

ASYMMETRY AND ENZYME ACTION

Nobel Lecture, December 12, 1975

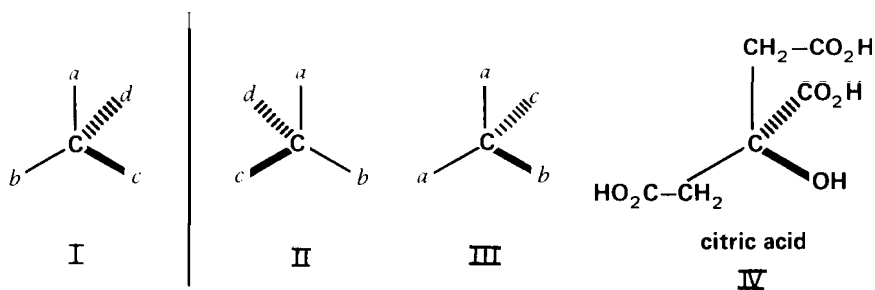
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It must, I think, be rare to be rewarded so generously for work that was so purely a pleasure in planning and execution. I shall try, in return, to impart some of that pleasure today.

In 1948, a short but historic note by Alexander Ogston appeared in the scientific magazine *Nature*, demonstrating the importance of a particular type of stereochemical thinking in relation to biochemical processes catalysed by enzymes. Up to that time I had, as an organic chemist interested in the synthesis of natural products, the same kind of feeling for stereochemistry that a motorist might have for a system of one-way streets - a set of rules forming one more obstacle on the way to a destination. But 1948 was a year in which as well as continuing collaboration with Robert Robinson on the total synthesis of sterols, I had begun to co-operate with biological scientists at the National Institute for Medical Research; so that Ogston's note was a seed that germinated the more readily in my mind.

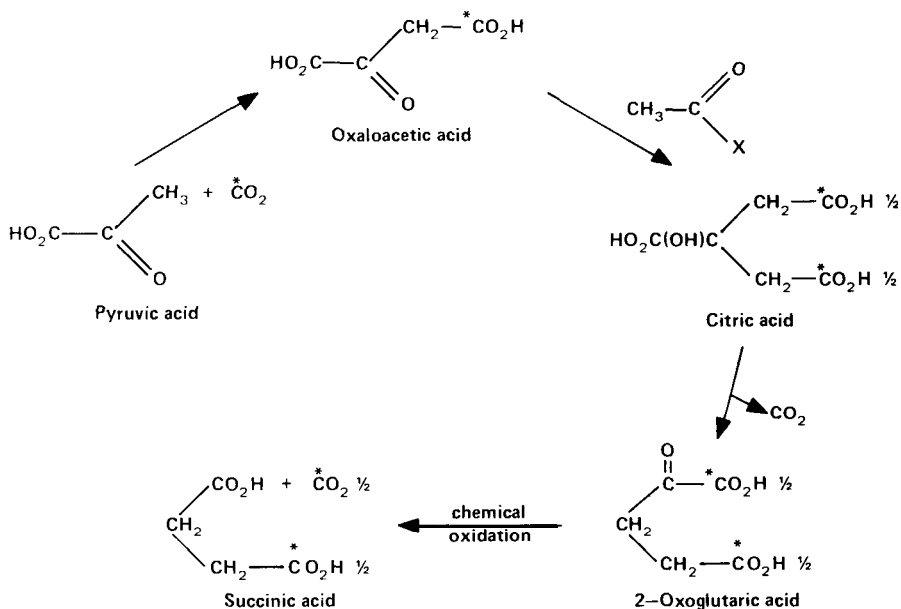
The essential principles of the three-dimensional structure of organic molecules had been correctly formulated by the first Nobel laureate in Chemistry, Jacobus van't Hoff, as early as 1874. In particular he (and independently Le Bel) gave a structural basis to Louis Pasteur's discovery that certain molecules can exist in two *optically active* forms that differ from each other in their effect on a beam of plane polarized light; the plane of polarization is rotated to the right when the beam passes through a solution of one form, and to the left when the other form is substituted. van't Hoff theorized that when a carbon atom in a molecule is attached to its maximum number of four other atoms, these occupy the four apices of a tetrahedron, with the carbon atom in the middle. Another way of saying the same thing is that the four atoms keep as far away from each other as they can, given that they are bound at fixed distances from the central atom. If these four atoms are all different, or at any rate if each forms part of a different group of atoms, they can occupy two distinct spatial arrangements that are *chiral*: their relationship is that of a right hand with a left hand and they are mirror images of one another (I, II).
mirror plane



The central carbon may then be called a centre of asymmetry. On the other hand, if two of the atoms or groups are indistinguishable from each other (III), only one arrangement about the central atom is possible.

