE BIOSYNTHESIS OF POLYISOPRENOIDS

J. W. Cornforth
National Institute for Medical Research, London, U.K.

To a chemist, a natural product may be a structural problem, an objective for synthesis, an exhibitor of chemical and physical properties, or perhaps mere raw material. The tendency to regard it as a sample of the living organism's synthetic power is more recent, and experimental investigation of that power is more recent still. Such experiments depend on the use of isotopic tracers, and they alter the traditional objectives and values in an interesting manner. Thus, before isolation of a natural product, the organism must be induced to make it from an isotopically labelled precursor; chemical degradations are directed not to elucidation of structure but to unambiguous location of labelled atoms; and complicated and novel syntheses may be needed to prepare a specifically-labelled intermediate. And, eventually, the student of biosynthesis may find himself looking on certain large classes of natural products not—as he ought to do—as rich collections of beautiful chemistry, but as conglomerations too variable to be of great importance in the fundamental chemistry of life, where universal occurrence of the same or very similar substances seems to be the rule.

And yet—it was the very existence of large classes of chemically related natural products that stimulated the first speculations on biosynthesis. As Robinson has pointed out[1], these hypotheses, of which his own on alkaloids and Ruzicka's on terpenes are the most notable examples, were based on structural grounds; indications of the exact chemical nature of precursors could not be precise. That, even so, they often came near the truth is a tribute to the chemical intuition of the pioneers, and perhaps also a sign that enzymic processes do come within the scope of an intuition developed by the contemplation of reactions *in vitro*. It is pleasant to record that a structural speculation, made long before it could have been verified, assisted the establishment of squalene as a crucial intermediate in the biosynthesis of many polyisoprenoids; but it is even more satisfactory to note how hypotheses both structural and mechanistic have blended with chemical and biochemical experiment in piecing together the story which I hope now to unfold.

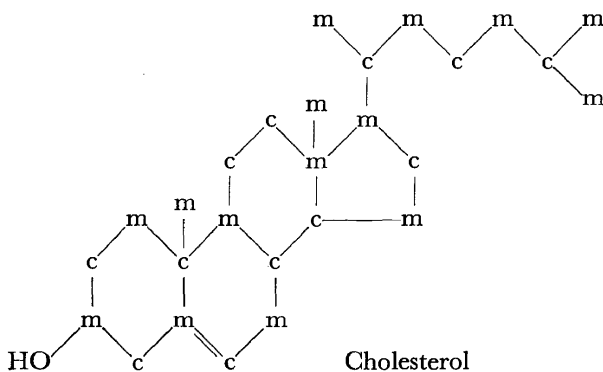
Actual work on the biosynthesis of polyisoprenoids began with the recognition that acetic acid is an important precursor of steroids. Sonderhoff and Thomas[2] found in 1937 that a large excess of deuterium is present in the unsaponifiable fraction—mainly ergosterol—of yeast grown in a medium containing trideuteroacetic acid. This result was extended to animal tissues by Bloch[3], whose studies, at first with Rittenberg and later at the head of his own group, outweigh all other contributions to knowledge of steroid biosynthesis.

When the carbon isotopes, especially ^{14}C, became available as tracers, it was quickly shown that both carbon atoms of acetate were incorporated

into cholesterol, the methyl carbon to a somewhat greater extent. But the prospect of obtaining more detailed information first became apparent when Little and Bloch[4] degraded cholesterol, biosynthesized from isotopic acetate, to fragments representing some individual carbon atoms of the side-chain. Those positions in cholesterol which were labelled when methyl-labelled acetate was the precursor were inactive when carboxyl-labelled acetate was used, and the converse was also true. Moreover, quantitative comparison of the radioactivities of cholesterol and of the fragments indicated that all 27 carbon atoms of cholesterol could originate from acetate; about 15 from methyl and 12 from carboxyl groups.

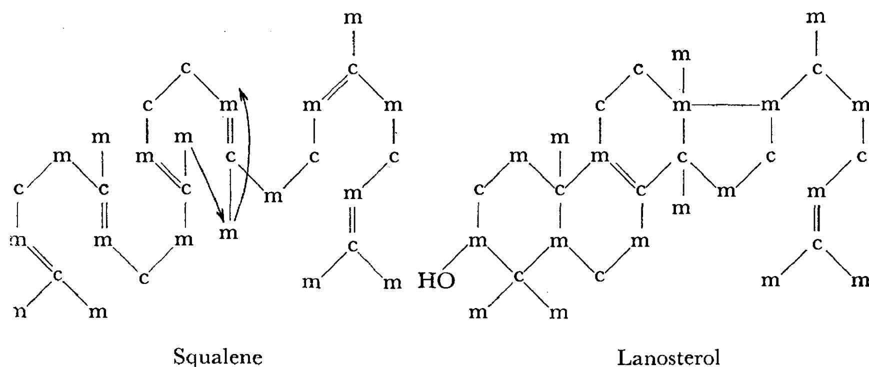
It was remarkable that from so simple a precursor an obviously complicated biosynthesis occurred without that mixing of labels which confused, for example, studies on biosynthesis of aromatic amino-acids and on the photosynthetic fixation of carbon dioxide. However, the opportunities in animal metabolism for acetate to mix its labels are limited. Methyl-labelled acetate can, indeed, be converted into carboxyl-labelled acetate by becoming involved in the citric acid cycle, and this effect seemed to operate to a small extent in our own preparations of cholesterol. Even so, the pattern of labelling in cholesterol appeared perfectly clear-cut, and the complete elucidation of that pattern a worthwhile object. The experimental method was to prepare two specimens of radioactive cholesterol, one biosynthesized from acetate labelled with ^{14}C in the methyl group, the other from acetate similarly labelled in the carboxyl group. The use of rat-liver slices and later of rat-liver homogenates for the biosynthesis made this stage relatively simple. Then, after suitable dilution, each specimen was degraded so as to obtain, in sufficient amount to measure radioactivity, fragments representing each carbon atom of the molecule. Bloch and his collaborators[5] identified the pattern in the eight side-chain carbons and at C–7 of the nucleus[6], and obtained evidence on the origin of two other nuclear carbons. Popják and I, with Hunter[7] and Youhotsky Gore[8], identified all the nuclear carbons except C–7. Dauben[9] and collaborators independently investigated C–7 and, by their extensive degradations of ergosterol[10] and eburicoic acid[11], went far to establish in these two substances the same pattern that emerged from the studies on cholesterol.

Thus the complete pattern of " methyl " carbons (m) and " carboxyl " carbons (c) in cholesterol biosynthesised from acetate was established:



But, long before this work was complete, the pattern of labelling in the side-chain had recalled to Bloch a suggestion put forward by Bonner and Arreguin[12] for the biosynthesis of the rubber hydrocarbon from acetate. The idea was wrong, but had enough truth in it to give for rubber a predicted distribution of methyl and carboxyl carbons similar to that found in the side-chain of cholesterol. This hint of a relationship between steroids and terpenes led Bloch to examine the old hypothesis that squalene is a precursor of cholesterol. With the help of ^{14}C, it was not too difficult to prove[13] that squalene was indeed produced from acetate *in vivo*, and that this squalene was further transformed quite efficiently into cholesterol.

Soon afterwards, an important contribution came from Woodward and Bloch[14], who postulated for squalene a mode of cyclization which could lead to the tetracyclic system of the sterols. A particularly interesting feature was that the triterpene alcohol lanosterol could be the first product of cyclization:

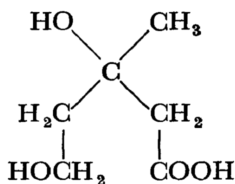


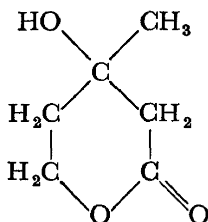
Squalene Lanosterol

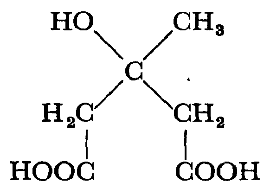
Evidence was produced, and later fully confirmed, that this mode of cyclization was consistent with the actual labelling pattern found in cholesterol biosynthesized from acetate, provided that the acetate units were associated in the expected manner to form squalene. Popják and I were able to show[15], by a total degradation of squalene biosynthesized from methyl-labelled acetate, that the pattern in squalene was in truth the expected one. The complete agreement of the two patterns, given the postulated mode of folding for cyclization, is evident. And, when Clayton and Bloch[16] showed that lanosterol is biosynthesized from acetate and converted into cholesterol *in vivo*, the sterols took what terpene chemists would consider their proper place as degenerate triterpenes.

