ROBERT ROBINSON LECTURE

The Logic of Working with Enzymes

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I enjoyed being asked to deliver this lecture because I could offer it, in gratitude and affection, to my teacher, colleague, and friend: Robert Robinson. The choice of subject was influenced by the nature of our friendship, which for more than thirty years has been a continuous sequence of differences of opinion; for I felt that if only I could persuade him that there is something in what I have to say, the task of convincing the rest of you would be trivial.

The word 'logic', as used in the title of this lecture, means 'the science and art of reasoning, as applied to a department of knowledge'. Nobody doubts the need for logic in chemistry but, as with virtue, its standards tend to be applied more strictly to the conduct of others. Few of us, I would venture to say, have received formal training in logic; and perhaps there is a feeling that with all the information within reach of us today the need to reason accurately about it is lessened.

But the ease with which it is possible, by many kinds of physical measurement, to obtain detailed information about a chemical substance or a chemical process, has not lightened the task of reasoning correctly from what is observed; it has made it much more complex. Statements like 'the substance is 99% pure by g.l.c.' are often made; and too often they really mean: 'when the substance was introduced into a certain g.l.c. column, in certain conditions of gas flow and temperature, 99% of the integrated response of a recorder to that portion of the substance or its impurities or decomposition products that reached and affected the detecting device in the time available was exhibited as a single peak'. It is even possible to pick a logical hole or two in the more exact statement: for example, it is not unknown for a chemical reaction to occur between an injected substance and the stationary liquid phase in a column, so that a part of what is detected may not be a part of what is put in. Yet the facile, illogical first statement will often be used as the basis of further reasoning. Some of you might like to try your hands at an exact definition of what 'molecular weight 216 by mass spectrometry' really means: and one could multiply examples of illogical deduction from the data of u.v., i.r., mass and n.m.r. spectrometers.

Things are rather worse on the frontier between chemistry and biochemistry, since the work tends to be done by people who do not fully understand the language of both disciplines, let alone the logical limitations of their methods. The purpose of this lecture is to help chemists and biochemists who work in this area to understand more exactly what they are doing, and to describe and assess

more accurately the significance of what they or others have done, or said, or written. This purpose does not strictly observe my terms of reference, which are to review progress in a branch of chemistry; I am more concerned to remove obstacles to further progress.

I want to begin by examining what people mean when they talk about an enzyme. The chemist tends to think of an enzyme as a chemical substance, a protein, able in the right conditions to catalyse a definable range of chemical reactions. It may have a large molecule, it may even be an association of sub-units, but in principle it is a compound substance like any other. The biochemist's view is subtly different, because historically he is conditioned to think of what enzymes do, not what they are. When he says he has isolated an enzyme he may mean no more than that he has a preparation that can catalyse a particular chemical reaction without any side-effects detectable by whatever methods of assay may have occurred to him. The fact that the same preparation may be capable of many other types of catalytic activity need not concern him until it is forced on his attention. And he has an unfortunate tendency to apply similar criteria to substances much simpler than enzymes.

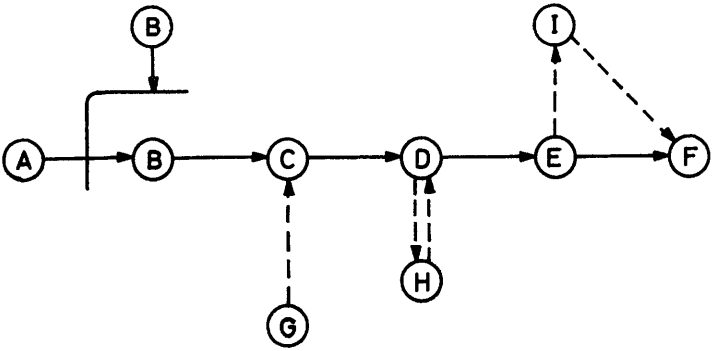
Both approaches have their advantages and limitations, but for most present purposes the biochemist's view is more constructive. Clifford, Donninger, Mallaby, and I recently[1] examined the stereochemistry of the reaction catalysed by what is called 'malic enzyme', or, more systematically, malate dehydrogenase (decarboxylating) E.C. 1.1.1.40. We made our enzyme preparation from chicken livers, and we got results that confirmed similar experiments by Rose[2] on enzyme preparations from pigeon livers and from *Escherichia coli*. Or was this a confirmation? It is rather more likely than not that the catalytically active proteins in the three preparations were not chemically identical substances, but differed to at least some extent in their amino-acid sequences. They all receive the same classification in the Enzyme Catalogue, for they all catalyse the transformation of the same substrates to the same products; and now we know that they all do so with the same overall stereochemistry.

Should we call them the same enzyme? In this state of the art we adopt something like Robert Boyle's approach to the chemical elements: we call them by the same name until we have proof that they differ. Thereafter, we should always distinguish between them; but this is not always done. Moreover, it is logically dangerous to combine results obtained with enzymes from different sources without knowing that one is discussing the same subject; and this is done too often. It will be a long time before we can replace the present functional description of most enzymes by a chemical description; and until that happens we reason about them as chemical substances at our peril.

It is also necessary to be careful about using that word 'enzyme' in the singular. True, there are some enzymes which have been purified enough, and worked with enough, to make it certain that one protein often catalyses one definable chemical

[1] K. Clifford, J. W. Cornforth, C. Donninger, and R. Mallaby, *European J. Biochem.*, 1972, **26**, 401.
[2] I. A. Rose, *J. Biol. Chem.*, 1970, **245**, 6052.

process or sequence. There are also plenty of enzymically catalysed processes that have been attributed to a single enzyme before the multiplicity of the catalysis was revealed. Plenty of people talked about 'squalene oxidocyclase', meaning a particulate preparation that could transform squalene into lanosterol, until squalene epoxide was discovered as an intermediate and the enzyme producing it was differentiated[3] from the enzyme—or enzymes—cyclizing it. Again, we have such entities as the crystalline preparation[4] that synthesizes, in 44 distinguishable operations of five different chemical types, palmitic acid from acetic and malonic acids in the form of their coenzyme A thiolesters. One enzyme or several? Clearly, the functional view is logically safest for the time being.



Scheme 1 *Metabolic pathway, with restricted access, side-track, blind alley, and alternative route*

A good deal of effort has gone into tracing sequences of enzymic reactions whereby substances important to life are synthesized and broken down. As a rule, such sequences are inferred in the first place by putting isotopic labels into possible intermediate products, and showing that the label appears in the substance regarded as the end of that sequence, when the labelled precursor is administered either to an intact organism or to a preparation made by slicing, pressing, pulping or what the Mandarin Shan Tien[5] summarized as 'a variety of less tersely describable discomforts'. It is surprising how many workers have tended to regard this appearance, in yields however miserable, as a sufficient demonstration, although the evidence may be equally compatible with the precursor not being on the principal pathway but merely degradable or otherwise transformable to a true intermediate that comes largely from a different precursor. If all the sterols that have been shown to generate cholesterol when added to liver homogenate were assembled, any pathway that could be drawn between them would look much like the plan of a maze.