Most molecules, including enzymes, that mediate the processes of life are optically active and have centres of asymmetry, but many molecules quite important in life processes have no centre of asymmetry and one of these is citric acid (IV), which was first isolated in 1784 by the great Swedish chemist Scheele: it is a *Caabc* compound, the two acetic acid groups attached to the central carbon being identical. When processes in living cells began to be studied with the help of radioactive and stable isotopes as tracers, an apparent anomaly arose that centred round this substance.

The biochemical course of events, which was outlined quite correctly on the evidence available at the time, was studied with preparations of pigeon liver and is expressed in Scheme 1. Pyruvic acid reacts with carbon dioxide to give oxaloacetic acid, and this condenses with "active acetate" (now known to be acetyl-coenzyme A) to yield citric acid. The citric acid then undergoes oxidation with loss of carbon dioxide to 2-oxoglutaric acid, which is further oxidized to a second molecule of carbon dioxide and succinic acid. All these reactions are catalysed by enzymes (though the last step can also be done chemically) but the original stereochemical reasoning about the sequence was based on what would happen if the same reactions were carried out nonenzymically. The reasoning went something like this: "citric acid is a symmetrical molecule of the type *Caabc*; there is no difference between the two a groups, which are both acetic acid residues; so that if we make one of them radioactive, for instance by using radioactive carbon dioxide in the



Scheme 1. Predicted distribution of isotopic label from carbon dioxide in 2-oxoglutaric acid

first step, then when the citric acid is broken down by way of 2-oxoglutaric acid to succinic acid and carbon dioxide the two residues will be affected indifferently and half the radioactivity will be in succinic acid and half will be in the second molecule of carbon dioxide liberated on oxidation”.

But experiment showed that *all* the radioactivity appeared in carbon dioxide and none in succinic acid, and no explanation (except the incorrect conclusion that citric acid took no part in the biochemical sequence) could be found before Ogston's note. Although Ogston clearly grasped the principle, I am giving the explanation in rather more general terms.

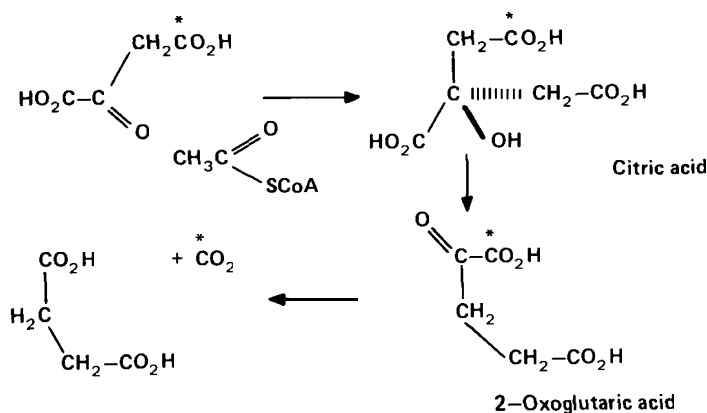
Enzymes catalyse chemical reactions by binding the reactant molecules (substrates) at a specific site in the enzyme molecule. Enzymes are proteins, and proteins are made up of a large number of asymmetric units, the amino-acids. There is no element of symmetry in an enzyme, or in its specific site. Moreover, each enzyme will characteristically accept very few molecular types as substrates: small changes in shape or size from the normal substrate may result in a very much slower reaction or in none at all. Emil Fischer had this in mind when he said, as long ago as 1894, that enzyme and substrate must fit each other like lock and key.

A lock and key must fit each other; but also, if the lock has no symmetry, the key has to be oriented three-dimensionally in a unique manner for introduction into the lock; and then the key has to be turned in a particular sense to operate the lock, so that one particular side of the key executes the actual operation of moving the lock's mechanism.