At this point, the first stage of the investigation could be called complete: there was a clear picture of a biosynthesis proceeding from acetate *via* squalene and lanosterol to cholesterol. It remained to fill in gaps between intermediates, to study the enzymic reactions mediating each stage, and thus to arrive at as complete an understanding of a biosynthetic process as is at present possible.

The longest gap lay between acetate and squalene, and attempts to bridge it were not very successful until 1956. A team at the Merck laboratories had been studying a growth factor which could replace acetate in the culture of *Lactobacillus acidophilus.* Folkers and his collaborators finally isolated the factor[17], and identified it as an optically-active 3,5-dihydroxy-3-methylvaleric acid, now known as mevalonic acid. What the *Lactobacilli* need it for is still, I believe, unknown. It is obviously similar in structure







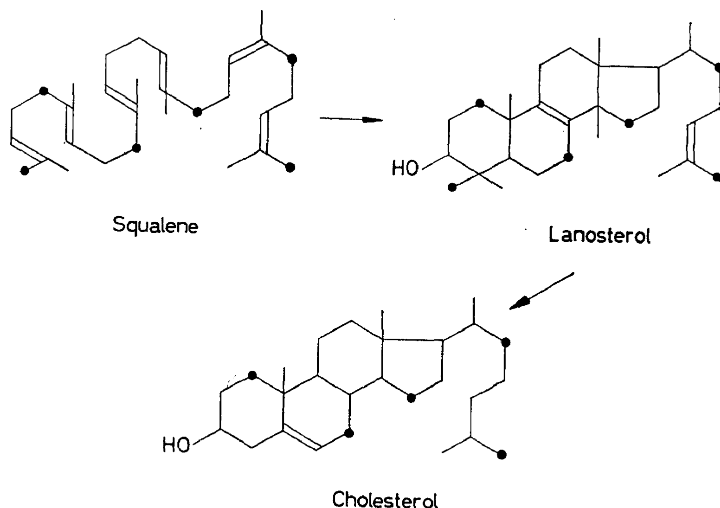
Mevalonic acid | Mevalonic lactone | 3-Hydroxy-3-methylglutaric acid

to 3-hydroxy-3-methylglutaric acid, which was known at the time to be built up, with the expected labelling pattern, from acetate in the liver, and to serve as a source of carbon for cholesterol, albeit a poor one, in the rat. Tavormina, Gibbs and Huff[18] were inspired by this structural similarity to prepare racemic mevalonate labelled at C–2 with ^{14}C and to try it as a precursor of cholesterol. In the event, over 40 per cent of the radioactivity of the acid was incorporated into cholesterol by a liver homogenate. Since it is now known that only one enantiomorph of mevalonate is used, and that one-sixth of the radioactivity of this is lost on the way to cholesterol, the yield first reported was, indeed, slightly more than 100 per cent, but the excess would be within experimental error. Soon afterwards, Tavormina and Gibbs[19] showed that radioactivity in the carboxyl group of mevalonate was not incorporated at all into cholesterol, but lost as carbon dioxide during biosynthesis.

To all workers on polyisoprenoid biosynthesis, these discoveries came like an answer to prayer. Here was a substance, obviously related to 3-hydroxy-3-methylglutarate and thence to acetate, which was capable of losing one carbon atom to give a branched C_5 unit. More, the loss of two additional molecules of water could give isoprene itself, the basis of Ruzicka's structural hypothesis on the biogenesis of terpenes.

It was, of course, necessary to be sure that these carbon atoms of mevalonate were being used without prior dissociation. Popják and I, with Mrs Cornforth and Youhotsky Gore, were able to show[20] that 2-^{14}C-mevalonate, when incubated with liver preparations in the absence of oxygen, was largely incorporated into squalene rather than into cholesterol, and a total degradation of the squalene thus synthesized revealed only six labelled positions, the positions predicted for integral incorporation of the C_5 unit into squalene. This is shown in the diagram below, black circles being

used to indicate the labelled positions in squalene, lanosterol and cholesterol biosynthesized from 2-^{14}C-mevalonate:



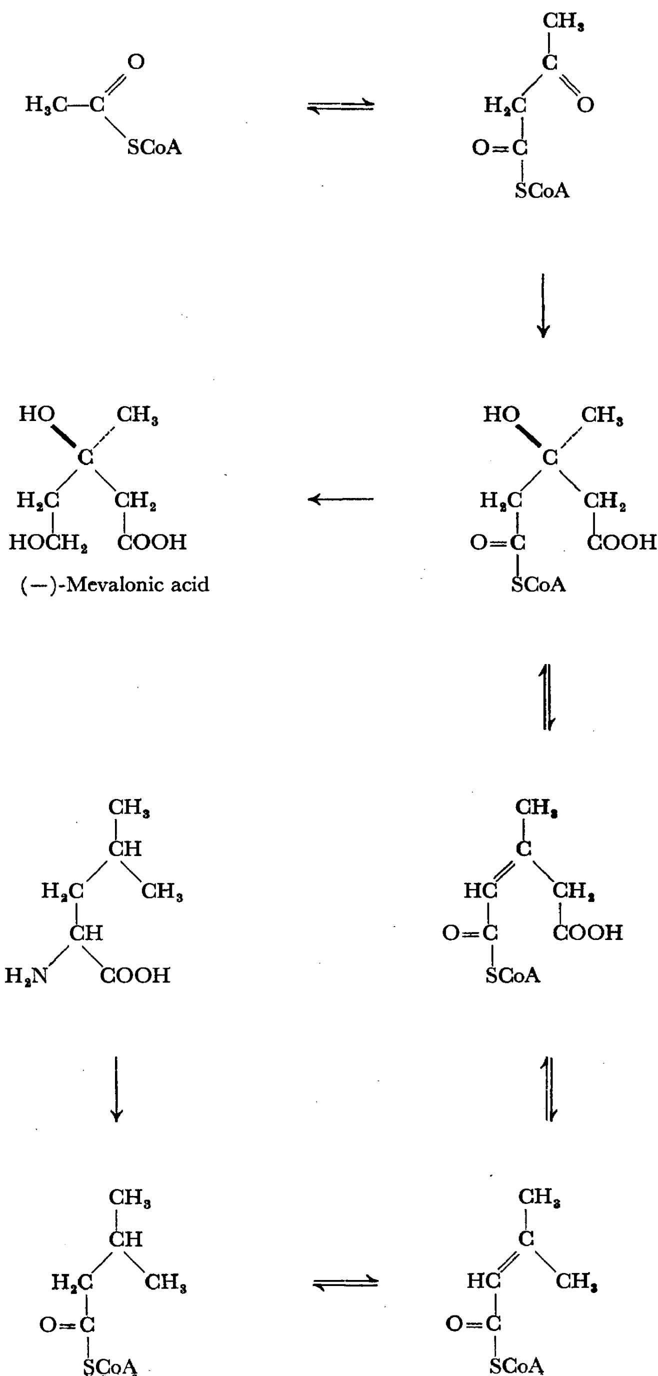
Dituri, Gurin and Rabinowitz[21] independently produced similar evidence, and Isler and this group[22] have done much the same thing for cholesterol biosynthesized from 2-^{14}C-mevalonate; here, measurements of radioactivity indicated five labelled positions of which three were found where expected. The sixth radioactive atom is incorporated into a ring A methyl group of lanosterol and is lost.

With mevalonate as a very conveniently placed landmark on the path of biosynthesis, the mapping of earlier and later stages was made easier, and it became profitable to use fractionated enzyme systems, mainly from liver and yeast, which could carry out only a limited number of stages. It is possible that every intermediate between acetate and lanosterol has now been identified, and many of the enzymes responsible have been extensively purified as well.

