PEDLER LECTURE

Exploration of Enzyme Mechanisms by Asymmetric Labelling

By J. W. Cornforth, F.R.S.

SHELL RESEARCH LTD., MILSTEAD LABORATORY OF CHEMICAL ENZYMOLOGY, SITTINGBOURNE, KENT

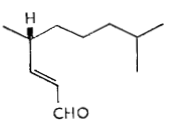
In thanking the organisers of this Symposium I am especially grateful that the occasion of this lecture is a joint meeting of the Chemical and Biochemical Societies. Much of the work to be described arose from close collaboration between an organic chemist and a biochemist, Professor George Popják. This collaboration persisted for some twenty years. Although it has been interrupted by Popják's return to the medical environment from which, with many a backward look, he emerged as a biochemist, neither of us has at all modified our conviction that the integration of organic chemistry and biochemistry can provide exceptionally powerful techniques for examining the processes of life.

In the earlier years we were principally engaged, along with Konrad Bloch and others, and with the help of our collaborators Gordon Hunter and Irene Gore, in finding out the pattern in which eighteen molecules of acetic acid are assembled, with the loss of six carboxyl groups and of three methyl groups, to form the molecule of cholesterol. The chemically interesting task of dissecting the cholesterol nucleus into its nineteen constituent carbon atoms depended, on the biochemical side, upon a supply of radioactive cholesterol biosynthesised from methyl-labelled or carboxyl-labelled acetate. The systems used for biosynthesis—live rats or sliced rat liver—could be crude in comparison with later preparations: the only consequent complication was that operation of enzymes of the citric acid cycle converted a small proportion of methyl-labelled acetate to the carboxyl-labelled variety before incorporation. At that stage, with so many intermediates undiscovered, few thoughts on detailed mechanism could be put to an experimental test. Nor did any point of stereochemical interest arise, except that the synthesis[1] of one degradation product, a C_{11} aldehyde (I), from (*R*)-citronellal (II) helped to establish the absolute configuration of the steroids.

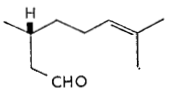
Things began to change with Bloch's discovery that the acyclic hydrocarbon squalene is, as some earlier speculation had forecast, an intermediate in the biosynthesis of cholesterol; soon afterwards, experiments from Bloch's labora-

[1] J. W. Cornforth, I. Youhotsky, and G. Popják, *Nature*, 1954, **173**, 536.

tory confirmed the further hypothesis, originated by Woodward and Bloch,[2] that lanosterol is an intermediate between squalene and cholesterol.

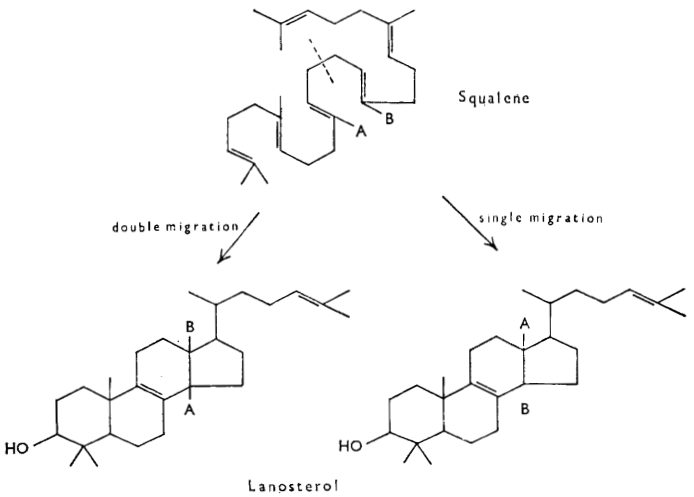


(I)



(II)

These discoveries immediately raised questions of detailed mechanism. From our laboratory came the demonstration of the pattern of radioactive labelling in squalene biosynthesised from acetate. This showed that the position of at least one methyl group of squalene must have changed on the way from squalene to lanosterol. The chemically most plausible mechanism, as elaborated by the Zürich school[3] and by Stork and Burgstahler,[4] entailed intra-molecular rearrangement of two methyl groups during cyclisation of squalene to lanosterol. The alternatives were intermolecular methyl transfer, or intramolecular rearrangement of a single methyl group.

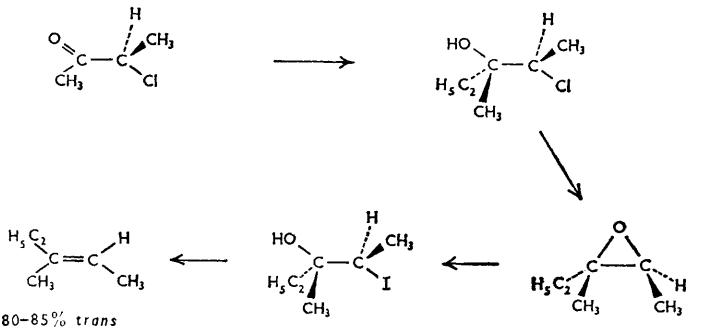


Scheme 1 Lanosterol

To decide between these alternatives by experiment, one must overcome the refreshing topological complication that the two methyl groups in question (A

[2] R. B. Woodward and K. Bloch, *J. Amer. Chem. Soc.*, 1953, **75**, 2023.
[3] A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, 1955, **38**, 1890.
[4] G. Stork and A. W. Burgstahler, *J. Amer. Chem. Soc.*, 1955, **77**, 5068.