[3] S. Yamamoto and K. Bloch, *J. Biol. Chem.*, 1970, **245**, 1668.
[4] D. Oesterhelt, H. Bauer, and F. Lynen, *Proc. Nat. Acad. Sci., U.S.A.*, 1969, **63**, 1377.
[5] E. Bramah, 'Kai Lung's Golden Hours', Richards Press, p. 263.

Equally insufficient is the technique of administering a labelled precursor and looking for labelled substances, other than the end-product, that chemically speaking can be regarded as intermediates. The fallacy in this approach is that such a labelled substance may have become detectable not because it is a normal intermediate, but because it is not. It may not be transformed at all into the final product, or it may be utilized more slowly than normal intermediates are.

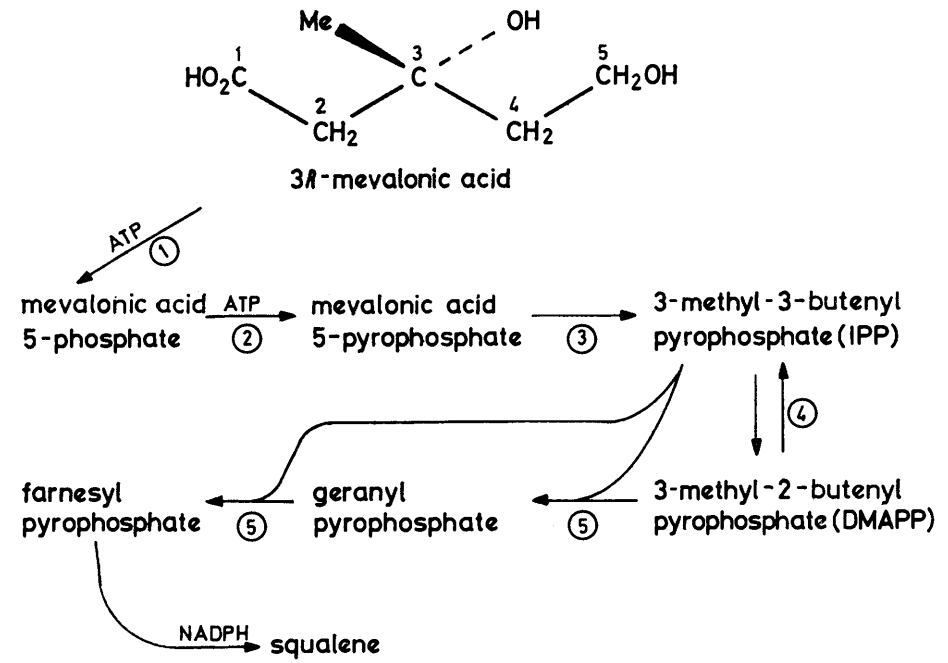
Still another method is that of the 'cold trap' in which a suspected intermediate, unlabelled, is given in large amount to a preparation that is executing the biosynthesis from an earlier, labelled precursor. Bloch[6] used this approach when he fed squalene to rats before administering labelled acetate: in this way, he got radioactive squalene and showed that mice would incorporate radioactivity from this squalene into cholesterol. As evidence that squalene is an intermediate on the normal pathway to cholesterol, this is still insufficient; but in this case it was to be supplemented by much more detailed studies.

The 'hot trap' is logically a better experiment than the 'cold trap', though it is seldom used because it requires more preparation. Here, a suspected intermediate labelled with one isotope is introduced into a system synthesizing the end-product from a precursor labelled with a different isotope. The incorporation of both isotopes into the end-product, and of the precursor's isotope into the intermediate, can be measured in a single experiment.

But suppose that an experiment of this kind reveals no incorporation of label from the precursor into the suspected intermediate, and no incorporation of the intermediate into the end-product; should one conclude that the pathway follows another course? No, not necessarily; if this were true, one could furnish proofs that mevalonic acid is not, in several tissues, an intermediate in the biosynthesis of polyisoprenoids. For a complex biosynthesis may require or use intact elements of the organism's structure, and these may incorporate membranes through which some intermediates cannot pass. A demonstration of this effect is probably seen in the work of Goodwin on the synthesis of carotenoids in intact chloroplasts of bean leaves; labelled mevalonic acid incubated with chloroplasts yielded no labelled carotenoid,[7] though labelled lycopene incubated with similar chloroplasts was incorporated readily into β-carotene.[8] But chloroplasts prepared by non-aqueous techniques will synthesize phytoene from mevalonate.[9]

At Milstead Laboratory we have done a good deal of work on the detailed mechanism (see Scheme 2), as revealed by a study of stereochemical changes, of the enzymic sequence leading from mevalonic acid to squalene.[10] Since I shall be referring later on to a part of this sequence, it is useful to review now the evidence from which it has been inferred.

[6] R. G. Langdon and K. Bloch, *J. Biol. Chem.*, 1953, **200**, 129, 135.
[7] L. J. Rogers, S. P. J. Shah, and T. W. Goodwin, *Biochem. J.*, 1966, **99**, 381.
[8] H. M. Hill and L. J. Rogers, *Biochem. J.*, 1969, **113**, 31P.
[9] J. M. Charlton, K. J. Treharne, and T. W. Goodwin, *Biochem. J.*, 1967, **105**, 205.
[10] G. Popják and J. W. Cornforth, *Biochem. J.*, 1966, **101**, 553.

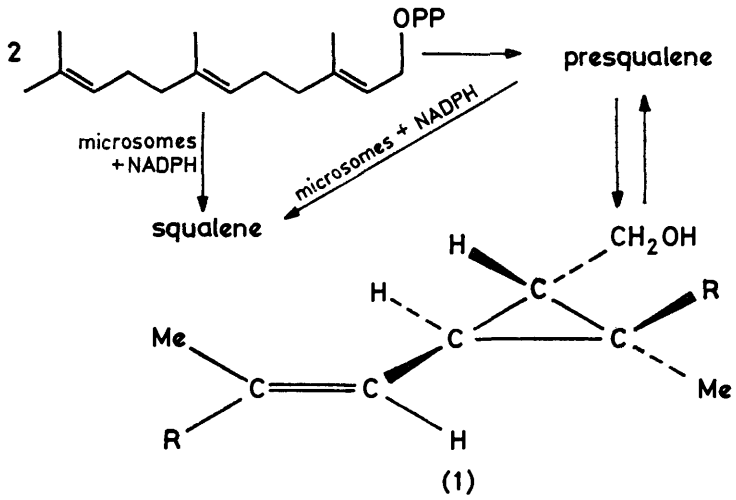


Scheme 2 *Biosynthesis of squalene from mevalonic acid*

It is not too difficult to make a homogenate from mammalian liver which, in the absence of oxygen and the presence of a few co-factors, will convert *R*-mevalonic acid, the biologically active enantiomer, into squalene with a yield not far short of the theoretical. If a soluble fraction is obtained by spinning the same liver preparation, and is further fractionated by precipitation with ammonium sulphate, the mixture of proteins so obtained cannot synthesize squalene but in the presence of ATP can convert mevalonic acid in high yield into farnesyl pyrophosphate. The fine-particulate (microsomal) fraction separable from the same liver preparation will not utilize mevalonic acid but, in the presence of reduced nicotinamide–adenine dinucleotide phosphate (NADPH) and the absence of oxygen, it will convert farnesyl pyrophosphate efficiently into squalene.