The first two stages (see *Scheme 1*), conjugation of acetate with coenzyme A and condensation to acetoacetyl-coenzyme A, are not peculiar to polyisoprenoid biosynthesis and are well-known. Both are reversible, and this seems to have defeated attempts[23] to demonstrate incorporation of acetoacetate into cholesterol as a C_4 unit.

The condensing enzyme which, from acetyl- and acetoacetyl-coenzyme A, produces 3-hydroxy-3-methylglutaryl-coenzyme A has been studied and partially purified by Rudney and Ferguson[24]. It is known that the molecule of coenzyme A liberated during condensation is the one present originally as acetyl-coenzyme A. Also, it is known that the product must have the absolute configuration shown, for Rudney demonstrated that only one-half of a synthetic preparation, but all of an enzymically produced specimen, could act as substrate for reduction to mevalonate. The condensation is not readily reversible, though there is another enzyme[25] which breaks down the product to free acetoacetate and acetyl-coenzyme A.

Scheme 1. Biosynthesis of mevalonic acid



3-Hydroxy-3-methylglutaryl-coenzyme A seems also to be involved in the metabolism of the amino-acid leucine (see *Scheme 1*). Oxidative breakdown of leucine can give isovaleryl-coenzyme A and thence 3-methylcrotonyl-coenzyme A. From work by Coon[26] and Lynen[27] it is known that this can be carboxylated enzymically, and that the glutaconic ester so formed can be hydrated by an enoyl hydrase (methylglutaconase) to hydroxymethyl-glutaryl-coenzyme A. Thus breakdown of leucine can be associated with biosynthesis of steroids and terpenoids, though to what extent this actually occurs is uncertain.

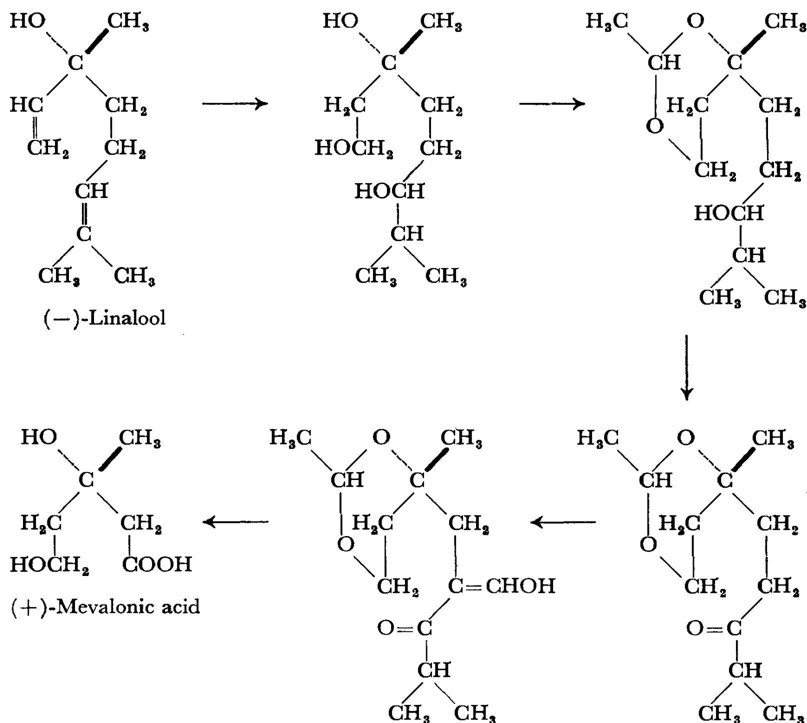
The next enzymic step produces mevalonic acid directly (see *Scheme 1*). The immediate reducing agent seems to be triphosphopyridine-nucleotide, the group operated on is the thiol ester, coenzyme A is liberated (never to participate again in the biosynthesis), and the process seems to be practically irreversible. One might expect a reaction of this sort to proceed *via* an aldehyde as intermediate, but attempts to trap this aldehyde, mevaldic acid, have been uniformly unsuccessful. The enzyme has been studied by the groups of Lynen[28] and Rudney[29]; its mechanism should be interesting to study, for irreversibility in a process of this kind is not usual; and further, the biosynthesis of polyisoprenoids in the tissues so far studied seems to be cut off at this point from other metabolic processes and to proceed almost undisturbed to its goal.

The chemistry of mevalonic acid, and of its lactone which is easily formed and hydrolysed, has been extensively studied; and six or seven syntheses have been devised, one or other of which would permit almost any type of labelling. Natural mevalonate, the growth factor for *Lactobacilli*, is optically active and gives a benzhydrylamide which is laevorotatory in ethanol. We have shown that, when racemic 2-^{14}C-mevalonate is fed to rats[30], approximately half the radioactivity is excreted in the urine, and that this is recoverable as a dextrorotatory mevalonyl-benzhydrylamide. Nearly all the retained radioactivity is found in cholesterol. Thus the enantiomorph which is the growth factor for *Lactobacilli* is also the precursor of steroids.

The absolute configuration of mevalonic acid has been deduced by Arigoni[31] by a chemical correlation with quinic acid, which had itself been correlated with D-glucose. Mrs Cornforth and I were recently able to make a further correlation by means of a synthesis (see *Scheme 2*) which was designed to obtain optically active mevalonic acid in quantity, resolution of the racemic acid being particularly difficult. We started from (—)-linalool which is commercially available. A Brown reaction of this with boron hydride in tetrahydrofuran was followed by oxidation with alkaline hydrogen peroxide, when the expected triol was formed in high yield. Two of the hydroxyl groups were " tied up " by reaction with acetaldehyde, and the third was then oxidized by chromic acid in pyridine to a ketone. This was formylated with methyl formate and sodium methoxide. The crude hydroxymethylene-ketone was smoothly converted with sodium periodate into mevalonic acid, isolated as the crystalline lactone.

We had expected, on the basis of the absolute configuration assigned to linalool by Prelog and Watanabe[32] in 1957, to obtain " natural " mevalonic acid from this synthesis, and it was an unpleasant surprise to find that the benzhydrylamide was dextrorotatory and the acid enzymically inert. The

Scheme 2. Synthesis of mevalonic acid from linalool



" unnatural " isomer had in fact been formed. Examination of the evidence showed that the assignment of configuration to (—)-linalool, which was founded on an asymmetric synthesis of tetrahydro-linalool, contained an error of interpretation, and that the configurations assigned to the two optical isomers must now be reversed[33]. It was satisfactory that the method of asymmetric synthesis then gave yet another result in agreement with that of direct chemical correlation; but we were not pleased, for (+)-linalool is hard to come by.

It has been found convenient by Bloch and by Lynen to study the stages between mevalonate and squalene in enzyme systems prepared from yeast, and Popják has now overcome the difficulties of demonstrating that the same processes also occur in liver. The first step is a phosphorylation of the primary alcoholic group in mevalonate (see *Scheme 3*). The " natural " enantiomorph is the only one attacked; the enzyme, called mevalonic kinase, has been extensively purified from yeast by Tchen[34] and from liver by Levy and Popják[35]. Adenosine triphosphate is the phosphorylating agent, and the product can readily be isolated. It is sometimes convenient to use biosynthetic 5-phosphomevalonate instead of racemic mevalonate in enzymic reactions where the presence of " unnatural " mevalonate would be a complication. To separate and identify the hydrophilic substances encountered in this region of the biosynthesis, paper chromatography and paper ionophoresis have been used extensively, and information on the