and B, Scheme 1) are indistinguishable because of the symmetry of ordinary biosynthetic squalene about its mid-point. At the time when the problem could first be formulated, a synthesis of squalene labelled in a special manner with ^{13}C seemed to offer the only hope of a solution, and here the organic chemist in me protested that although four of the double bonds in squalene were capable of geometrical isomerism and were all in the *trans* form, no method then existed for stereospecific synthesis of trisubstituted olefins. The ensuing olefin synthesis,[5] ably worked out by my wife and by Dr. K. K. Mathew, relies on the discovery that organometallic reagents (and some metal hydrides) add to α-chloroketones in a notably stereoselective manner. It was not possible to proceed stereospecifically from the resulting chlorohydrins to olefins in one step: two stereospecific steps led from chlorohydrins to iodohydrins, and a somewhat exotic new reagent—a mixture of stannous chloride, pyridine and phosphoryl chloride—was developed for the stereospecific reduction of iodohydrins to olefins. Thus the stereoselectivity in the initial reaction is reflected without loss in the geometrical nature of the olefin, and trisubstituted olefins predominantly either *cis* or *trans* can thus be made (Scheme 2). The process was applied successfully to a stereoselective synthesis of *all-trans* squalene.[6] This synthesis of halohydrins, epoxides and olefins of predetermined configuration has since been used by others, a notable recent example being the final stage of an elegant synthesis of the Röller juvenile hormone from W. S. Johnson's laboratory.[7]



Scheme 2 Stereoselective synthesis of olefins

It took some time to work out the synthesis of squalene, and developments occurring elsewhere at the same time made the synthesis unnecessary for its original purpose. Chief among these developments was the discovery of mevalonic acid as a growth factor for *Lactobacilli*, and the inspiration which led

[5] J. W. Cornforth, R. H. Cornforth, and K. K. Mathew, *J. Chem. Soc.*, 1959, 112.
[6] J. W. Cornforth, R. H. Cornforth, and K. K. Mathew, *J. Chem. Soc.*, 1959, 2539.
[7] W. S. Johnson, Tsung-tee Li, D. J. Faulkner, and S. F. Campbell, *J. Amer. Chem. Soc.*, 1968, **90**, 6225.

Tavormina, Gibbs and Huff[8] to the experiment showing that this acid was converted far more efficiently into cholesterol by liver preparations than was any precursor then known.

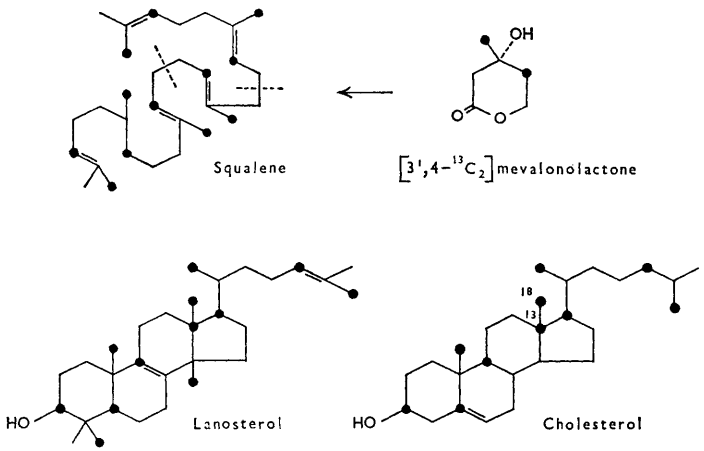
Degradation of squalene[9] and of cholesterol[10] biosynthesised from labelled mevalonate soon confirmed the expected pattern of incorporation: six molecules of mevalonate form one molecule of squalene and thence one molecule of cholesterol. This allowed a solution of the problem of methyl migration in the cyclisation of squalene by a type of asymmetric labelling of mevalonate which we have never used since: a specimen of mevalonate was heavily labelled with ^{13}C at two positions, and the preparation was arranged so that the proportion of molecules having two ^{13}C atoms, and of those with no ^{13}C atoms, greatly exceeded the statistical abundances which would have obtained had the isotopic atoms been distributed at random over the labelled positions of the whole specimen. One could call this statistically asymmetric labelling.

This device was effective because the discovery of mevalonic acid as a precursor of cholesterol had simplified the problem: if the predicted pair of intramolecular 1 : 2-shifts occurs during cyclisation of squalene, one of them is a shift within a single mevalonate-derived C_5 unit of the structure. If, on the other hand, the methyl rearrangement is intermolecular or occurs by a single 1 : 3 shift, the methyl group migrates from one such unit to another one. If a 'statistically-labelled' specimen of mevalonate is used as precursor, all mechanisms give the same distribution of isotopic atoms in the steroid product, but the lopsided distribution which we provided for our mevalonate gives, by the intramolecular 1 : 2 shift, larger proportions of cholesterol molecules having a ^{13}C atom at both positions, 13 and 18. The short reason for this is that when rearrangement is within a single unit derived from the precursor (delineated by dotted lines in squalene, Scheme 3), the proportion of label residing in molecules doubly labelled at positions 13 and 18 depends only on the initial molecular composition of the precursor, whereas methyl migration between different units depends not only on this composition but on the chance that two suitably labelled units were assembled side by side when squalene was synthesised (or on transfer between two appropriately labelled molecules).