In the soluble enzyme fraction, five enzymes (and I am using this word in its functional sense) have been demonstrated and each has been obtained free from the others. Their substrate and co-factor requirements have been explored and their products have been fully identified. The least fully characterized intermediate is geranyl pyrophosphate because it is always transient, being converted into farnesyl pyrophosphate by (so far as is at present known) the same enzymes, in association with one of the same substrates, that were used to synthesize it. No

enzyme has been demonstrated in the liver preparation that will affect mevalonic acid or any of these intermediates in a manner that could be interpreted as an alternative route to polyisoprenoids, though there are phosphatases that can irreversibly hydrolyse the prenyl pyrophosphates, and other enzymes that can degrade the alcohols so formed.



Scheme 3 *Relations between farnesyl pyrophosphate, presqualene, presqualene alcohol* (1), *and squalene* (*R* = *homogeranyl*)

The logical situation with respect to squalene synthesis from farnesyl pyrophosphate is at the present time so interesting that I make no apology for discussing it here. The process, as I have said, requires NADPH, and we have shown[11] that the *pro*-4*S* hydrogen of this reduced nicotinamide is incorporated stereospecifically into one half of the squalene molecule. If the coenzyme is omitted no squalene is formed, but something else accumulates and can be separated. This 'something else', which we shall follow Rilling in calling presqualene, may be a single compound, though proof of homogeneity is at present lacking. When incubated with a fresh microsomal preparation supplied with NADPH, it forms squalene. When, instead, it is incubated with a phosphatase or cleaved by lithium aluminium hydride, an alcohol (1) can be isolated which certainly has the structure shown in Scheme 3; this is confirmed both by analytical procedures[12,13] and by synthesis,[14–16] and there is good evidence that the relative

[11] J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Ryback, and G. J. Schroepfer, jun., *Proc. Roy. Soc.*, 1966, **B163**, 436.
[12] W. W. Epstein and H. C. Rilling, *J. Biol. Chem.*, 1970, **245**, 4597.
[13] J. Edmond, G. Popják, S.-M. Wong, and V. P. Williams, *J. Biol. Chem.*, 1971, **246**, 6254.
[14] L. J. Altman, R. C. Kowerski, and H. C. Rilling, *J. Amer. Chem. Soc.*, 1971, **93**, 1782.
[15] R. V. M. Campbell, L. Crombie, and G. Pattenden, *Chem. Comm.*, 1971, 218.
[16] R. M. Coates and W. H. Robinson, *J. Amer. Chem. Soc.*, 1971, **93**, 1785.

stereochemistry is also as shown, though the absolute stereochemistry is still debated. When the natural or synthetic alcohol is phosphorylated by trimethylamine phosphate–trichloroacetonitrile, a preparation can be separated which has chromatographic properties exhibited also by presqualene, and which yields squalene rather slowly on incubation with NADPH and yeast microsomes.[13,14] Presqualene is therefore formulated as the pyrophosphate of the alcohol (1).

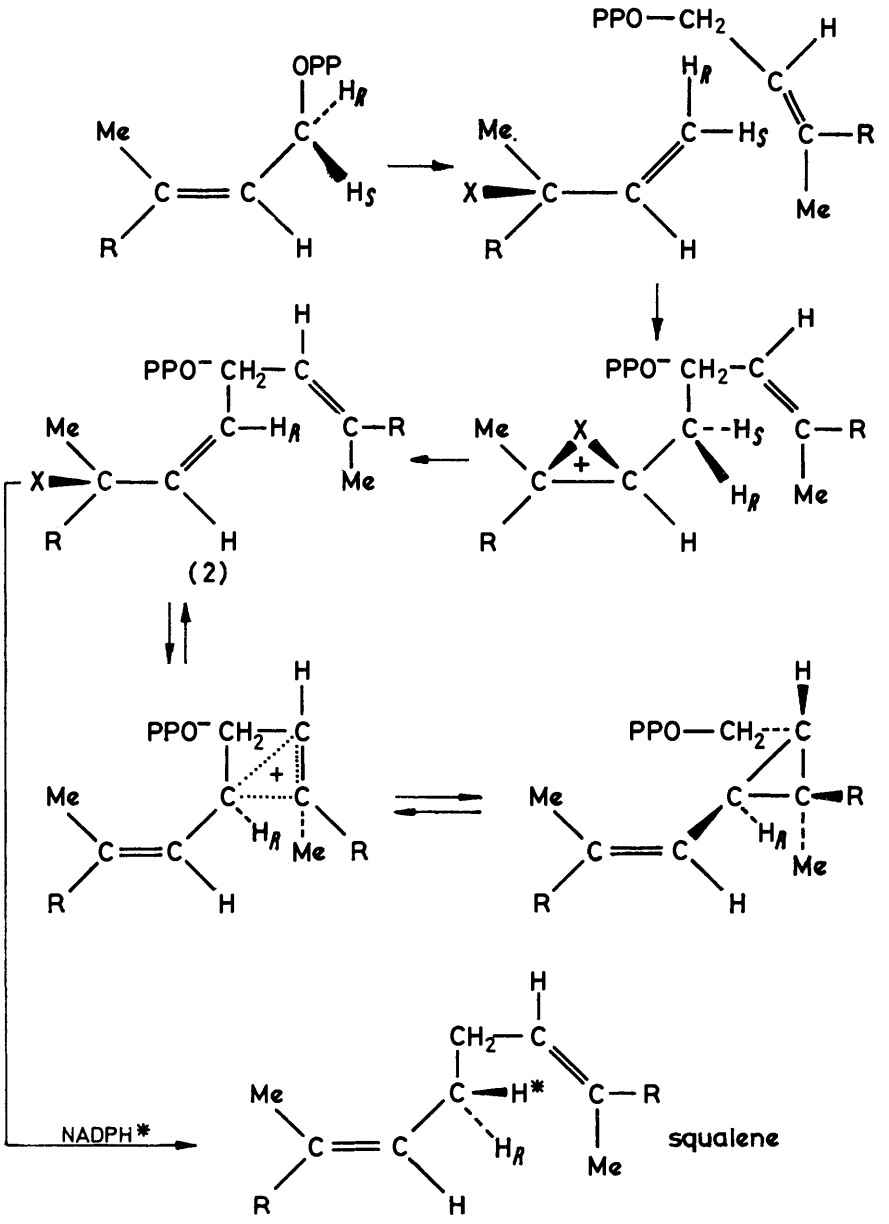
Accepting this not conclusively proved assumption, is presqualene an intermediate on the normal pathway to squalene? If the system synthesizing squalene from farnesyl pyrophosphate consists of at least two enzymes—one to make presqualene, one to reduce presqualene to squalene—then the presumption is strong that it is. But if the squalene synthetase is one enzyme, then presqualene may represent a stable product formed from a true intermediate by processes that are at least to some extent reversible when presqualene is bound again to the enzyme in the presence of NADPH.