Scheme 3. Enzymic conversion of mevalonate to iso-pentenyl pyrophosphate

HO CH$_3$
C
H_2C CH_2
HO—CH_2 COOH

+ATP → −ADP

HO CH$_3$
C
H_2C CH_2
H_2O_3P—O—CH_2 COOH

+ATP ↓ −ADP

HO CH$_3$
C
H_2C CH_2
$H_3O_6P_2$—O—CH_2 COOH

+ATP ← −ADP

H_2O_3P—O CH$_3$
C
H_2C CH_2
$H_3O_6P_2$—O—CH_2 C
O O$^-$

↘

$H_2PO_4^-$ + CH$_3$ + CO_2
C
H_2C CH_2
$H_3O_6P_2$—O—CH_2

Isopentenyl pyrophosphate

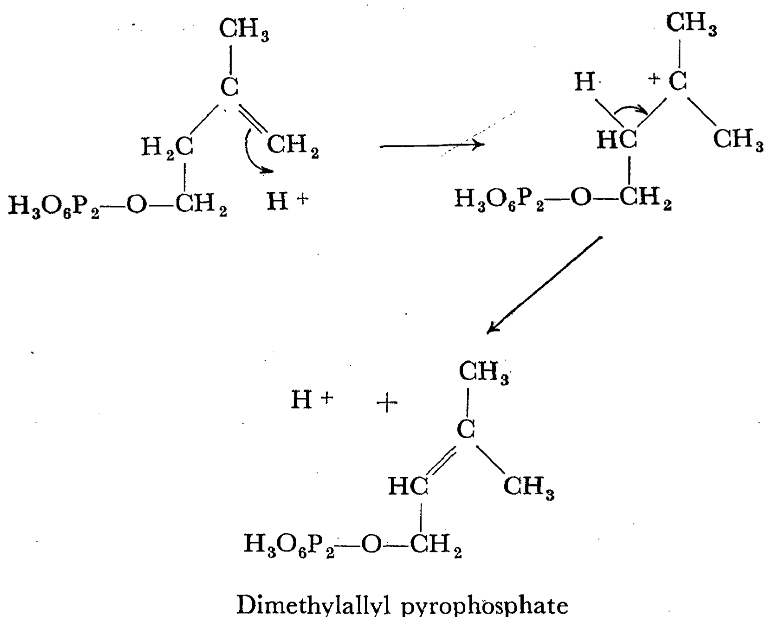
nature of intermediates was also gained by using ^{32}P in conjunction with ^{14}C. Thus, it was shown[36, 37] that the first phosphorylation is immediately followed by another, to give a pyrophosphoric ester. 5-Phosphomevalonate has so far been synthesized only as a racemate. Enzymically formed 5-phosphomevalonate is laevorotatory, $[\alpha]_D = -6{\cdot}1°$.

It is in the next stage that the superfluous carboxyl group of mevalonate is lost. The process can be formulated as a dehydration–decarboxylation of a not unfamiliar type; however, adenosine triphosphate is a necessary cofactor, and it was shown[38], by using isotopic phosphorus in its terminal phosphate group, that this group is set free as orthophosphate during this reaction. Bloch[39] has recently prepared diphosphomevalonate labelled at the tertiary hydroxyl group with ^{18}O, and has shown that the isotope appears in this inorganic phosphate. The presumption is that ATP first phosphorylates the tertiary hydroxyl group, and that a concerted elimination of orthophosphate and carbon dioxide then occurs. That the process is concerted and not an elimination followed by a decarboxylation seems to follow[38] from experiments indicating that tritium is not incorporated from the medium during the enzymic reaction.

The product of this elimination is isopentenyl pyrophosphate, an intermediate discovered and synthesized by Bloch's and Lynen's groups[36, 40, 41].

It will be noticed that this substance is isoprene + pyrophosphoric acid, and it can, in fact, be regarded as the actual chemical basis of Ruzicka's isoprene rule. Its conversion into squalene and cholesterol by suitable enzyme preparations without ATP has been demonstrated. With the newer methods of phosphorylation now available to make its synthesis more convenient, it may well become the precursor of choice for experiments on terpenoid and carotenoid biosynthesis in plants.

Isopentenyl pyrophosphate has a terminal isopropenyl group, whereas squalene, rubber, and many acyclic polyterpenoids have terminal isopropylidene groups. It was, therefore, reasonable for Lynen[42] and colleagues to postulate a prototropic change to dimethylallyl pyrophosphate as a preliminary to the association of C_5 units, and he was able to find in yeast an enzyme able to effect this change reversibly with artificially introduced radioactive substrate:

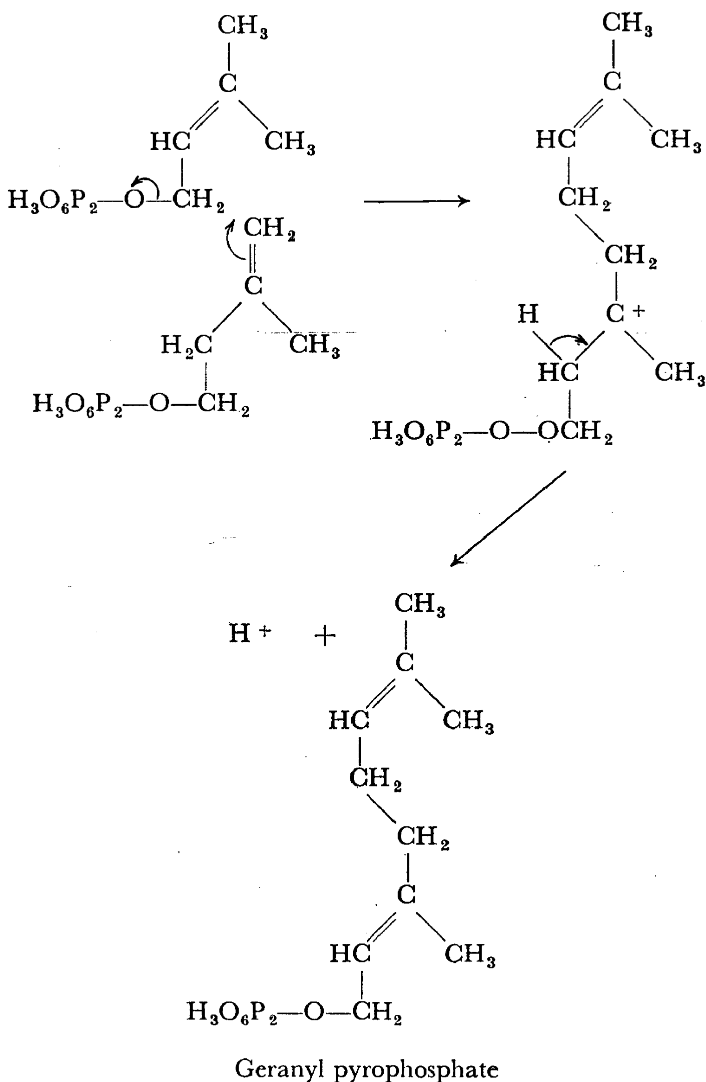


Dimethylallyl pyrophosphate

That the change was an essential one for biosynthesis of squalene could also be demonstrated[43], for the enzyme happened to depend for activity on a sulphydryl group which could be blocked by iodoacetamide. An enzyme preparation so treated could not synthesize squalene from isopentenyl pyrophosphate alone, but could do so when dimethylallyl pyrophosphate was also added. Thus it was shown not only that isopentenyl pyrophosphate is isomerized to dimethylallyl pyrophosphate, but that isopentenyl pyrophosphate is required again at a later stage.