The pair of atoms at positions 13 and 18 of cholesterol was separated as acetic acid by Kuhn–Roth oxidation, along with acetic acid originating from other parts of the molecule. In analysing the isotopic composition of the acetic acid, which of course was done by mass spectrometry, allowance had to be made for this extraneous acetic acid. Unusual problems tend to stimulate invention: two new syntheses of mevalonate had to be worked out, along with new methods for purifying acetic acid in centigram amounts: and a special new inlet for the mass spectrometer had to be devised to minimise the tendency of acetic acid to react with metallic surfaces. Further, the liver preparations used to convert

[8] P. A. Tavormina, M. H. Gibbs, and J. W. Huff, *J. Amer. Chem. Soc.*, 1956, **78**, 4498.
[9] J. W. Cornforth, R. H. Cornforth, G. Popják, and I. Youhotsky Gore, *Biochem. J.*, 1957, **66**, 10P; 1958, **69**, 146.
[10] O. Isler, R. Ruegg, J. Würsch, K. F. Gey, and A. Pletscher, *Helv. Chim. Acta*, 1957, **40**, 2369.

mevalonate to sterol had to be freed as much as possible from cellular cholesterol, and the biosynthesis had to be sustained for unusually long times, in order to raise the proportion of newly-synthesised sterol. With the help of our collaborators Rita Cornforth, Marjorie Horning and Andrew Pelter, the whole rather complex experiment was brought to a successful conclusion,[11] and the proportion of doubly-labelled acetic acid confirmed the intramolecular 1 : 2-shift.



Scheme 3 Biosynthesis of squalene, lanosterol and cholesterol from mevalonolactone. The black circles mark positions of ^{13}C labelling

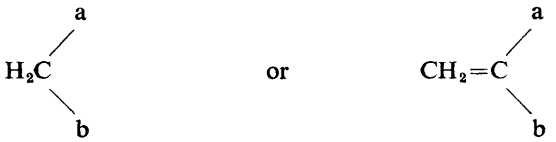
This result was reported at a Ciba Symposium on biosynthesis of terpenoids,[12] a meeting seminal of much work in years to come. Already at that time I was captivated by the thought of using other kinds of asymmetric labelling to explore enzyme mechanisms, and the possibility of such experiments was coming nearer as techniques, chiefly physical and biochemical, advanced.

The growth in general knowledge of enzymic reactions was reinforcing the view that these had much in common with organic chemical reactions *in vitro*. Now some of the best evidence for the mechanism of organic chemical reactions is stereochemical in nature—it is only necessary to recall the decisiveness, in the history of nucleophilic substitution, of the experiment[13] which showed that the total rate of stereochemical inversion of optically active *sec*-octyl iodide by radioactive iodide ion is equal to the total rate of incorporation of radioactivity.

Stereospecific labelling with hydrogen isotopes has unusual advantages in examining enzymic reaction mechanisms. The radioactive and the stable isotopes

[11] J. W. Cornforth, R. H. Cornforth, A. Pelter, M. G. Horning, and G. Popják, *Tetrahedron* 1959, **5**, 311.
[12] 'Biosynthesis of terpenes and sterols', ed. G. E. W. Wolstenholme and M. O'Connor, Churchill, London, 1959.
[13] E. D. Hughes, F. Juliusburger, S. Masterman, B. Topley, and J. Weiss, *J. Chem. Soc.*, 1935, 1525.

of hydrogen are both readily available and relatively cheap, so that the problems of synthesising labelled substrates, or of inducing an enzyme to label a product, are simplified. Above all, it becomes possible to study the stereochemistry of reactions at methylene groups of the types



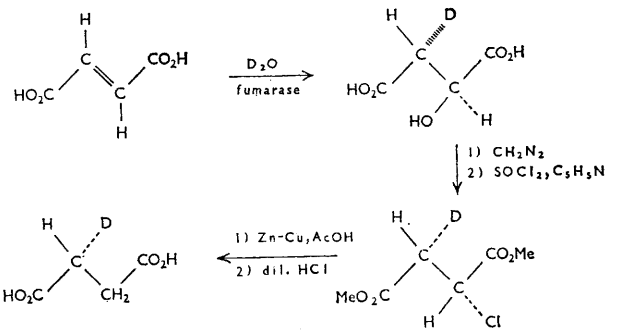
a particularly useful feature. Even when hydrogen isotope effects have to be reckoned with, it is generally possible to arrange the experiment so that these either do not affect the result or give, indeed, additional information.

When tritium is the isotope, the only practical way to trace and measure it is by counting disintegrations; but with deuterium we have used two other methods. Molecular mass spectrometry was, of course, one; the other was spectropolarimetry.

At the time when this work was planned, not much was yet known of optical activity due to asymmetric substitution of hydrogen by deuterium; but the classic work of Westheimer and Vennesland on alcohol dehydrogenase had shown that 1-deuterio-ethanol, at least, had a specific rotation of a few tenths of a degree at the D line.[14] This could be measured easily with an ordinary polarimeter, given enough deuterioethanol to fill a polarimeter tube. The question was whether such specific rotations could be measured with specimens of a few milligrams—which was all we were likely to have, even after scaled-up enzymic reactions. Naturally, an early thought was that rotations might be exaggerated by the Cotton effect, since anomalous rotatory dispersion can often lead to increases of two orders of magnitude in rotatory power. However, such increases occur invariably near an absorption band, and too much absorption is just as effective a bar to polarimetry as is too little rotation. Consideration of typical cases convinced us that little was likely to be gained by modifying substances to produce Cotton effects—though it might have become necessary to try. More promising it seemed to look at transparent substances in the ultra-violet, where optical activity also rises steeply. For this, a spectropolarimeter having an extremely high sensitivity maintained far into the ultra-violet was essential. Someone who knows about optics told us that the prototype of such an instrument was under development at the National Physical Laboratory. Our first measurements, by the kindness of Dr. R. J. King, were made on this machine. A reference substance, asymmetrically labelled with deuterium and with known absolute configuration, was another essential. *R*-Monodeuteriosuccinic acid was peculiarly suitable for the work to be done; and it has served both ourselves and others well in defining absolute configurations. Addition of water to fumaric acid by the enzyme fumarase was known[15,16] to be a *trans* addition, and to give