So that if a molecule of oxaloacetic acid is considered as a key, then only one particular side (above or below the plane of the paper) of its ketone carbonyl group can react with the other substrate, acetyl-coenzyme A. The other side *cannot* be fitted to the enzyme without changing the whole orientation of the substrate, as if one tried to fit the wrong end of a key, or a key upside down, into a lock. This is quite different from the reactions of the same carbonyl group in free solution, where both sides are equally open to attack by a reagent.

If the oxaloacetic acid carries a radioactive label, as it does when it has been made from pyruvic acid and radioactive carbon dioxide, then the citric acid which is formed on the enzyme carries its labelled acetic acid residue in a particular orientation that is distinct from the orientation of the other acetic acid residue - the one that originates from acetyl-coenzyme A. Since the next reaction in the biochemical sequence produces a change in one of the acetic acid residues, it is obvious that *all* citric acid molecules presented to the enzyme that alters them will have the labelled residue altered, or else they will all have the unlabelled residue altered: this is a necessary consequence if the citric acid molecules must be presented to the enzyme in a particular orientation. As it happens, the labelled residue is the one altered; and the relevant stereochemistry of the process, as elucidated much later by Kenneth Hanson and Irwin Rose, is as shown (Scheme 2).

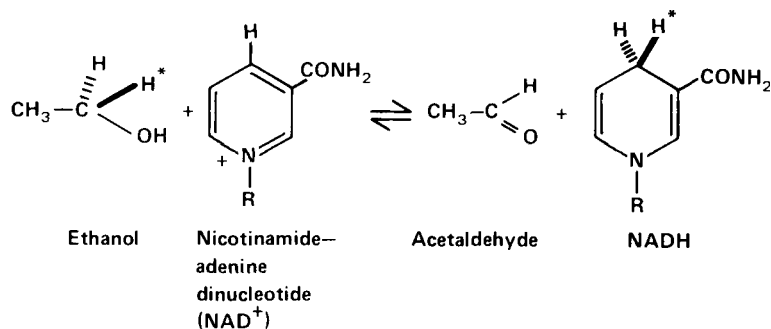
Asymmetric synthesis is not unknown to organic chemists: for example a reaction in free solution that produces a new centre of asymmetry by bringing



Scheme 2. Actual stereochemistry of citric acid enzymic synthesis and degradation

together a symmetrical and an unsymmetrical molecule will often produce an excess of one of the two chiral forms. But here was something of a different order: two reactions promoted with complete specificity by an asymmetric catalyst. And in both reactions the asymmetry of the catalysis is *hidden*: if it had not become possible to place the experimenter's private mark, in the shape of an isotopic label, on one of the two acetic acid residues, the Ogston effect, as it has come to be known, might have remained unsuspected for many years. In this field of work, the use of isotopes as markers is almost indispensable: replacement of an atom in a substrate molecule by one of its isotopes makes very little change in shape or chemistry; and an enzyme will always accept a labelled substrate, though it may transform it a little slower.

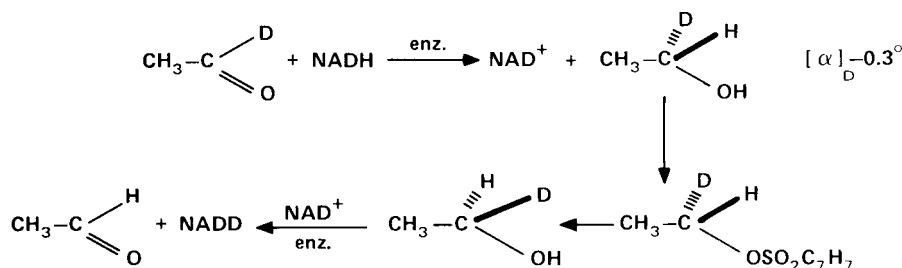
In 1953, Frank Westheimer, in collaboration with the biochemists Frank Loewus and Birgit Vennesland, studied yeast alcohol dehydrogenase. This enzyme catalyses the reversible transfer of a hydrogen atom between a molecule of ethanol and a molecule of a coenzyme, nicotinamide-adenine dinucleotide. The transfer neither creates nor destroys a centre of asymmetry, but nevertheless, the two hydrogen atoms on the oxygenated carbon of ethanol, and the two sides of the nicotinamide ring in the coenzyme, are stereochemically distinct in the Ogston sense. A simple test of this is to look at the rest of the molecule from the viewpoint of each hydrogen atom in turn, or from each side of the