Clearly, the movement of the double bond to form an allyl pyrophosphate would facilitate the separation of pyrophosphate ion either by ionization or by nucleophilic attack—for example, attack by the available electrons

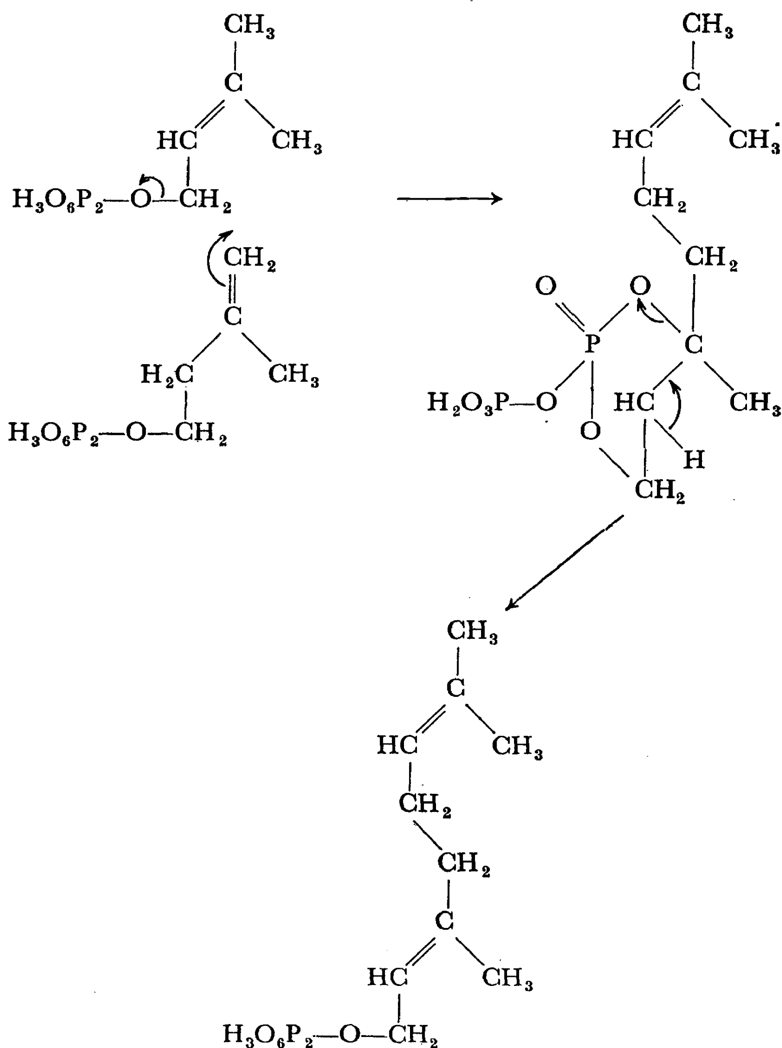
from the double bond of isopentenyl pyrophosphate. The product, which could also, of course, be formed from the olefin and a dimethylallyl cation, might then lose a proton to give geranyl pyrophosphate:



Geranyl pyrophosphate

and Lynen[43] has recently obtained evidence that this substance, which he has synthesized, is indeed formed as an intermediate by his yeast enzyme system. Condensation of geranyl pyrophosphate with a third C_5 unit would then lead to farnesyl pyrophosphate. This also was identified as an intermediate by Lynen[40], and it was recently synthesized by Cramer[44] using a modification of his excellent trichloroacetonitrile method.

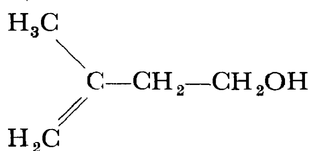
The precise mechanism by which C_5 units are associated is still open to discussion. Johnson[45] recently put forward the interesting suggestion that the pyrophosphate group on the isopentenyl pyrophosphate also facilitates the reaction by an effect of the same kind as is observed, for instance, in the formation of bromo-lactones from bromine and some unsaturated acids. According to this idea, a cyclic phosphate is a stabilized intermediate, and is later cleaved to generate the double bond in a new position. The postulated mechanism may be represented thus:



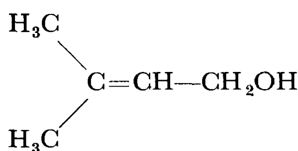
In fact, a solvolysis of benzhydryl chloride in the presence of a suitable unsaturated acid was shown to give carbon–carbon bond formation with concurrent lactonization.

Attractive though it is, the idea does seem to overlook the relatively slight tendency of phosphate ions towards nucleophilic attack on carbon. Perhaps the negatively-charged pyrophosphate residue may rather act electrostatically to polarize the double bond and at the same time to assist removal of the allylic hydrogen. This appears stereochemically possible.

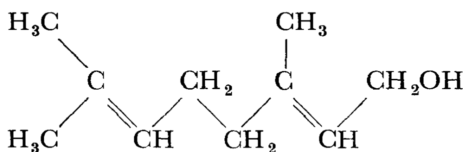
In geranyl pyrophosphate, in farnesyl pyrophosphate and in squalene, there are double bonds capable of geometric isomerism. Natural squalene as the clathrate in thiourea is undoubtedly the all-*trans*-form, as first shown by the X-ray crystallographic data of Nicolaides and Laves[46]. Since natural geranyl pyrophosphate and farnesyl pyrophosphate are precursors of squalene, the presumption is that they also are *trans*-, and this is supported by the available evidence. Synthetic metabolically, active geranyl pyrophosphate was made from natural geraniol, which Jackman and Weedon[47] have conclusively proved to be *trans*-, by methods which would not be expected to alter the geometry of the double bond. As for natural farnesyl pyrophosphate, the evidence that this is exclusively in the *trans,trans*-form is also strong. Popják has obtained a soluble enzyme system from rat liver which will convert radioactive 5-phosphomevalonate to a range of isoprenoid pyrophosphates. The free alcohols can be liberated enzymically by various phosphatases and examined by gas–liquid radiochromatography[48], a method which permits automatic recording of the retention time, height and total radioactivity of each peak as it emerges from a column of high resolving power. In this way isopentenol, 3,3-dimethylallyl alcohol, geraniol and farnesol were detected:



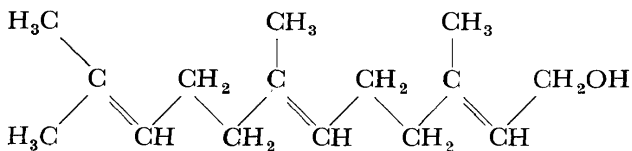
Isopentenol



3,3-Dimethylallyl alcohol



Geraniol



Farnesol

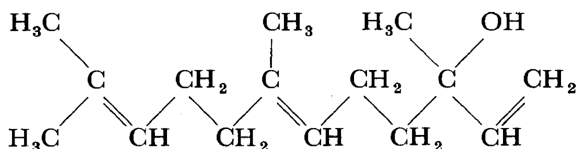
as well as a radioactive peak corresponding in retention time to the C_{20} compound geranylgeraniol. Both geraniol and farnesol appeared as single

peaks, though the column was easily able to resolve geometrical isomers of these alcohols. Geraniol was present as the slower-moving *trans*-isomer and the farnesol peak corresponded to the slower-moving peak of a synthetic product consisting of *cis,trans*- and *trans,trans*-farnesol[49].

The principle of construction of polyisoprenoid chains revealed in all these studies is presumably applicable to the biosynthesis of such natural products as the carotenoids, though evidence on the point is still confined to the demonstration of mevalonate as a precursor. And it is also significant that, with substances such as the ubiquinones and vitamins K_2, the polyisoprenoid chain is attached to an aromatic nucleus by the " tail " carbon. If supplied as an allyl pyrophosphate, this carbon, of course, would be open to nucleophilic substitution of the same type as in the chain-extending process. In long-chain alcohols such as solanesol, in rubber and in gutta-percha, we presumably have examples of the continued operation of this extension. It is true that in rubber the double bonds are *cis*-, but the stereochemical outcome of the synthesis is determined by the spatial orientation of each new C_5 unit added.

The exact nature of the process by which two C_{15} units are united to give the C_{30} compound squalene is not yet determined, and has been the subject of some recent work by Goodman and Popják with Mrs Cornforth and myself. Farnesyl pyrophosphate is undoubtedly a precursor of squalene, conversions having been attained by Lynen with a particulate preparation of yeast, and in especially high yield by Popják with a preparation of rat liver microsomes. The process is a reduction, two electrons being required for synthesis of each molecule of squalene, and for this a reduced pyridine nucleotide, preferably TPNH, is required.