[14] H. R. Levy, F. A. Loewus, and B. Vennesland, *J. Amer. Chem. Soc.*, 1957, **79**, 2949.
[15] O. Gawron and T. P. Fondy, *J. Amer. Chem. Soc.*, 1959, **81**, 6333.
[16] F. A. L. Anet, *J. Amer. Chem. Soc.*, 1960, **82**, 994.

exclusively *S*-malic acid. It followed that addition of deuterium oxide to fumaric acid gives 2*S*,3*R*-deuteriomalic acid, whence by chemical removal of the hydroxyl group *R*-deuteriosuccinic acid is easily derived (Scheme 4). It turned out to have



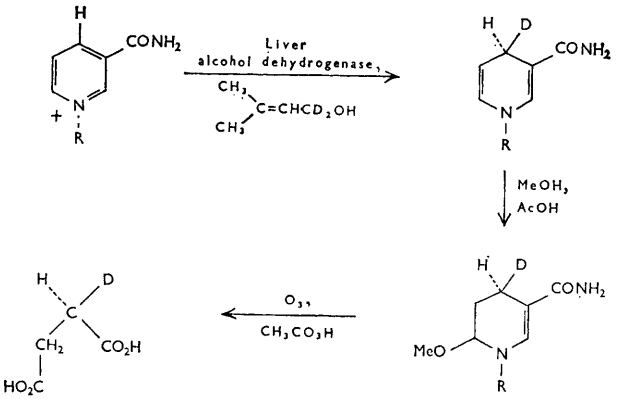
Scheme 4 Preparation of *R*-monodeuteriosuccinic acid

quite a high specific rotation (−17° at 250 nm) at wavelengths where even a saturated aqueous solution is comparatively transparent. With the type of spectropolarimeter available, accurate measurements of its optical activity required not much more than a milligram—and usually we have had more than this. Of course, the optical activity is still small compared with that of most asymmetric compounds, and therefore the purification of the succinic acid needed careful attention. Procedures that we have used include chromatography, sublimation of the anhydride and distillation of the methyl ester. Final purification has usually been by crystallisation from about two parts of water in a capillary tube.

The stereochemical problem of the nicotinamide coenzymes proved to be referable to the asymmetry of deuteriosuccinic acid. It was known that an enzyme ransferring hydrogen from a substrate to the 4-position in the nicotinamide ring of a coenzyme generally transferred it to a particular side of the ring, and that different enzymes used different sides; it was not known which side was which. We and our colleagues Cyril Donninger, George Ryback, and George Schroepfer attacked the problem.

To prepare the stereospecifically labelled reduced coenzymes, it was not necessary to have an enzyme of each type. It was sufficient to use the same enzyme-liver alcohol dehydrogenase—and to present it first with a deuteriated substrate and a normal coenzyme, and then with a normal substrate and a deuteriated coenzyme. Thus, two epimeric reduced and deuteriated coenzymes were formed, and each of these was converted by chemical degradation to deuteriosuccinic acid (Scheme 5).

The two specimens so obtained proved, as expected, to be enantiomeric, and one of them had an optical rotatory dispersion curve identical with that of the

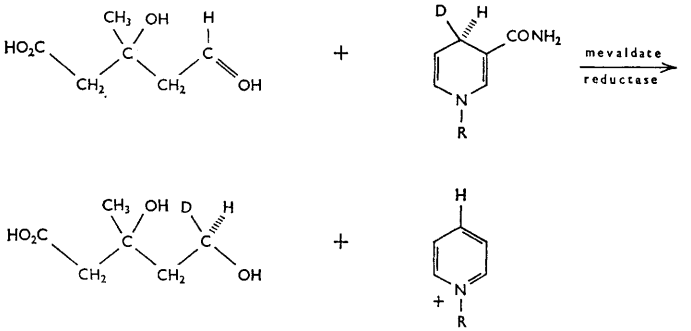


Scheme 5 Principle of determining stereochemistry of hydrogen transfer to nicotinamide coenzymes

reference substance. The absolute stereochemistry of the hydrogen transfer then followed by simple deduction.[17,18]

In this exercise, we had used enzymes together with a source of isotopic hydrogen to generate asymmetric methylene groups, the chirality of which was then ascertained by degradation to a reference substance. To study the enzymes of polyisoprenoid biosynthesis, however, we could not leave all the asymmetric synthesis to the enzymes. In *R*-mevalonic acid there are three methylene groups, and the substitution at each of them is changed at least once on the way to polyisoprenoids. Asymmetric labelling of the 5-methylene group, work in which Cyril Donninger and George Ryback took part, was achieved with the help of an enzyme, mevaldate reductase: the aldehyde of which mevalonic acid is the alcohol was reduced on this enzyme by a deuteriated or tritiated dihydronicotinamide nucleotide. The absolute stereochemistry of the reduction was defined by correlation[19,20] with the substrate stereochemistry of liver alcohol dehydrogenase, and it was found that mevaldate reductase has the same stereospecificity of hydrogen transfer: our experiment, therefore, gave 5*R*-labelled mevalonate (Scheme 6). Singularly, the enzyme reduces both the *R*- and *S*- forms of mevaldate; so that if one presents it, as we did, with racemic mevaldate the product is racemic mevalonate. This is not an obstacle to employment of the labelled mevalonate for studies of polyisoprenoid synthesis: the first enzyme of the series, mevalonic kinase, acts as an efficient stereochemical filter by accepting only the 3*R*-form for phosphorylation. This fortunate circumstance can be put to good use in labelling stereospecifically the methylene groups at positions 4