Scheme 3. Substrate and coenzyme stereochemistry of yeast alcohol dehydrogenase

ring in turn: if the two views are different (as they are) an enzyme can, and probably will, concern itself with only one of the atoms or only one of the sides. By using the hydrogen isotopes deuterium or tritium as marking labels it was indeed possible to show that the hydrogen that is transferred occupies a unique stereochemical position in both substrate and coenzyme. A hydrogen not occupying one of these positions is not transferred at all. The stereochemistry has been worked out since then in a number of laboratories, including mine, and it is as shown (Scheme 3).

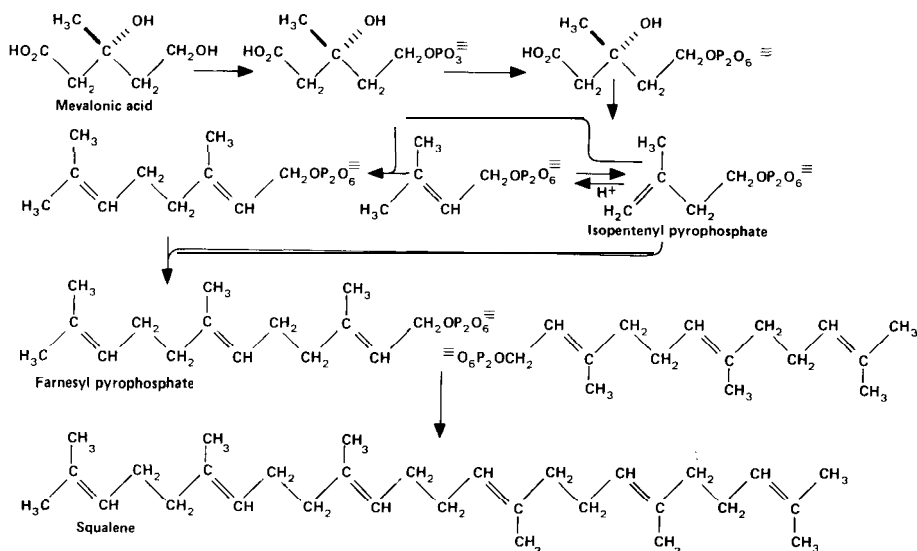
Further, when hydrogen was transferred from an unlabelled coenzyme to acetaldehyde in which the aldehydic hydrogen had been replaced by deuterium, the alcohol formed showed measurable (though small) rotation of polarized light, which made possible the correlation of its stereochemistry with this physical property. Finally, when the deuteriated ethanol was submitted to a purely chemical procedure: hydrolysis of its toluene-4-sulphonyl ester, a new specimen of deuteriated ethanol was obtained which, unlike its precursor, transferred deuterium and not hydrogen to the coenzyme in the presence of yeast alcohol dehydrogenase (Scheme 4).



Scheme 4. Stereochemical inversion of 1-deuterioethanol

Now although this last experiment was not carried out for the purpose, it can be regarded as proving that the hydrolysis of a typical sulphonic ester of a primary alcohol proceeds with *inversion of configuration* at carbon; which was something never demonstrated before, although it had been shown to be true of secondary alcohols in which the asymmetry owed nothing to isotopic substitution. Knowledge of the stereochemistry of a chemical reaction is one of the most useful guides in elucidating the correct mechanism and in excluding alternatives.

I had been following this work with much interest, and perceived some of its potential importance for studying enzymic mechanisms; but I was engaged at the time with my biochemical colleague George Popják and our collaborators, on a problem of biosynthesis: by chemical degradation of cholesterol synthesized in rat liver preparations from acetic acid, we were showing the pattern of incorporation of the precursor into the ring structure of the sterol. Later, and especially when mevalonic acid emerged as the parent substance of steroids and terpenoids, we were able to plan experiments of greater subtlety, using mevalonic acid specifically labelled with carbon isotopes to decide details of the molecular rearrangement that takes place when the steroid ring

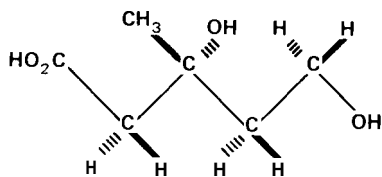


Scheme 5. Biosynthesis of squalene from 3R-mevalonic acid

structure is formed. At the same time, Konrad Bloch and Feodor Lynen were identifying the intermediate stages leading from mevalonic acid to the sterols in yeast, and Popják was demonstrating that the same intermediates were formed in rat liver. The sequence from mevalonic acid to squalene, the precursor of all steroids and triterpenoids, was mapped out as shown (Scheme 5).