A possible clue to the mechanism was found by Goodman and Popják[49]. When farnesyl pyrophosphate is hydrolysed by acid—this happens very easily—the major product is not farnesol but the tertiary alcohol nerolidol:



Nerolidol

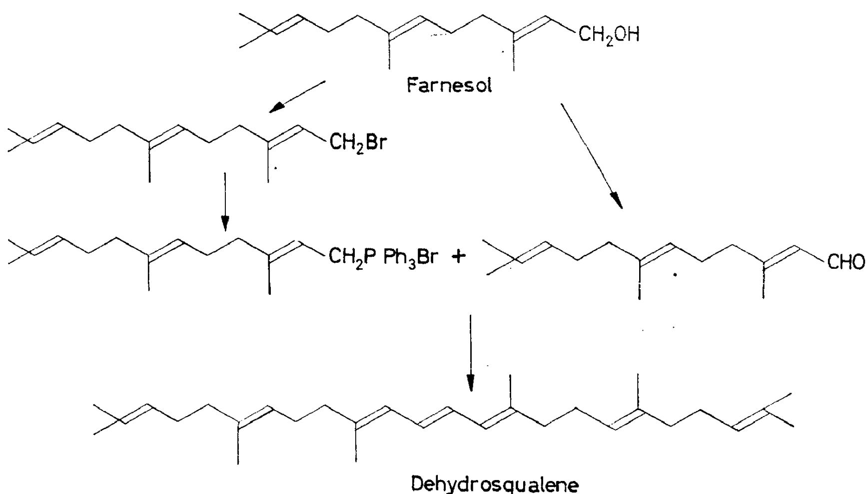
accompanied by both *cis,trans*- and *trans,trans*-farnesol. This observation is easily understood as an allylic rearrangement, but nerolidol also appears, though in much smaller proportion, when the natural pyrophosphates are hydrolysed enzymically at neutral or alkaline pH; nerolidol was also found after fission of the phosphate ester by lithium aluminium hydride. The possibility thus raised, that farnesyl pyrophosphate is naturally accompanied by nerolidyl pyrophosphate, conceivably produced from it by rearrangement, led us to put forward[50] a hypothesis that the coupling of C_{15} units is simply another alkylation of a terminal double bond, here that of nerolidyl pyrophosphate, by an allyl pyrophosphate, here farnesyl. Loss of a proton from the resulting cation would lead to a C_{30} pyrophosphate still of allylic type, which could be expected to lose pyrophosphoric acid with ease to

give a conjugated triene, dehydrosqualene, which might then be reduced. The postulated reaction is as shown in *Scheme 4* pp. 622-3.

In putting forward this mechanism, we were influenced by some work by Rilling and Bloch[51] on the uptake and exchange of hydrogen during biosynthesis of squalene from mevalonate. This work may be summarized thus: first, that squalene, biosynthesized from mevalonate in which both hydrogens on C-5 have been replaced by deuterium, contains not twelve deuterium atoms but only about ten; and when this squalene is degraded by ozonolysis, the succinic acid from the four central carbon atoms contains not four deuterium atoms, but only about two. Secondly, squalene biosynthesized from ordinary mevalonate in a deuterium oxide medium contains 3·5 to 4 deuterium atoms, and in a parallel experiment in tritiated water half the tritium introduced into squalene was found in the succinic acid, the other half being furnished by the two hydrogens which are necessarily taken up in forming the terminal isopropylidene groups.

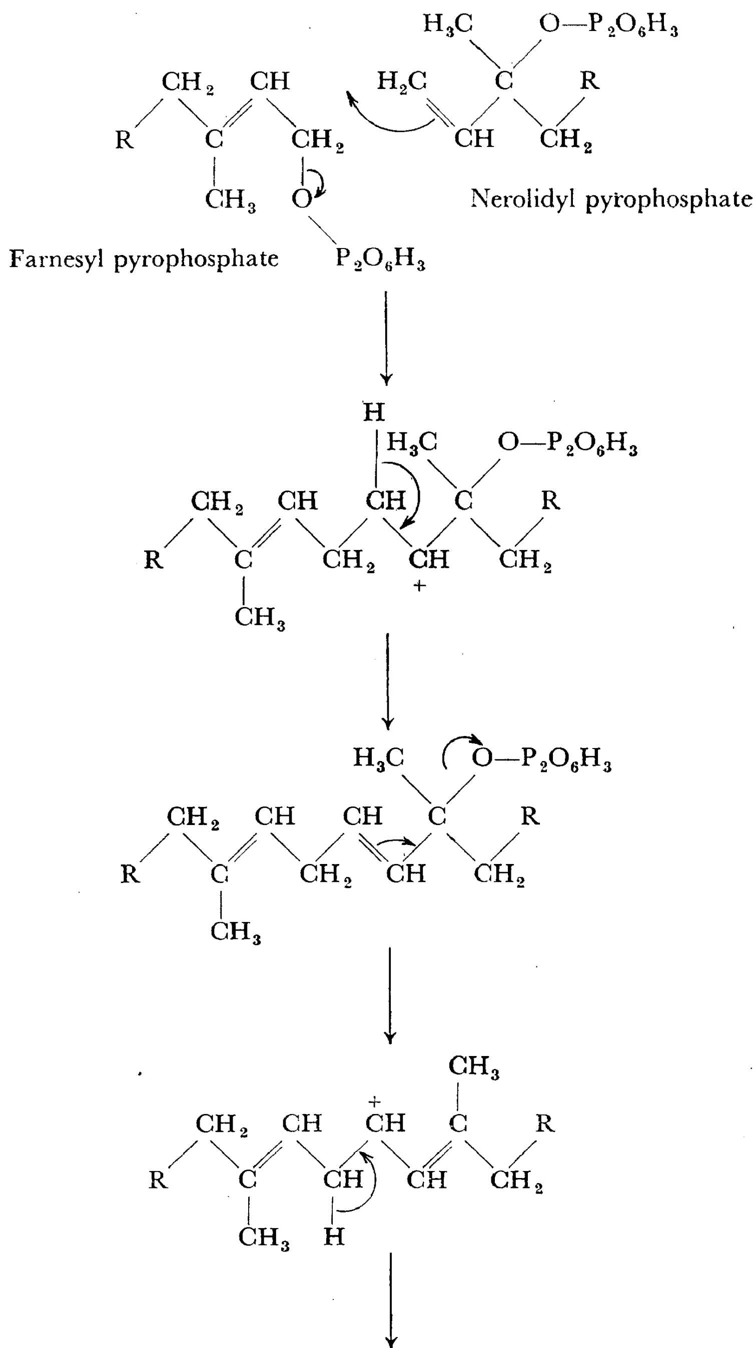
All this seemed clear evidence that two hydrogen atoms, both originally attached to the oxygenated carbon of farnesyl pyrophosphate, are replaced by other hydrogen atoms during biosynthesis of each molecule of squalene; if dehydrosqualene were an intermediate such a replacement would, of course, be inevitable.

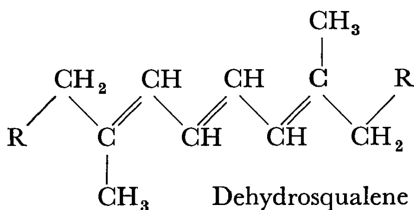
We therefore devised some experiments to examine the detailed mechanism of squalene biosynthesis. A chemical synthesis of dehydrosqualene was one objective, and Mrs Cornforth achieved this quite simply. In the first step, farnesol was converted into farnesyl bromide by Rydon and Landauer's[52] method with triphenyl phosphite benzylobromide. The undistilled bromide was immediately treated with triphenylphosphine, when there was formed a phosphonium salt which with farnesal and sodium ethoxide gave dehydrosqualene by a Wittig synthesis. The reaction scheme is illustrated as follows:

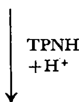


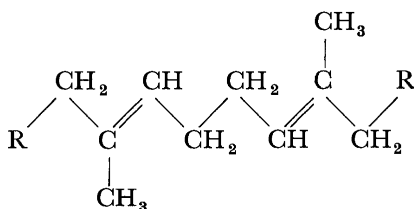
The ultra-violet absorption spectrum of this substance corresponded closely with that of an analogously constituted natural product, the C_{40} compound

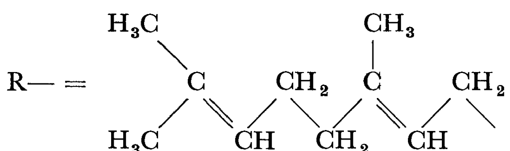
Scheme 4. Postulated biosynthesis of squalene