It turns out to be possible (see Scheme 4) to formulate an intermediate which (*a*) can go directly to squalene by reaction with NADPH, and (*b*) can give presqualene by processes that are chemically unremarkable and predictably reversible.

The formation of the intermediate (2) by a solvolysis of farnesyl pyrophosphate in the presence of a nucleophilic group X, followed by alkylation of the rearranged double bond by a second molecule of farnesyl pyrophosphate leading to a cation from which a proton is lost, is in accordance with an earlier hypothesis. So also is the reduction of this intermediate to squalene by NADPH.[17] If the latter is missing, reversible rearrangements of the homoallylic cation formed by elimination of X^- can result in reattachment of the pyrophosphate anion originally eliminated from the second molecule of farnesyl pyrophosphate: this anion can be presumed to be available in the correct position for reattachment if it is still bound to the enzyme. This process would lead to formation of presqualene; and reattachment of presqualene to the enzyme in the presence of NADPH could lead naturally to the regeneration of the intermediate (2) and its consequent reduction to squalene.

The argument shows, I believe, that the new mechanism is logically possible. To distinguish between this mechanism and several others that have been proposed for the normal formation of squalene *via* presqualene is not easy. All one can say is that the presqualene formed by the new mechanism must have suffered cleavage and re-formation of its C—O bond; for formation of presqualene by the other mechanisms this is not necessary. A proof that this C—O bond has broken and re-formed would therefore be suggestive though not quite conclusive. It should be mentioned that the stereochemical arrangement of the four distinguishable hydrogen atoms at the two central methylene groups of squalene, which has been fully determined for the complete enzyme system,[17] has not been checked for squalene derived from isolated presqualene. For the new

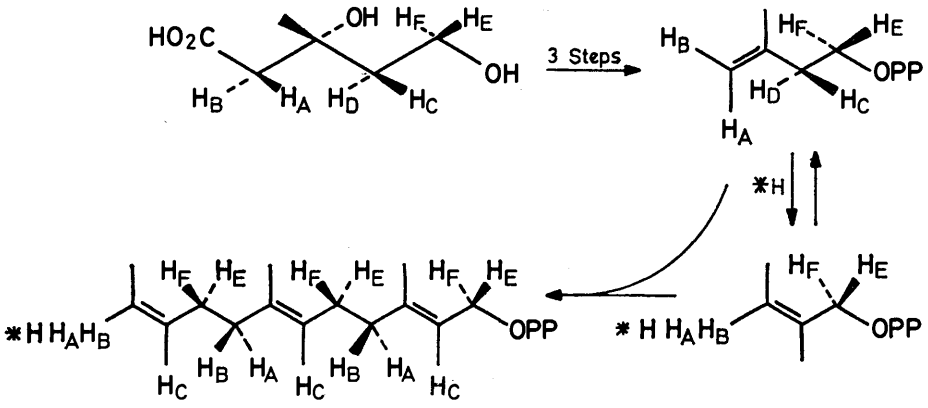
[17] J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popják, *Proc. Roy. Soc.*, 1966, **B163**, 492.



Scheme 4 *Hypothetical relationship of squalene and presqualene*

mechanism, this arrangement could rather unfortunately be the same whether the excursion to presqualene is taken or not.

Having said (I hope) enough about the perils of postulating intermediates, I want to continue by describing a piece of work that Clifford, Mallaby, Phillips, and I recently executed[18] on the stereochemistry of an enzymic process. It happens to be one of those experiments in which one does apparently simple things for complicated reasons, and from which it is a complicated process to draw apparently simple conclusions.



Scheme 5 *Stereochemistry of farnesyl pyrophosphate biosynthesis*

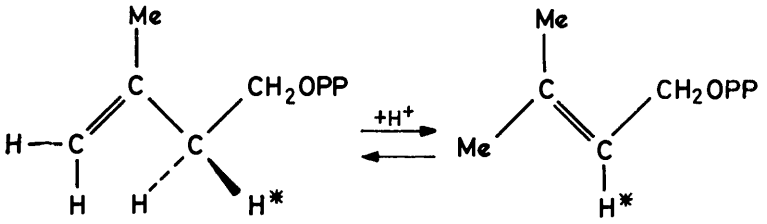
In the assembly of a molecule of farnesyl pyrophosphate from three molecules of *R*-mevalonic acid, chemical changes occur at all but one of the nine methylene groups. The exception is of course C-1 of the pyrophosphate, which is still (or so we believe) bound to the same four atoms that were connected to it in the mevalonic acid molecule from which it came. Of the remaining eight methylene groups, Popják and I with our co-workers were able to trace the stereochemistry of the overall changes to seven of them. This was done by labelling in a stereospecific manner each of the three methylene groups in mevalonic acid with a hydrogen isotope, and developing methods for demonstrating the presence, absence, or absolute stereochemistry of the isotope in farnesol.

In one of these experiments, both the *pro*-4*R* and the *pro*-4*S* positions in mevalonic acid were labelled with deuterium and with tritium. When 4*S*-mevalonate so labelled was incorporated into farnesyl pyrophosphate by soluble liver enzymes, the derived farnesol contained no hydrogen isotope. When 4*R*-mevalonate was the precursor, all the hydrogen isotope was retained in farnesol.[17] When the tritiated mevalonates were used as precursors of the rubber hydrocarbon in *Hevea* latex, the reverse was true: 4*S*-mevalonate retained all tritium,

[18] K. Clifford, J. W. Cornforth, R. Mallaby, and G. T. Phillips, *Chem. Comm.*, 1971, 1599.

4*R*-mevalonate lost it all.[19] We suggested[10] that the mechanism of the reaction leading to hydrogen elimination could be the same in both cases, a different orientation of the substrate on the enzyme leading to *cis*-double bonds and 4*R*-elimination in rubber biosynthesis, and to *trans*-double bonds and 4*S*-elimination when farnesyl pyrophosphate is formed *via* dimethylallyl pyrophosphate and geranyl pyrophosphate.

Later, Hemming[20] and collaborators found for a number of polyprenols, alcohols of the general formula $H[CH_2C(Me)=CHCH_2]_nOH$ containing some *cis*- and some *trans*-double bonds, a numerical coincidence between the number of *cis*-double bonds indicated by n.m.r. measurements and the number of tritium atoms retained when 4*S*-mevalonate was the precursor. So far as the observations go, they support an extension of our hypothesis to this class of polyisoprenoids; but there the evidence ends. This looks to be a rather shaky foundation for the now rather general assumption that if the hydrogen of a *cis*-double bond in a polyisoprenoid originates from the *pro*-4*R* position of mevalonate, the double bond must originally have been *trans*. It may be so; but one would like more proof and, until then, better logic.