In 1960 we were checking on the formation of squalene from two molecules of farnesyl pyrophosphate. This reaction looks like a symmetrical coupling of two identical halves: in fact, we found that the process is attended by the exchange of one, and only one, hydrogen atom from one of the carbon atoms that become joined together in squalene. This non-symmetrical synthesis of a symmetrical molecule roused further my curiosity about the mechanism of the whole process, from mevalonic acid to squalene.

Mevalonic acid has asymmetry of the ordinary kind, with *Cabcd* substitution at the central carbon atom, but this type of asymmetry is soon lost in the biochemical sequence. But mevalonic acid has three *Caabc* centres (Scheme 6) and all of these undergo changes in bonding on the way to squalene. Each of these six hydrogen atoms in these three groups is stereochemically distinct in the Ogston sense, and so it was possible in principle to follow the fate of a hydrogen atom from any one of these six positions, until it



Scheme 6. Mevalonic acid and its six methylene hydrogens

was either lost in the aqueous medium of reaction or came to a specific, and stereospecific, destination in a molecule of squalene. This fate is uniquely determined by, and therefore throws light on, the stereochemistry of the enzymic reactions in the biosynthetic sequence.

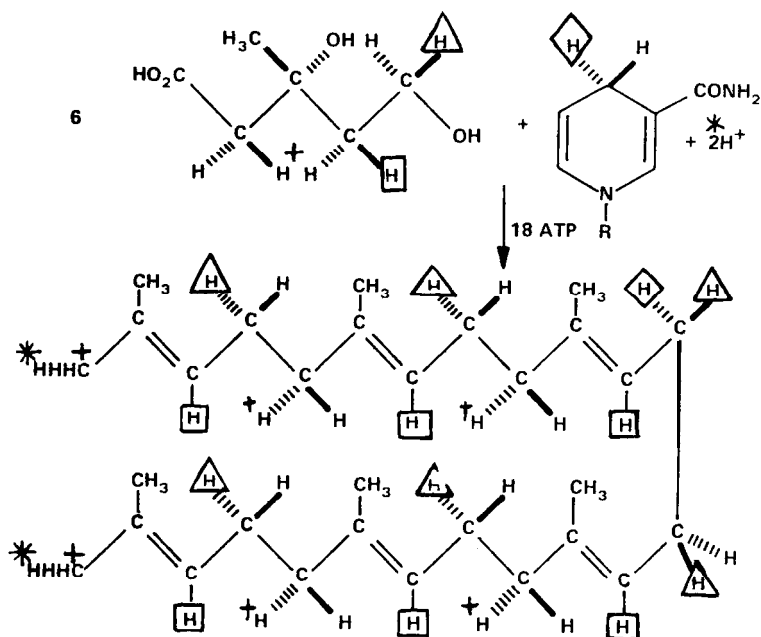
Thus it became necessary to place a distinguishing mark on each of these six hydrogen atoms in turn, and this could not be done except by replacing normal hydrogen by one of its isotopes: the stable deuterium or the radioactive tritium. This problem was solved to a large extent by drawing on the vast store of organic chemical knowledge, especially that part which concerns the stereochemistry of reactions. In this way, the individual labelling of four out of the six hydrogen atoms was achieved by non-enzymic processes, and an organic reaction of known stereochemical preference was employed to define the stereochemistry of an enzyme - mevaldate reductase - which was used to generate a label on the fifth hydrogen: the sixth was also labelled, eventually, with the help of enzymes of known stereochemistry.