[17] J. W. Cornforth, G. Ryback, G. Popjàk, C. Donninger, and G. J. Schroepfer, jun., *Biochem. Biophys. Res. Commun.*, 1962, **9**, 371.
[18] J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Ryback, and G. J. Schroepfer, jun., *Proc. Roy. Soc.*, 1966, *B*, **163**, 436.
[19] C. Donninger and G. Popjàk, *Proc. Roy. Soc.*, 1966, *B*, **163**, 465.
[20] C. Donninger and G. Ryback, *Biochem. J.*, 1964, **91**, 11P.



Scheme 6 Preparation of 5*R*-5[D_1]mevalonic acid

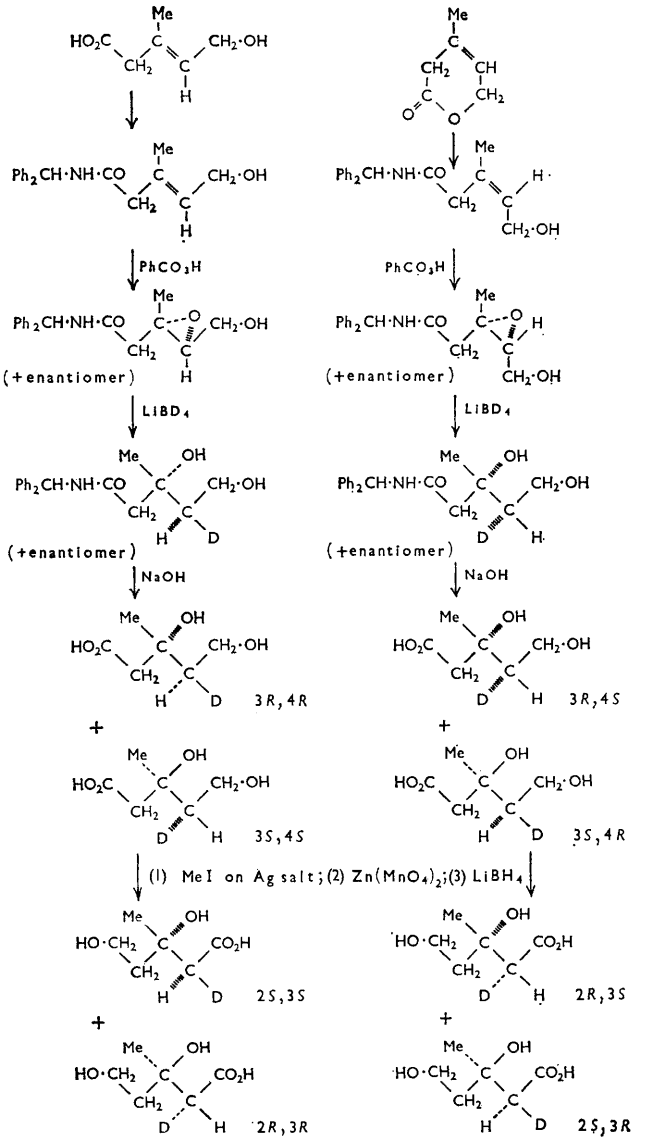
and 2. The associated chemistry (Scheme 7), worked out by Rita Cornforth, is of some interest.[21,22]

The two starting-points for the synthesis were a *trans*-olefinic hydroxy-acid and the lactone of the related *cis* acid.[9] The lactone was converted into a benzhydrylamide, which was epoxidised with peroxybenzoic acid. The epoxide was then reduced with lithium borodeuteride or lithium borotritide, and the benzhydrylamide was hydrolysed by alkali to give a 4-labelled mevalonic acid. Essentially the same procedures were then applied to the *trans* hydroxy-acid.

Epoxidation of double bonds by peroxy-acids is a *cis* addition of oxygen; and reduction of epoxides by metal hydrides proceeds with inversion of configuration at the carbon atom to which hydrogen is added. Thus in each product there is a fixed stereochemical relationship between the two asymmetric centres produced; and this relationship is different in the two specimens of mevalonate because the synthesis started from two different geometrical arrangements about the double bond. Effectively, we were using existing knowledge of the mechanism of organic chemical reactions to construct two diastereoisomeric specimens of racemic mevalonic acid. Mevalonic kinase could be relied on to do the rest, and to treat each specimen as though it consisted of 3*R*-mevalonic acid, labelled in the 4*R* or the 4*S* position according to whether it originated from the *trans* hydroxy-acid or the *cis* lactone. In theory, stereochemically homogeneous specimens could be made without enzymic aid by optical resolution of the mevalonates, but in practice no convenient non-enzymic method for resolving mevalonic acid has yet been found.

Moreover, there was positive advantage in having racemic specimens, for we were to use them directly to prepare mevalonates stereospecifically labelled in the 2-position. Mevalonic acid is both a primary alcohol and a carboxylic acid. If these functions are interchanged, one still has mevalonic acid, but the atoms at positions 4 and 5 in the precursor are at positions 2 and 1 in the product;

[21] J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popják, *Proc. Roy. Soc.*, 1966, *B*, **163**, 492.
[22] J. W. Cornforth, R. H. Cornforth, G. Popják, and L. Yengoyan, *J. Biol. Chem.*, 1966, **241**, 3970.