Scheme 6 *Isopentenyl pyrophosphate isomerase*

Our own work does show, however, that the liver enzyme isomerizing isopentenyl pyrophosphate to dimethylallyl pyrophosphate eliminates what was a *pro*-4*S* hydrogen of mevalonate. But this is not the only methylene group of mevalonate involved in this isomerization: the 4-methylene group of isopentenyl pyrophosphate, which was the 2-methylene group of mevalonate, acquires a proton from the aqueous medium and becomes a methyl group (Scheme 6). Since the two faces of the plane-trigonal $=CH_2$ group in isopentenyl pyrophosphate are spatially distinguishable and can be called the *re* and *si* sides according to Hanson's convention,[21] addition of this proton may *a priori* be from either side; and the stereochemical relation between the added and abstracted protons is of interest in discussing possible mechanisms for the enzymic reaction. The direction

[19] B. L. Archer, D. Barnard, E. G. Cockbain, J. W. Cornforth, R. H. Cornforth, and G. Popják, *Proc. Roy. Soc.*, 1966, **B163**, 519.
[20] F. W. Hemming, *Biochem. Soc. Symp.*, 1970, **29**, 105.
[21] K. R. Hanson, *J. Amer. Chem. Soc.*, 1966, **88**, 2731.

of addition of the proton was the one problem that Popják and I could not solve in our investigations of the stereochemistry of squalene biosynthesis.

The enzyme—and again I am using the word in the biochemical sense—isopentenyl pyrophosphate isomerase has been partially purified from baker's yeast[22] and from pig liver.[23,24] Its properties from both sources are similar. It catalyses the reversible interconversion of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the concentration at equilibrium being about 9 : 1 in favour of DMAPP. Catalysis by the enzyme seems to require the presence of at least one sulphydryl group, since iodoacetamide brings about rapid irreversible inactivation.

Since the overall reaction catalysed by the enzyme is reversible, all the stages into which it can be divided are also reversible. Let us consider what such stages could be. We have to consider four reagents (DMAPP, IPP, and two protons) as well as the enzyme, if we regard the magnesium and/or manganous ions that are required for maximum activity as part of the enzyme. Taking the direction IPP → DMAPP, it is clear that a molecule of IPP must become bound to the active site of the enzyme [step (*a*)]. Here it encounters a proton, which will not be free but part of a water molecule or some other acidic group. The binding of this proton or proton source to the enzyme can be recognized as another possible step, which we can call step (*b*) without implying that it follows step (*a*). Step (*c*) is the addition of the proton to IPP or to some species related to IPP. Step (*d*) is the hypothetical formation of a covalently bound intermediate between IPP, or a derived product, and the enzyme. Step (*e*) is the dissociation of this intermediate. Step (*f*) is elimination of the proton from C-2 of IPP, step (*g*) is the merging of this proton with water of the aqueous medium, and step (*h*) is dissociation of DMAPP from the enzyme—a total of eight entirely reasonable reversible processes, each with its own equilibrium and velocity constant.

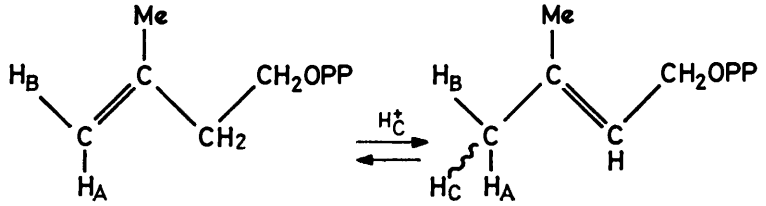
The only mechanism for the isomerization that is claimed to have experimental support is that of Shah, Cleland, and Porter,[23] who postulated that the isomerization proceeds by way of a covalent enzyme–substrate compound, 'possibly a thioether'. It is instructive to examine the description, headed 'Isolation of Enzyme-bound Intermediate', of the experiments on which this postulation is based. Radiaoctive IPP labelled with ^{14}C was incubated for five and for seven seconds at unspecified concentrations with a partially purified preparation of the enzyme from pig liver. Protein was then precipitated by addition of perchloric acid, centrifuged, and washed five times with dilute acetic acid. The final washings were non-radioactive. The precipitate was then dissolved in alkali and the radioactivity, about 0.3% of that originally added, was measured. These experiments, it was said, 'clearly establish that a covalent bond is formed between isopentenyl pyrophosphate and protein'.

I have already mentioned the perils of postulating intermediates, and it has to be acknowledged that these authors were exceptionally courageous. But their

[22] B. W. Agranoff, H. Eggerer, U. Henning, and F. Lynen, *J. Biol. Chem.*, 1960, **235**, 326.
[23] D. H. Shah, W. W. Cleland, and J. W. Porter, *J. Biol. Chem.*, 1965, **240**, 1946.
[24] P. W. Holloway and G Popják, *Biochem. J.*, 1968, **106**, 835.

choice of perchloric acid to precipitate protein was a little unfortunate: allylic pyrophosphates such as DMAPP are notoriously sensitive to acids and the carbonium ions generated by acid cleavage are reactive enough to combine indiscriminately with nucleophilic groups that happen to be around. Thus even if the radioactivity in the precipitated protein were covalently bound, this is not evidence that its presence is due to normal catalytic action at the active centre; and failure to wash a small amount of radioactivity from an amorphous precipitate could, to say the least, be attributed to causes other than covalent binding.

In postulating a covalent intermediate, these authors rejected a concerted mechanism for the isomerization—that is, a mechanism in which a proton is added and another proton abstracted without a recognizable intermediate stage. This rejection was based on an experiment in which tritiated water was added to a pre-equilibrated solution of DMAPP, IPP, and the enzyme; and it was stated that nearly all of the tritium initially entering IPP was attached to C-4 rather than to C-2. We have shown elsewhere[25] that the experimental results reported cannot be explained on the assumptions made by Shah, Cleland, and Porter in arguing against a concerted mechanism, and we therefore approached our own problem without preconceptions about mechanism.



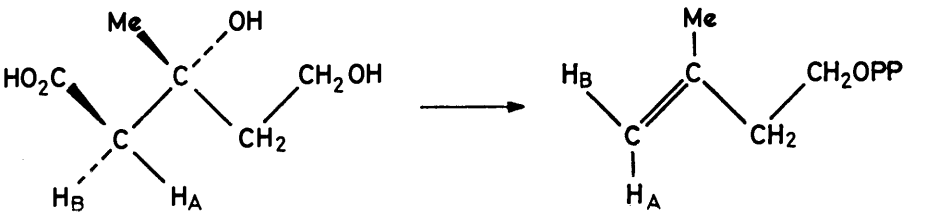
Scheme 7 *Generation of chiral methyl group on isomerase*

Now the isomerization of IPP to DMAPP is one of those enzymic reactions in which a methyl group is generated from a methylene group. If such a reaction is stereospecific, an asymmetric methyl group can be formed. One then needs IPP in which the two hydrogen atoms at C-4 are two different isotopes of hydrogen, each isotope occupying only one specific geometrical position of the two possible. The isomerization must then be carried out in a medium containing the third hydrogen isotope, when chiral methyl groups will be generated (Scheme 7), and the absolute configuration of these groups will be determined by the geometry of the hydrogen isotopes in IPP and the direction of addition of the third isotope.