These labelled mevalonic acids were introduced into enzyme preparations made from rat or pig liver. According to the nature of the preparation and to the presence or absence of co-factors or inhibitors, it was possible to execute the whole sequence from mevalonic acid to squalene or to stop at various intermediate stages; phosphomevalonic acid, isopentenyl pyrophosphate, or farnesyl pyrophosphate (see Scheme 5). Especially in experiments where the label was deuterium, unusually large amounts of product - typically, 50 milligrams - had to be accumulated from these enzymic incubations, and Popjak and I became familiar with the dialogue "How much can you make?" "How little do you need?"

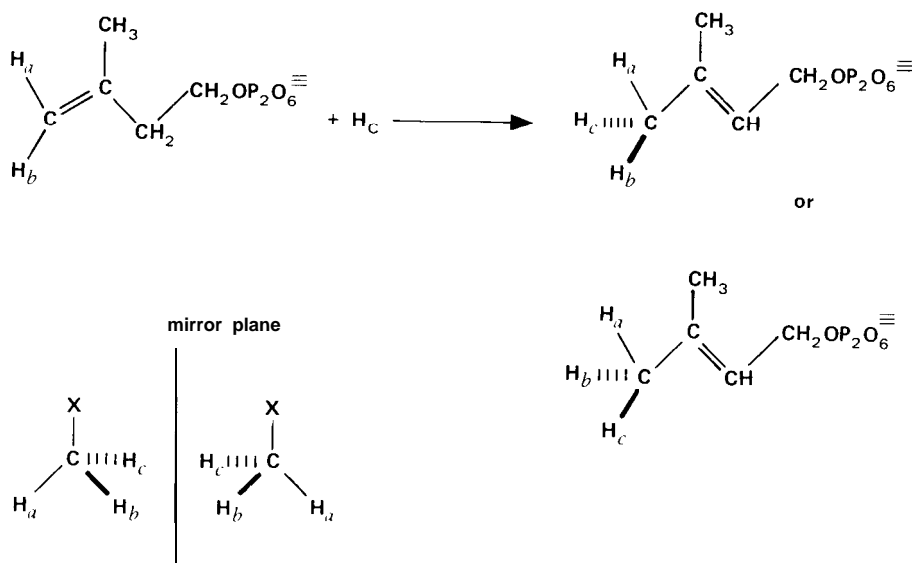
After the enzymic transformations, the products were examined to find out what had happened to the labelling isotope. When it was a question of whether the isotope had been lost from, or retained in, the product the procedure was relatively simple; one examined the product in a mass spectrometer for the presence or absence of deuterium, or one measured the radioactivity assignable to tritium. When the absolute stereochemistry at a labelled position was needed, it was necessary to use deuterium as the label and to degrade the product chemically, by reactions that either left the labelled centre undisturbed or altered it in a well-defined manner, to a substance suitable for examination by polarimetry or mass spectrometry. It was fortunate that this work coincided with the development of polarimeters sensitive enough to measure, in favourable cases, optical activity due solely to substitution of hydrogen by deuterium in specimens of a few milligrams. Our first measurements of this sort were, indeed, made on a prototype machine at the National Physical Laboratory. Since contamination by optically active material of the usual type could have been damaging, we had to develop a technique for recrystallization in capillary tubes. This permitted the recrystallization of succinic acid, for example, in milligram quantities from about two parts of water. Once the optical rotation was known it could be compared with the rotation of a sample into which a known absolute configuration had been built by enzymic and chemical synthesis.

Thus the work required in unusual measure the harmonious blending of stereospecific synthesis, isotopic labelling, enzymology, chemical degradation on the centigram scale, and sensitive physical methods of analysis, into a single experimental sequence. In the end, we succeeded in demonstrating stereospecificity for all but one of the enzymic steps then known for squalene biosynthesis; the fate of individual hydrogen atoms was as shown in Scheme 7. This was about as far as we could get by introducing asymmetry into CH_2 groups by isotopic substitution of one of the hydrogens, but the availability of these specifically labelled mevalonic acids was to prove, in our hands and in others', of considerable use in mapping the pathways of terpenoid biosynthesis in general.