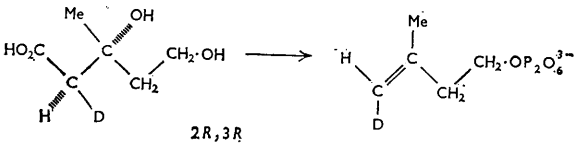


Scheme 7 Preparation of mevalonates stereospecifically labelled with isotopic hydrogen at positions 4 and 2

and a 3*R* configuration in the product is derived from a 3*S* precursor. The necessary interchange of functional groups was done chemically: the somewhat unstable methyl mevalonate was prepared from silver mevalonate and methyl

iodide, and oxidised with zinc permanganate in ice-cold acetone. This gave a half ester of hydroxymethylglutaric acid, which was purified by partition chromatography and reduced, as its lithium salt, with lithium borohydride. Thus we again had two specimens of mevalonic acid, each labelled stereospecifically in the 2-position; and in each of these, only one molecular species was utilisable in biosynthesis. In both theory and practice, homogeneous specimens of both 2- and 4-labelled 3*R* mevalonates could be made by allowing mevalonic kinase to phosphorylate the 3*R* portion of the 4-labelled mevalonates, and then inverting the residual 3*S* portions to 3*R* mevalonates labelled in the 2-position. Perhaps some future application of the labelled mevalonates will require this procedure; for our own purposes it has not yet been necessary. The optical isomerism of the 2-labelled mevalonates is converted into a geometrical isomerism of the enzymically derived isopentenyl pyrophosphates: according to the stereospecificity of the elimination reaction which produces the double bond, a 2*R* (for example) deuteriated or tritiated mevalonate will give a *cis* or a *trans* relation of the isotopic hydrogen to the methyl side chain according to whether the elimination is *cis* or *trans*. Leon Yengoyan took part in the work to determine this point.[22] The biosynthesis of polyisoprenoids from mevalonate on soluble enzymes from liver or yeast can be arrested at isopentenyl pyrophosphate by poisoning the preparations with iodoacetamide: deuteriated isopentenyl pyrophosphates were prepared in this way from the mevalonates labelled at position 2, and the geometry of the isotope in the related isopentenols was deduced by applying existing knowledge of organic chemical reaction mechanisms: specifically, that the addition of bromine to a double bond and the bimolecular-type elimination of hydrogen and bromide ions from adjacent carbon atoms both proceed predominantly in the *trans* sense. In this way it could be shown that the enzymic elimination of the elements of carbon dioxide and water, which leads to the formation of isopentenyl pyrophosphate, is also a *trans* elimination. This means that 2*R*-deuterio-(or tritio-)mevalonates give isopentenyl pyrophosphates in which the isotopic hydrogen is *trans* to the methyl side-chain. With this information in hand, it was also possible to use these mevalonates as precursors in more extended biosyntheses, in which the labelled methylene group again becomes a centre of asymmetry—and beyond that may again participate in a double bond (Scheme 8).

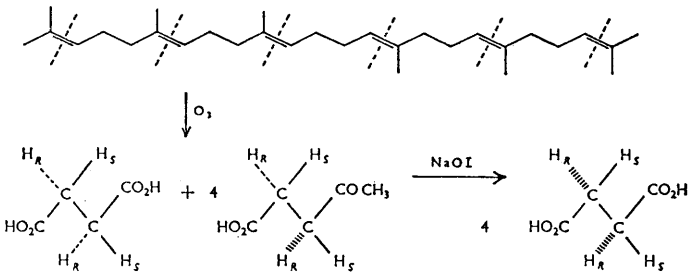
We studied the incorporation of these stereospecifically labelled mevalonates into farnesyl pyrophosphate, or sometimes into squalene, using preparations of rat-liver enzymes.



Scheme 8 Enzymic synthesis of *trans*-4[D_1]isopentenyl pyrophosphate from 2*R*,3*R*[2D_1]-mevalonate

With the 4-labelled mevalonates, it was simply a question of finding out if the isotopic hydrogen was retained or lost when the carbon atom became a terminus for a new double bond. This question could be answered equally well either by mass spectrometry of farnesol or squalene biosynthesised from deuteriated precursors, or by counting radioactivity in the same products derived from the tritiated mevalonates. It turned out that in the biosynthesis of a molecule of squalene, the 4*S* hydrogen is lost from all six mevalonate molecules participating; the 4*R* are all retained.

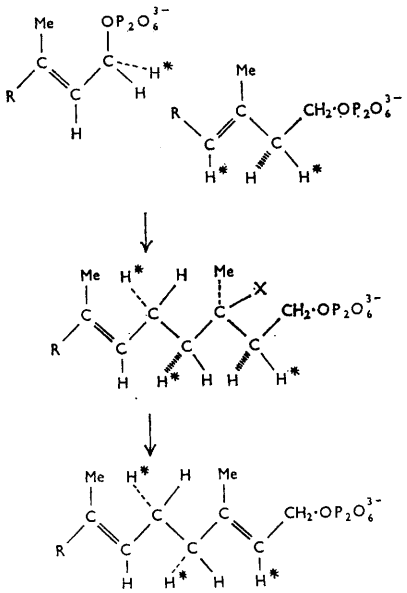
Both farnesol and squalene are easily degraded to succinic acid. Careful ozonolysis of squalene (Scheme 9) gives an acid originating predominantly from the four central carbon atoms of the molecule. The other products are acetone