Professor Hermann Eggerer and I, with our collaborators at Milstead and in München, have reported on the configurational assay of asymmetric methyl groups that can be brought into the form of acetic acid,[26,27] and Arigoni and Rétey[28]

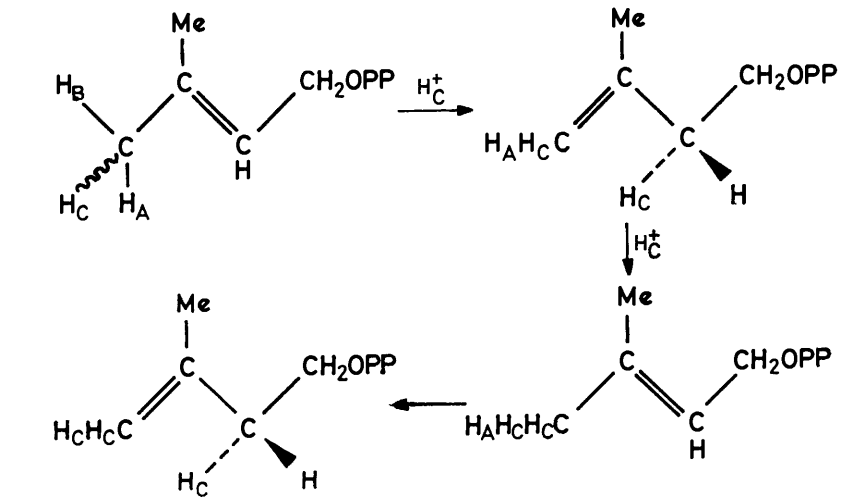
[25] J. W. Cornforth, K. Clifford, R. Mallaby, and G. T. Phillips, *Proc. Roy. Soc.*, 1972, **B182**, 277.
[26] J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature*, 1969, **221**, 1212.
[27] J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *European J. Biochem.*, 1970, **14**, 1.
[28] J. Lüthy, J. Rétey, and D. Arigoni, *Nature*, 1969, **212**, 1213.

independently devised a similar method. Thus if methyl groups generated in the way I have just described can be made, without loss of chirality, into acetic acid, their predominant absolute configuration can be determined too.



Scheme 8 *Stereospecific C-4-labelling of isopentenyl pyrophosphate*

The first part of the problem—to obtain IPP labelled stereospecifically at C-4 by a hydrogen isotope (Scheme 8)—has already been solved: 2*R*[2-$^2H_1$]-mevalonate has been shown[29] to generate *Z*-[4-$^2H_1$]IPP by the sequential action of three enzymes from liver, whereas 2*S*[2-$^2H_1$]mevalonate produces the *E*-isomer. It can be taken for granted that the same applies to the stereospecifically labelled 2-tritiated mevalonates which are now commercially available. The isomerization of the IPP would then have to be executed in a deuterium oxide



Scheme 9 *Destruction of chiral methyl group by isomerase*

[29] J. W. Cornforth, R. H. Cornforth, G. Popják, and L. Yengoyan, *J. Biol. Chem.*, 1966, **241**, 3970.

medium; earlier work of Rilling and Bloch[30] on the synthesis of squalene from mevalonate in deuterium oxide shows that this is possible in principle, since the isomerization is a necessary step in the enzymic sequence leading from mevalonate to squalene.

A complication remains—the reversibility of the reaction on the isomerase. Since the methyl group in DMAPP must be presumed capable of free rotation in solution, and perhaps even while DMAPP or a covalently bound intermediate is bound to the enzyme, regression to IPP can result in the loss of any one of its three hydrogens. If a normal hydrogen isotope effect is operative in this loss, there is not even one chance in three that chiral DMAPP would be regenerated from this IPP (Scheme 9). Neither of the two other possible species is chiral, and one of them—the predominant one for a normal isotope effect—contains tritium that would desensitize the assay.

If the conclusion reported by Shah, Cleland, and Porter[23] were valid—that IPP on the enzyme exchanges hydrogen at C-4 much faster than it isomerizes to DMAPP—our experiment had little chance of success. But if exchange of hydrogen at C-4 were a consequence of multiple isomerizations, it would obviously be prudent to remove DMAPP continuously from participation in the equilibrium; and fortunately the means to do this lay ready to hand—instead of using separated isomerase we used the soluble enzyme fraction from pig liver that is able to convert mevalonate into farnesyl pyrophosphate. The condensing enzyme assembling geranyl pyrophosphate from one molecule each of DMAPP and IPP would then at least keep the concentration of DMAPP at a lower level than it would otherwise attain. Moreoever, mevalonate stereospecifically tritiated at C-2 could then be used without the need to isolate the derived IPP.

The simple sequence of operations was then (1) introduce each stereospecifically labelled 2-tritiated mevalonate into a soluble enzyme fraction from pig liver, prepared in deuterium oxide, and supplemented with magnesium and manganous ions and with adenosine triphosphate for the phosphorylations, (2) hydrolyse the resulting farnesyl pyrophosphate enzymically to farnesol and separate the latter, (3) convert farnesol into acetic acid containing methyl groups originating, without intermediate hydrogen exchange, from that one methyl group of farnesol which is derived from C-2 of mevalonate, (4) assay that acetic acid for chirality by the method already devised. If the acetic acid is chiral, its chirality is determined by the direction of addition of the proton to IPP on the isomerase.

Let us now examine this conclusion for hidden assumptions. The first and most important is that the chemical reaction on the isomerase is the only process generating that methyl group in farnesol. The evidence for this is still to some extent negative—the pathway to farnesol from mevalonate is clearly demonstrated, and no other pathway has been detected. The second assumption, granting the first, is that stereospecifically labelled mevalonates do generate IPP stereospecifically labelled as we have previously concluded. The evidence for this is based on

[30] H. C. Rilling and K. Bloch, *J. Biol. Chem.*, 1959, **234**, 1424.

interpretations of stereoselective chemical (not enzymic) reactions, and forms part of the general fabric of organic stereochemistry.

All one can say there (as in most similar cases) is that organic chemists in general have been satisfied that no alternative explanation is reasonable. The third assumption, granting the first two, is that C-2 of mevalonate generates only one methyl group in farnesol. This is supported by numerous degradations of farnesol and of squalene, interpreted by the ordinary structural generalizations of organic chemistry. The fourth assumption is that our assignments of chirality to acetic acid from the results of our assay are correct; this rests again on the general correctness of organic stereochemical theory. It is not necessary to add that we are also assuming our experimental results to be free from error, for anyone may now repeat the experiments; and the other assumptions are at least supported by evidence.