One step in terpenoid biosynthesis is the (reversible) isomerization of isopentenyl pyrophosphate into dimethylallyl pyrophosphate (see. Scheme 5). The addition of a proton from the aqueous medium to the terminal methylene group, if it is stereospecific, is to one side only of the terminal methylene group. Thanks to the work already done, labelled mevalonic acids were available which were known to give isopentenyl pyrophosphate having a geometrically defined deuterium or tritium label at either of the two hydrogens of the methylene group; but if this group was converted in a normal incubation to a methyl group, free rotation about its C-C bond would give indistinguishable products whatever the initial geometry of the label and whatever the direction of addition of the proton. The only way in which it seemed possible to retain the individuality of the three hydrogen atoms concerned was to use all three isotopes of hydrogen - protium, deuterium and tritium - in proper sequence. Then, if the isopentenyl pyrophosphate was stereospecifically la-



Scheme 7. Partial stereochemistry of squalene biosynthesis from 3*R*-mevalonic acid

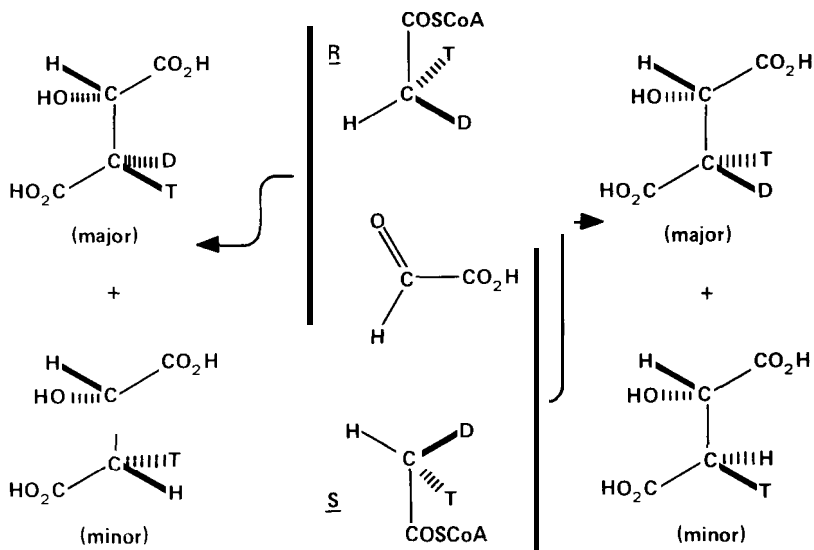


Scheme 8. Generation of a chiral methyl group

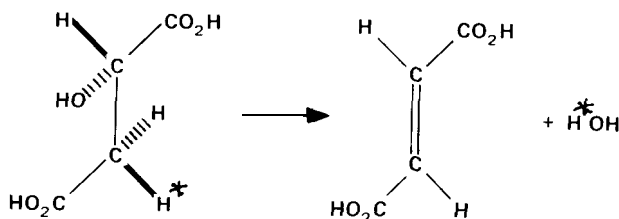
belled with two hydrogen isotopes and the third isotope was supplied in the water of incubation, a stereospecific addition of hydrogen from the water would give a *chiral methyl group*, the chirality of which would be determined by, and diagnostic of, the direction of proton addition (Scheme 8).

Chiral methyl groups were unknown at the time, and it was not obvious how their absolute configuration could be determined: optical rotation was an unlikely candidate for measurement since a substance having tritium in atomic proportion (instead of the usual small labelling concentration) would have a specific radioactivity of some 30,000 Curies per mole, and the rotatory power would probably be so small as to require large specimens having this order of radioactivity.

The solution of the problem grew from a suggestion made by Hermann Eggerer in 1967. He had been studying the enzyme malate synthase, which makes malic acid, an asymmetric substance having sinistral or *S* chirality according to the convention of Cahn, Ingold and Prelog, from glyoxylic acid and acetyl-coenzyme A; and he was led to favour a mechanism for the reaction which predicted a particular stereochemical relation between the hydrogen atom that is lost from the methyl group and the glycollic acid residue that replaces it (Scheme 9). If this mechanism was correct and if the reaction was attended by a normal "isotope effect", then protium should be displaced from a chiral methyl group more often than deuterium. Thus, the molecules of malic acid that contained tritium should comprise a larger proportion of the species containing deuterium and tritium than of the species containing tritium and protium; and in these two species the stereochemical location of the tritium must be different. An analytical method for determining this location was already available: the enzyme fumarase was known to catalyse the stereospecific *anti* elimination (Scheme 10) of water from *S* malic