Scheme 9 Degradation of squalene for identification of stereochemistry of deuterium substitution

and laevulinic acid, and the latter acid (which also of course can be made by ozonolysis of farnesol) can be oxidised to succinic acid by hypoiodite. This might be thought an unsafe procedure to adopt when an asymmetrically deuteriated methylene group lies next to the keto group and can have its asymmetry destroyed by enolisation. The point is that enolisation is the rate-limiting step in base-catalysed iodination. All molecules of laevulinic acid, therefore, that enolise towards the methylene group are iodinated. Since in these conditions iodination is practically irreversible, none of these molecules can generate succinic acid, and the acid that is isolated reflects, in its asymmetrically labelled methylene group, the chirality of the laevulinic acid and thence of the original squalene or farnesol. Polarimetric measurements on such succinic acid specimens, combined with the information already secured, gave a fairly complete picture of the stereochemical changes consequent on polyisoprenoid biosynthesis. There are, for example, three such changes in the reaction that adds successive C_5 units to a polyisoprenoid chain. In squalene biosynthesis, we have the inversion of configuration when pyrophosphate ion is displaced to form the new carbon-to-carbon bond, the attack on the sinistral side of the double bond of isopentenyl pyrophosphate, and the elimination of an *R* hydrogen (corresponding to the 4*S* hydrogen of mevalonate) to form the new double bond. The relationships between these chiralities, we think, are best explained by supposing that the

reaction occurs in two stages (Scheme 10): an electron-donating group X participates in an addition to the existing double bond and then in an elimination of HX to form the new one.



Scheme 10 Postulated mechanism of association of C_5 units in polyisoprenoid biosynthesis

Eventually, I trust, it will be possible to understand fully what happens at the active site of an enzyme. At present, fragmentary knowledge can be acquired in a number of different ways. The molecular structure of a crystallised enzyme may be determined by *X*-ray methods, and a three-dimensional map of the active site, at least as it is in the crystal, can be drawn with some accuracy. This, however, is a static picture at best. Kinetic methods can unravel the sequence of events at the active site, and can indicate intermediate stages. Unnatural substrates can help to explore the limits of the active site, and may on occasion reveal intermediates which are too transient in the normal reaction. Scraps of additional information can be gleaned by a number of other, chiefly physical, techniques. The information that can be got by studying the detailed substrate stereochemistry, with natural substrates or products asymmetrically labelled, is complementary to all these methods. We think it may contribute, directly or indirectly, to the first accurate and complete description of events at an active site.

Meanwhile, the availability of asymmetrically labelled precursors and products has proved useful for investigating other enzymes and other biosynthetic sequences. Optically active succinic acid has served as a reference substance for

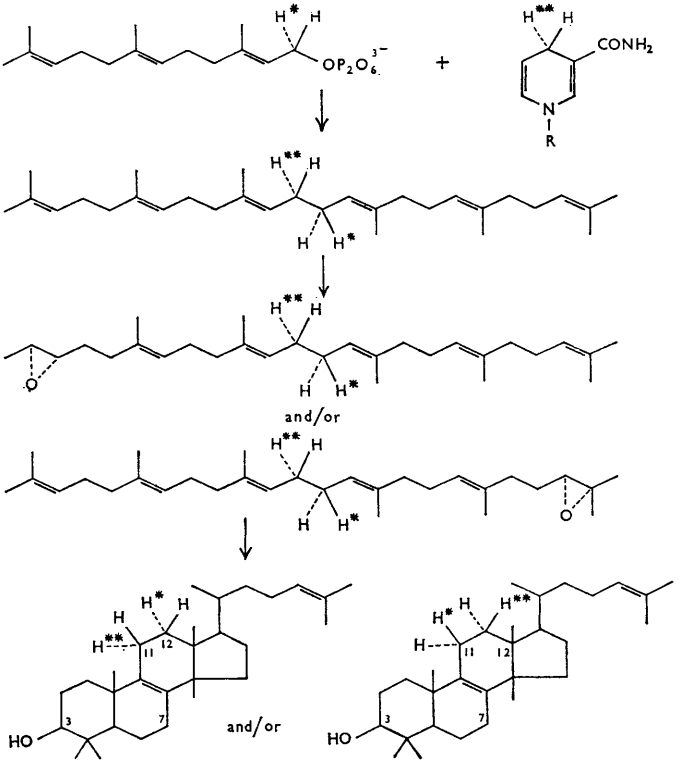
studies on several enzymes. Asymmetrically labelled mevalonates can be used, now that the stereochemistry of the first stages of their transformation has been worked out, as delicate probes to study the detail of more complex biosyntheses. One of the first essays of this kind was the demonstration[23] that although squalene biosynthesised from 4*R*-tritiated mevalonate retains a tritium label in all six C_5 units, the number is reduced to three in cholesterol; and only one of these is in the ring structure, at position 17α, where it has arrived by rearrangement. This relatively simple experiment confirmed several indications of the biosynthetic pathway from squalene to cholesterol. In other hands, the 2-labelled mevalonates have recently been instrumental in the discovery of a new intermediate stage on that pathway.

A duty of the Pedler lecturer is to speak of future developments, as well as of results achieved. Accordingly, I shall discuss some work still incomplete, which endeavours to use asymmetric labelling to look for organisation in systems of particle-bound enzymes. When a homogenate is made from yeast or liver cells, the 'soluble' fraction can support sterol biosynthesis from mevalonate only as far as farnesyl pyrophosphate: the remaining stages occur on particle-bound enzymes. It is perhaps no coincidence that at this point the intermediates also cease to be water-soluble. The question then arises: is the sequence of enzymes organised, in the cell, in such a way that the product of one enzyme passes to the active centre of the next enzyme in a spatially controlled manner, or does it find the next enzyme after wandering at random?