We went, then, to our experiment. A soluble enzyme fraction from pig liver was prepared in deuterium oxide by dialysis against heavy water. Though not so active as the original preparation, it was able to produce farnesol, after hydrolysis of farnesyl pyrophosphate by alkaline phosphatase, from mevalonate in satisfactory yield. The farnesol was extracted with pentane and purified by thin-layer chromatography. Farnesol so synthesized from [2-^{14}C]mevalonate when analysed by gas–liquid chromatography showed only one radioactive peak, and that coincident in retention time with the peak given by *trans,trans*-farnesol. Thus (sticking to logic) if there were any impurities derived from C-2 of mevalonate they were extractable with pentane, ran with farnesol on thin-layer chromatography, and were either involatile or ran with farnesol on gas–liquid chromatography.

The mass spectrum of farnesol so synthesized and purified was of particular interest to us as indicating the extent of multiple isomerizations or other causes of hydrogen exchange; and additional evidence for the essential homogeneity of our farnesol was thereby provided, the mass spectrum showing no peak that could not readily be attributed to deuteriated farnesols by comparison with the spectrum of the unlabelled alcohol (Figure).

The mass spectrum was that of a mixture of deuteriated farnesols. Very little undeuteriated farnesol was present: this spoke for the high deuterium content of the aqueous medium and the absence of endogenous farnesol derivatives in the enzyme preparation. The most prominent molecular ion was that of monodeuteriofarnesol, but it was accompanied by ions diminishing in intensity up to a mass/charge ratio corresponding to heptadeuteriofarnesol. This is a result from which the occurrence of multiple exchange on the isomerase could be inferred: complete exchange of the C-4 hydrogens of IPP would lead to heptadeuteriofarnesol. But the monodeuterio-species was still the most abundant, and this species can only arise from molecules that have suffered no exchange, though no doubt their number is swelled slightly by molecules in which a deuterium originally added to IPP was removed and replaced, necessarily without change in the chirality of the resulting methyl group if the process is stereospecific, by another deuterium.

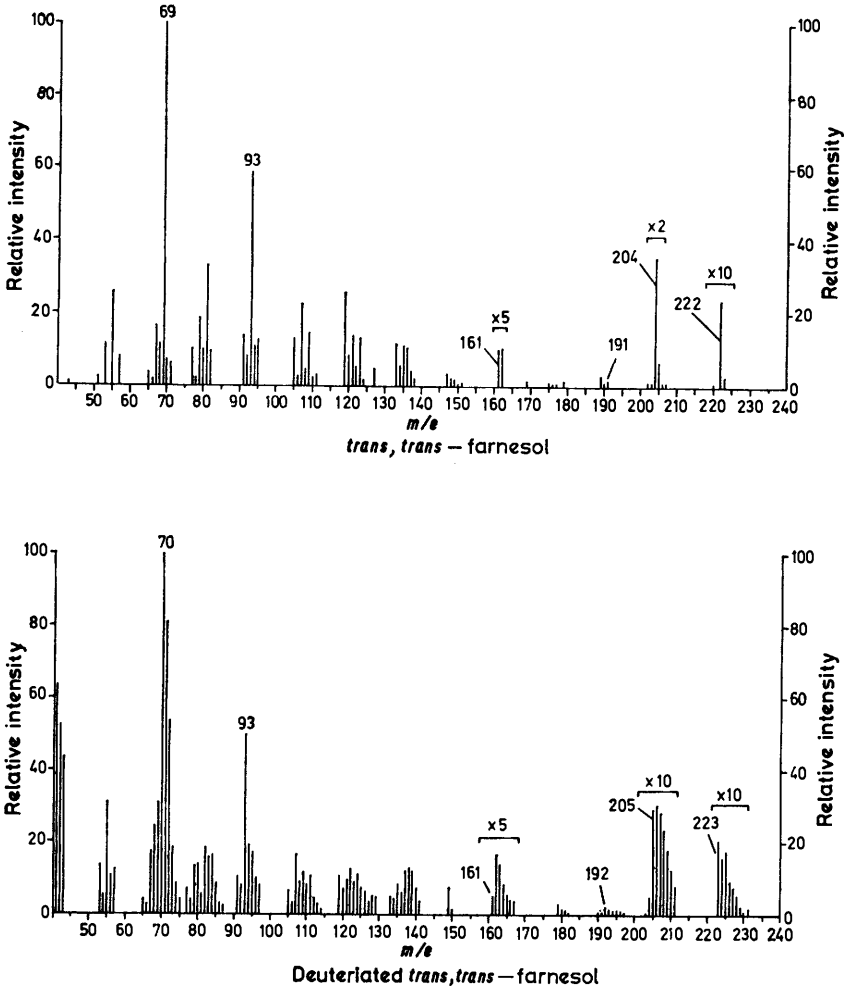
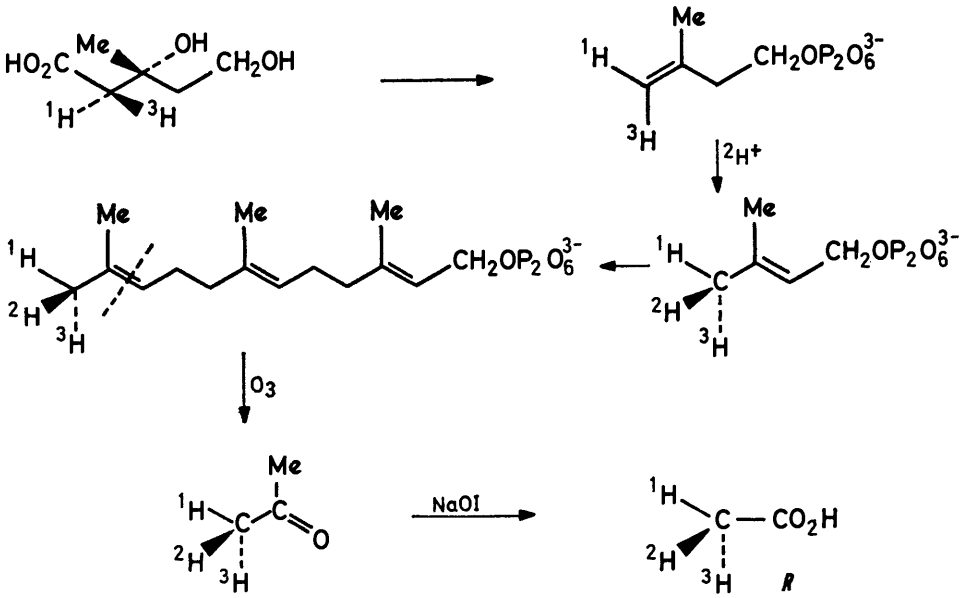


Figure *Comparison of mass spectra of normal and deuteriated* trans,trans-*farnesol*

The base peak in the mass spectrum of farnesol (at least in our spectrometers) is at a mass/charge ratio of 69. We attribute this peak to a dimethylallyl cation formed by cleavage of the terminal C_5 unit, because (*i*) this cleavage should be energetically favourable, (*ii*) no other part of the molecule can give such an ion by simple cleavage, (*iii*) if the ion originated by multiple cleavage *plus* rearrangement of hydrogen from another part of the molecule, it is not conceivable that so few ions of *m/e* 69 should appear in the spectrum of the deuteriated farnesol preparation. This spectrum shows in fact peaks at 70, 71, and 72, with the peak at 70,

corresponding to a monodeuteriated dimethyllallyl cation, the most prominent. The smaller contribution from 71 and 72 confirms the conclusion from the molecular ions: most IPP molecules rearrange to DMAPP without exchange of their C-4 hydrogen on the enzyme. Thus the experiment to determine the direction of hydrogen addition was 'on'.