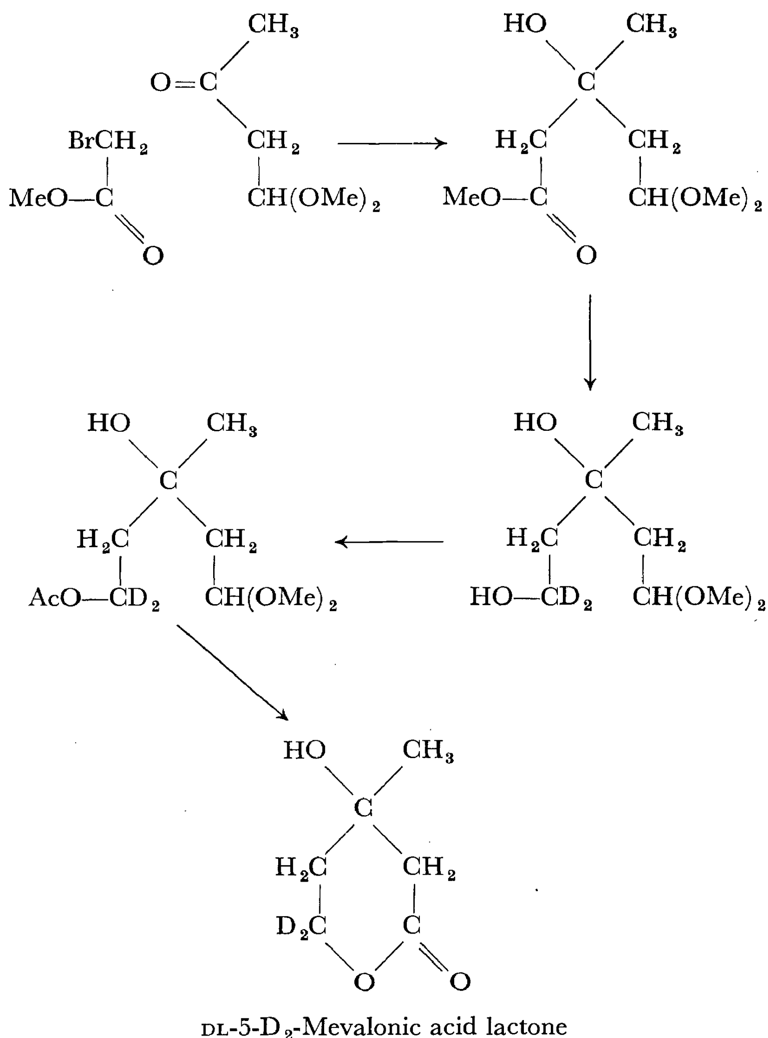


phytoene. The synthetic product was a mixture of geometrical isomers. Preparation of a radioactive dehydrosqualene has not yet been completed, for reasons which will become apparent.

We also wished to check the findings of Rilling and Bloch and to determine, if these findings were confirmed, whether replacement of two hydrogens occurred at one carbon atom or two: in other words, whether the central methylene groups in squalene biosynthesized from 5-D_2-mevalonate (or from normal mevalonate in deuterium oxide) were —CHD—CHD— or —CD_2—CH_2—. To do this, the degradation of squalene would have to be done without the usual dilution with unlabelled carrier.

Two dideuterosuccinic acids were prepared, the symmetrical isomer by adding sodium amalgam to a solution of maleic anhydride in deuterium oxide, the unsymmetrical by heating ethane tricarboxylic ester with deuterium oxide at 150°. Succinic anhydrides prepared from these acids gave excellently resolved infra-red absorption spectra in potassium chloride micro-plates. The two spectra were completely different, and it was demonstrated that 120 micro-grams of either anhydride mixed with three times its weight of unlabelled anhydride could be identified with certainty. In the event, this method did not have to be used, but it showed that deuterated succinic acids suffer little exchange of deuterium on conversion to anhydrides. Later, tetradeuterosuccinic acid was made by heating deuterium oxide with ethane tetracarboxylic ester.

The dideuteromevalonate was prepared by adapting an earlier synthesis of ours. 4,4-Dimethoxybutan-2-one with methyl bromoacetate and zinc gave an acetal-ester which was reduced by lithium aluminium deuteride (" 98 per cent D "). The product with hydrogen peroxide in acetic acid containing a trace of sulphuric acid gave crystalline mevalonic acid lactone containing 1·96 atoms of D per molecule, as determined by combustion of a diluted specimen of benzhydrylamide. The reaction scheme is given as follows:



DL-5-D_2-Mevalonic acid lactone

This mevalonate was mixed with a little 2-^{14}C-mevalonate before biosynthesis; the radioactivity was needed to measure the amount of squalene biosynthesized *de novo* in the enzyme system, and also for a check on the origin of the succinic acid obtained from the squalene by oxidation. Squalene

was also made from non-deuterated ^{14}C-farnesyl pyrophosphate in (i) a deuterium oxide medium, and (ii) a tritiated water medium. Another specimen of squalene was made from non-isotopic mevalonate in normal water with tritiated TPNH.

The biosynthetic squalene was purified by repeated separation as the clathrate in thiourea. A portion was burned after suitable dilution with non-isotopic squalene; the remainder was ozonized without dilution by adding it to excess of ozone in ethyl chloride at − 80°. The ozonide was decomposed with hydrogen peroxide and acetic acid, and, after destruction of excess peroxide, the acids were extracted into ether and the succinic acid crystallized by adding a little chloroform to the evaporated extract. Since 10–20 mg of squalene was available for ozonolysis, the amount of succinic acid did not exceed 1–2 mg, and this had to be recrystallized before conversion into succinic anhydride. A technique was therefore devised which may be of general interest.

The succinic acid in ether was allowed to flow slowly from a fine capillary into a melting-point tube tapered at the bottom and held in a bath at 50–60° (see *Figure 1*). When the transfer of succinic acid was complete, a

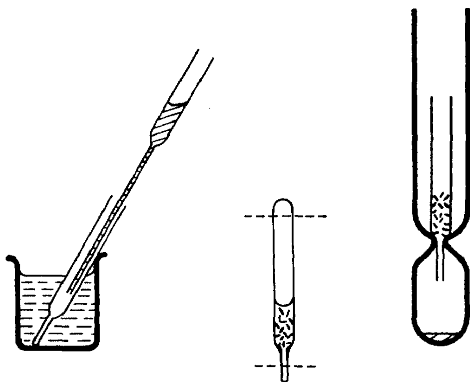


Figure 1. Micro-manipulations of succinic acid

capillary containing 2 μl of water per mg of succinic acid was rested at the top of the tube. Brief spinning in a centrifuge forced the water into the melting-point tube, which was sealed, heated in boiling water until the solid dissolved, spun whilst still hot to collect any sediment at the extreme tip, then cooled and allowed to crystallize. The tube was opened at the top and broken off near the tip. It was then rested on a constriction made in a larger tube, and spun. The crystals could not follow the mother liquor through the narrow orifice and they were washed *in situ* with about 2 μl of ice-water (temperature control of this assembly is not difficult to arrange). After being dried, the crystals were loosened with a fine gold wire and tapped into a new tube, where they were weighed. About twice their weight of acetyl chloride was spun down on them; the tube was sealed and heated for about 2 minutes in a paraffin bath at 90°; reaction was then complete. The liquid was spun into the tapered

end and allowed to crystallize, the mother liquor was removed as above and the succinic anhydride crystals were washed with cold ether. One crystal was then reserved for melting-point determination. Some manipulative losses due to inexperience were encountered, and in our best experiment 470 μgm of succinic anhydride were obtained from 1·43 mg of crude succinic acid.

The samples of succinic anhydride from squalene, and also from the three synthetic deuterated succinic acids, were examined in a high-resolution mass spectrometer, at the Karolinska Institutet in Stockholm, by Dr Ryhage. We are most grateful to him and to Professor Bergstrom who arranged for this examination.