The biosynthesis of squalene provides a possibly unique opportunity for examining this question, and this year Hassan Etemadi made the first experiments.[24] Our earlier work had shown that the process whereby a molecule of squalene is synthesised from two molecules of farnesyl pyrophosphate—outwardly, the construction of a whole from two equal halves—is asymmetric in that an *S* hydrogen atom in one, and only one, of the farnesyl moieties is replaced by a hydrogen originating from the 4*S* position of a reduced nicotinamide-adenine dinucleotide phosphate. The other farnesyl moiety suffers no change, except an inversion of configuration when the new carbon–carbon bond is formed (Scheme 11).

It follows that if the squalene is biosynthesised from farnesyl pyrophosphate labelled in the 1*S* position, a position corresponding to the 5*S* position in mevalonate, or if ordinary farnesyl pyrophosphate is used but labelled hydrogen is put in from the NADPH, the squalene will be labelled in one half of the molecule and not in the other. If this squalene is passed on to the next enzyme—presumably the oxidase forming squalene epoxide[25]—in a spatially defined manner, then one or other of these halves will suffer oxidation; but if the migration is at random, both halves will be oxidised equally: no isotope effect could conceivably

[23] J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, Y. Shimizu, S. Ichii, E. Forchielli, and E. Caspi, *J. Amer. Chem. Soc.*, 1965, **87**, 3224.
[24] A. H. Etemadi, G. Popják, and J. W. Cornforth, *Biochem. J.*, 1969, **111**, 445.
[25](a) E. J. Corey, W. E Russey and P. R. O. de Montellano, *J. Amer. Chem. Soc.*, 1966, **88**, 4750; (b) E. E. van Tamelen, J. D. Willett, R. B. Clayton, and K. E. Lord, *ibid.*, p. 4752.



Scheme 11 Biosynthesis of lanosterol from farnesyl pyrophosphate

operate to favour one half or the other. In any subsequent enzymic stage, no random acceptance of the substrate is possible since it is now asymmetric in the ordinary sense. Therefore, the distribution of label between the positions 11 and 12 of any steroid product from the squalene is a direct index of the randomness of squalene transfer: if labelling at these two positions is not equal, then transfer is not random.

We used a pig liver homogenate prepared according to Tchen and Bloch[26] to make lanosterol from mevalonate, lightly labelled with ^{14}C, in the presence of labelled NADPH, generated continuously from 1-tritiated glucose-6-phosphate on the related dehydrogenase. The tritiated lanosterol was carefully freed from accompanying dihydrolanosterol (which would probably have additional tritium in the side-chain), diluted with unlabelled material, hydrogenated and acetylated. The dihydrolanosteryl acetate was oxidised to the 7,11-dione by chromic acid in acetic acid. Control experiments showed that this procedure caused no exchange of hydrogen with the medium. This oxidation necessarily removed all

[26]T. T. Tchen and K. Bloch, *J. Biol. Chem.*, 1957, **226**, 921.

hydrogen from the 11-position. Hydrogen at position 12 was then removed by base-catalysed enolisation (sodium methoxide in methanol). The $^{3}H/^{14}C$ ratio of dihydrolanosteryl acetate was compared with those of enedione before and after the alkaline treatment.

In the event, we found that rather more tritium was lost in the oxidation than in the subsequent equilibration; the ratio of the two losses was 1·28. However, the experiment was unsatisfactory for two reasons. First, even after the equilibration the product retained some tritium, not removed by repeating the equilibration or by oxidising to a 3,7,11-triketone, for which we could not account. Secondly, we had to use a somewhat damaged preparation of particles in order to get lanosterol at all, and in some of the preparations an appreciable proportion of the radioactivity remained as squalene. This suggested that in the homogenates there may have been some accumulation of a squalene pool, and this of course could be fatal to stereospecific transfer. In the intact rat, on the other hand, squalene is highly transient: Bloch had to dose his animals heavily with squalene in order to recover from them a significant amount of squalene biosynthesised from acetate. It would not, however, be practicable to use in an intact animal the same method of asymmetric labelling with tritium. We are therefore going to synthesise 5*S*-tritiated mevalonate, the sole stereospecifically labelled mevalonic acid that is yet to be made. At the cost of some additional difficulties, which do not seem insurmountable, we hope to use this precursor to examine the question of spatially controlled transfer in the most favourable of environments—the cells of a living animal. Thus for good reasons, accumulated over fifteen years of research, we shall return in this experiment to the crude biological systems of the early days. Perhaps, too, the new labelled precursor may prove useful in other applications: experience certainly suggests that this will be so.

The organic chemist tends to look on asymmetry as something a little extraneous to his scheme of things, as when the successful synthesis of a racemic natural product is incomplete without an often tedious or capricious optical resolution. Perhaps this is why there has so often been speculation on the origin of optical activity as an attribute of life—it seemed to need a special explanation. But there are no racemic molecules; and if the replication characteristic of life is seen in modern terms as an event at the molecular level, it is much more difficult and complicated to imagine either the origin or the continuance of a 'racemic' life than of the lopsided variety that we enjoy. In all of the work I have described, the object has been to detect hidden asymmetry, chirality, stereospecificity, in life processes by superimposing an asymmetry that we were able to perceive. That, too, has not been without enjoyment.