The enzymic incubations were accordingly repeated with the two stereospecifically labelled 2-tritiated mevalonates, supplemented with [2-^{14}C]mevalonate to provide an internal standard for measurements of tritium content. Here, we needed only to prepare approximately one micromole of each purified farnesol for the mevalonates were of high specific radioactivity—*ca.* 60 μCi μmol^{-1} for tritium. But because the assay of asymmetric methyl groups ignores molecules containing no radioactivity, we were able to dilute the farnesol to the large bulk of 45 μmol with pure *trans,trans*-farnesol before chemical degradation.



Scheme 10 *Conversion of* 2*R*,3*R*-[2-$^{3}H_1$] *mevalonate into* R-*acetate*

No plausible method for obtaining the terminal methyl group of farnesol as the methyl group of acetic acid occurred to us other than routes *via* acetone. The fact that it would be impossible to convert acetone into acetic acid containing only the methyl group originating from C-2 of mevalonate was of no concern: the other methyl group is unlabelled and non-radioactive acetic acid does not affect the subsequent assay. It was important, though, to make sure that the intermediate acetone did not suffer hydrogen exchange by enolization; and this need dictated the experiment. Farnesol was ozonized in ethyl chloride at − 70 °C.

Potassium tri-iodide solution, for both the reduction of ozonides and the subsequent oxidation, was added below room temperature, and sodium carbonate was immediately put in. The iodoform reaction proceeded slowly but smoothly, and acetic acid was recovered (after removal of iodine, iodoform, and iodide ion) by steam distillation and was purified by partition chromatography. Overall yields were 50—70%.

It may seem odd to avoid hydrogen exchange in acetone by using a cleavage that depends on enolization. The point is that in base-catalysed iodination of acetone, enolization is rate-limiting and halogenation is fast. Thus, any enolization involving a hydrogen on a chiral methyl group would be followed by halogenation of that group: and since iodination is irreversible in basic conditions that group could not thereafter become a methyl group of acetic acid. The logic of this conclusion seemed impeccable—but because we could check it, we did. Unlabelled farnesol was ozonized; potassium tri-iodide in deuterium oxide was added; and the mass spectrum of the 4-bromophenacyl ester of the resulting acetic acid was examined for indications of excess deuterium content: no enrichment of deuterium was found.

From the evidence of the mass spectrum of our deuteriated farnesol, we could then assume that most of the tritiated molecules in our acetic acids contained a deuterium and a hydrogen atom on the same methyl group; these would be accompanied by a proportion of molecules containing a tritium and two deuterium atoms. Some methyl groups which had suffered exchange of tritium for deuterium on the enzyme were no doubt present in the acetic acid, but for the purposes of the assay they had vanished.

The assay of acetic acid for chirality consists of a composite first step in which three enzymes consecutively transform acetate to acetyl phosphate, to acetyl-coenzyme A, and finally to *S*-malate by condensation with glyoxylate on malate synthase. *S*-Malate is isolated and incubated with fumarase; after equilibrium between *S*-malate and fumarate has been established for some time, the mixture of acids is separated and counted for radioactivity.

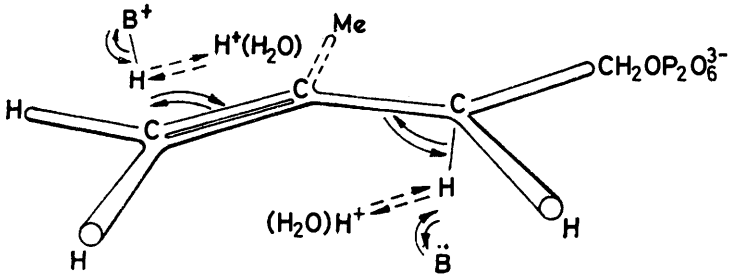
Table $^{3}H : ^{14}C$ *ratios in the sequence mevalonate → fumarate*

Substance	*From 2R,3R-* [2-$^{3}H_1$]MVA	*From 2S,3R-* [2-$^{3}H_1$]MVA
Mevalonyl benzhydrylamide	14.04	8.17
Farnesol	11.78	7.21
Acetate	12.2	7.71
Malate	10.25	6.60
Malate-fumarate after fumarase	6.50	2.41

Now irrespective of what happens in this complex sequence, it is an observed fact that when *R*-acetic acid is put through the procedure the *S*-malate retains 76% of its carbon-bound tritium on incubation with fumarase, whereas malate from *S*-acetic acid retains 24% and malate from tritiated acetic acid that is not chiral retains 50%. The only hidden assumption in this assay is, as already

stated, that the absolute configurations of synthetic *R*- and *S*-acetic acid, both made by two independent groups using different procedures, were correctly assigned.

Ratios of radioactivity at the initial, intermediate, and final stages are shown in the Table. In the event we found that malate from acetate from farnesol from 2*R*-[2-$^3H_1$]mevalonate retained 63.4% of its tritium with fumarase, and malate similarly derived from 2*S*-[2-$^3H_1$]mevalonate lost 63.5% of its tritium. These figures do not correspond to optically pure acetates but to acetates in which about one-half of the tritium is in molecules of a particular chirality, the other half being in non-chiral acetate. This is in no way an indication that the addition of a proton to IPP on the isomerase is not completely stereospecific; multiple isomerizations indicated by the mass spectra of the deuteriated farnesols would lead to the presence of non-chiral acetate in about this proportion.



Scheme 11 *Hypothetical concerted mechanism for isopentenyl pyrophosphate isomerase*

The completed experiment thus shows that, in the isomerization of IPP to DMAPP, proton addition as well as proton removal is a stereospecific process. The relationship between the stereochemistry of the two processes is that which would be expected of a concerted reaction, in which the new double bond is formed as the old one is saturated (Scheme 11). This does not at all prove that such a mechanism is operative; it does, however, mean that if a covalent intermediate exists in which C-3 of the pyrophosphate is bound to the enzyme, it is produced by a *cis* addition and decomposed by a *trans* elimination when the isomerization is running in one direction, with *trans* addition followed by *cis* elimination characterizing the reverse sequence. Summing up: we have, I submit, captured here a small piece of the truth about an enzyme by having due regard to the logical requirements of our experiment and the logical limitations on our conclusions.

There is said to have been a performance of the opera 'Don Giovanni' in which the Don's climactic descent to nether regions was checked when he stuck halfway through the trapdoor. A member of the audience—it might be unsafe to

guess at his nationality—cried 'May all the saints be praised! Hell's full at last!' I shall be well rewarded if some of my auditors or readers are led to question their conclusions, however attractive; and perhaps to avoid some trapdoors through which the significance of their experiments may suddenly disappear.