The molecular peaks of succinic anhydride in this spectrometer were small, but the cracking pattern provided the necessary information. The principal mode of fission is to carbon dioxide and a fragment of mass 56 (see *Table 1*). In normal succinic anhydride, the peaks at masses 57–60 are

Table 1. Mass spectra of succinic anhydrides at m/e 56–60. For each sample, the highest peak in the group is given the arbitrary value 100 and values for the other peaks are calculated on this basis

Sample of succinic anhydride	m/e				
	56	57	58	59	60
1. From normal succinic acid	100·0	4·72	0·34	0·28	0·20
2. From normal squalene ozonide decomposed in 1-D-acetic acid	100·0	4·4	0·37	0·07	0·13
3. From 2-D_2-succinic acid (synthetic)	3·78	15·0	100·0	18·9	1·71
4. From 2,3-D_2-succinic acid (synthetic)	4·83	24·0	100·0	4·24	0·5
5. From 2,3-D_4-succinic acid (synthetic)	1·25	2·29	5·48	23·0	100·0
6. From mixture of 1 and 5	100·0	7·18	4·11	14·08	53·3
7. From squalene biosynthesized from 5-D_2-mevalonate	10·5	9·60	46·2	100·0	3·91

small relative to 56, but it is otherwise in the specimen from squalene prepared from deuteromevalonate. Mass 59, a trideuterated species, is seen to predominate, and the proportion of mass 60, which would contain the tetradeuterated species, is scarcely larger than would be provided by the normal content of ^{13}C in the trideuterated fragment. The same thing could be seen in the other major mode of fission, to carbon dioxide, carbon monoxide and ethylene. Mass spectra of the three synthetic deuterated succinic anhydrides are shown in *Table 1* for comparison.

The proportion of succinic acid originating from other parts of the squalene molecule could be measured from the radioactivity of the sample, and was shown to be about 20 per cent of the total. Some, but not all, of the dideuterated species is thus accounted for. We examined the possibility of exchange of deuterium for hydrogen during ozonolysis, but unlabelled squalene ozonide, when decomposed by hydrogen peroxide in deuterated or tritiated water, gave succinic acid having no significant isotopic excess. Succinic acid recovered (with some dilution) from the mother-liquors of the original experiment, also showed the trideuterated species to be predominant when assayed as dimethyl succinate by mass spectrometry.

These results are entirely incompatible with the hypothesis that the central carbon atoms of squalene biosynthesized from mevalonate carry only two hydrogen atoms originating from mevalonate, since most molecules clearly contain three. Comparison of the total deuterium content of our squalene with that of the mevalonate from which it was made indicated that each squalene molecule contained eleven of the twelve deuterium atoms in the six molecules of precursor (see *Table 2*). Like the mass spectrometry, this indicates exchange of one hydrogen atom, but the origin of the

Table 2. Deuterium content of squalene biosynthesized from 5-D_2-mevalonate

	Atoms per cent excess of D
Found	0·266
Corrected for dilution	20·9
Calculated for 11 hydrogen (+ deuterium) atoms originating from C–5 of mevalonate (95·1 atoms per cent excess of D)	20·9
Calculated for 10 atoms	19·0
Calculated for 12 atoms	22·8

new hydrogen remains obscure, for squalene biosynthesized from farnesyl pyrophosphate by the squalene synthetase system in deuterium oxide gave succinic anhydride showing no excess whatever of deuterated species; and the incubation of normal mevalonate with the enzyme system and tritiated TPNH gave squalene containing very little tritium.

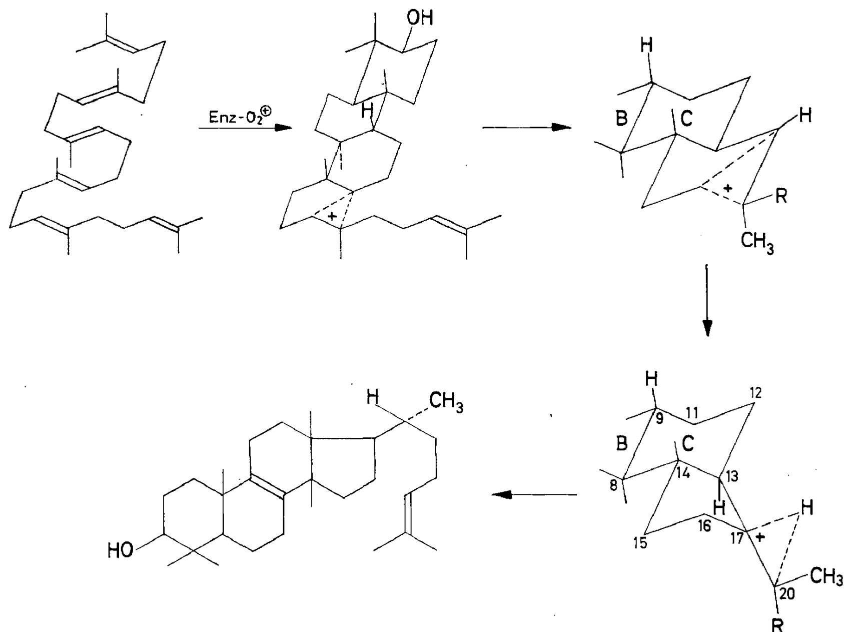
We cannot explain the discrepancy between these results and those of Rilling and Bloch*. However, our hypothesis of the biosynthesis of squalene by way of dehydrosqualene is proved incorrect and must be withdrawn. And, if the data indicating exchange of one hydrogen atom during biosynthesis are accepted, no mechanism yet proposed (*e.g.* by Lynen *et al.*[40]) is consistent with the facts. It would seem that the coupling of C_{15} units is a more complex process than had been thought.

Finally, we come to the cyclization of squalene; and here, in contrast, chemical prediction has constantly anticipated the results of biochemical experiment. In 1955, not long after squalene was established as a precursor of cholesterol, Ruzicka and his colleagues published a paper[53] which might be termed the apotheosis of the isoprene rule. Essentially, the cyclizations of squalene were pictured as reactions of an electron-deficient centre with the available electrons of a double bond. In chemistry of this sort, 1,2-shifts of hydrogen and alkyl groups are common. Some well-grounded assumptions were made about the stereochemistry of these additions and rearrangements. Detailed mechanisms for the biosynthesis from squalene of all known types of triterpenes could then be formulated, while observing the added condition of non-stop reaction: that is, the initiating electron-deficiency was not lost at any intermediate stage.

This theory, as applied to the biosynthesis of lanosterol, postulates an initiating species equivalent to a hydroxyl cation. A concerted cyclization

* This anomaly has since been resolved (G. Popják, De W. S. Goodman, J. W. Cornforth, R. H. Cornforth and R. Ryhage. *Biochem. Biophys. Research Communs.*, **4**, 138 (1961). The source of the "new" hydrogen atom is shown to be TPNH.

then leads to an intermediate in which the electron-deficiency has been transferred to what will be C–20. A rearrangement occurs, the effect of which is to invert the configuration of the carbonium ion. A series of four 1,2-shifts is then possible, two hydrogen atoms and two methyl groups migrating; and finally the positive charge is lost as a proton from C–9, leaving lanosterol. This cyclization of squalene to lanosterol is represented (after Eschenmoser *et al.*[53]) as follows:



All experimental facts so far available are consistent with this view. In particular, Bloch's group[54] and our own[55] have examined the rearrangements of the two methyl groups. This was a difficult problem, because the symmetry of squalene made it impossible to label only one of the groups, and both solutions were based on the fact that a mass spectrometer can distinguish molecules containing one isotopic atom from those containing two. We were able to show that the C–18 methyl group of cholesterol had arrived there by intramolecular migration from C–14, and our Harvard colleagues showed that the methyl group on C–14 of lanosterol is not the group occupying a corresponding position in squalene but has also arrived there by migration. All this is in strict accord with theory.

Theoretically, the position of squalene as the mother of triterpenes seems unassailable, but lanosterol is still the only triterpene of which squalene is actually proved to be the precursor. Arigoni's demonstration[56] that soyasapogenol B is biosynthesized from acetate and from mevalonate seemed also to show that the labels in both these precursors had become somewhat mixed. No doubt the tracing of a biosynthetic pathway in a higher plant will raise difficulties, and will require more workers in this field than are

yet available. Perhaps I may end on this note, for if even one of those present should be moved to experiment by the story I have tried to tell, then your patience in hearing it might be rewarded.

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