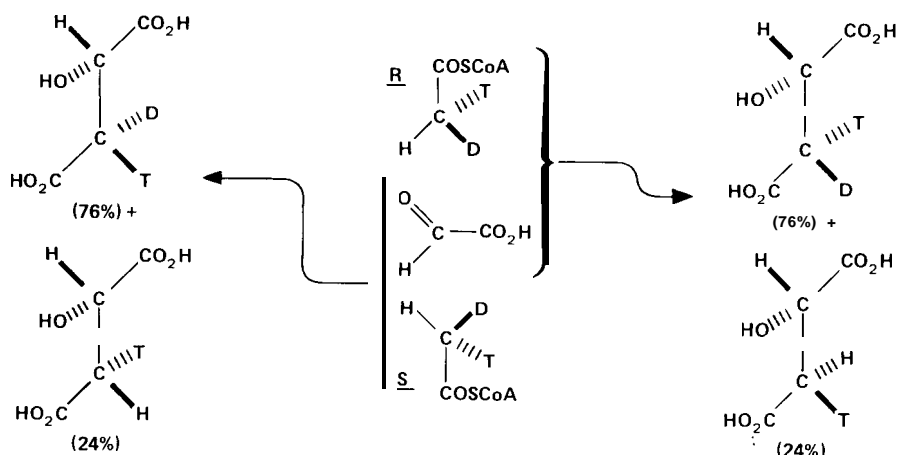
Scheme 9. Predicted distribution of tritium in *S* malate made from chiral acetates and glyoxylate on malate synthase, assuming retention of configuration at the chiral methyl group



Scheme 10. Stereochemistry of loss of carbon-bound hydrogen from *S* malate on the enzyme fumarase

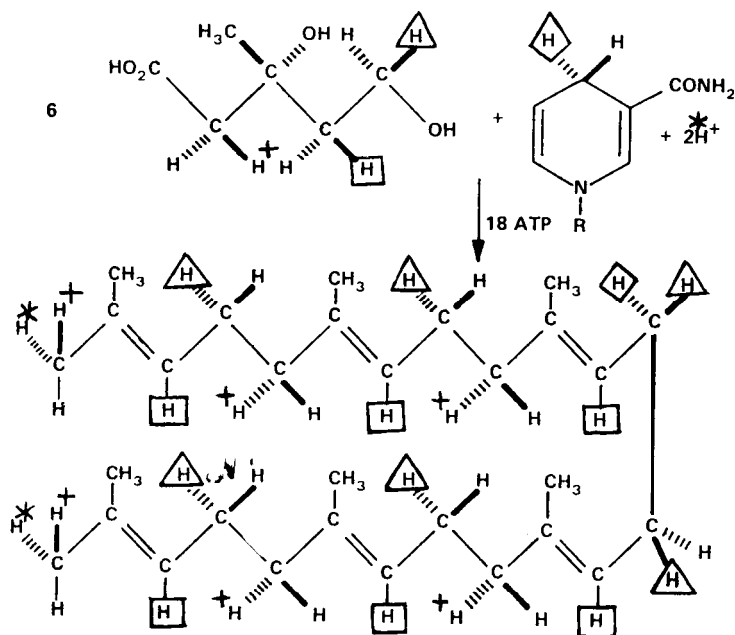
acid. So that if one carried out the sequence chiral acetate \rightarrow acetyl-coenzyme A \rightarrow malate \rightarrow fumarate and measured the percentage loss of tritium in the last stage, this percentage should be different for the two chiral forms of acetate: the percentage retention for the one should equal the percentage loss for the other. And if the stereochemical mechanism for malate synthase was assumed correct, one could infer the chirality of the acetate from these measurements of radioactivity alone.

The final plan (Scheme 11) did not depend on this assumption (which was just as well, for it turned out to be wrong). Instead, we were able at Milstead to synthesize, purely by chemical methods, potassium acetates the chirality and absolute stereochemistry of which were defined rigorously by the method of synthesis. When these were put through the sequence acetate \rightarrow acetyl-coenzyme A \rightarrow malate \rightarrow fumarate in Eggerer's laboratory, the malate derived from *S* acetate lost over three-quarters of its tritium on incubation with fumarase. In complementary contrast, malate derived from *R* acetate retained more than three-quarters of its tritium. Thus, without making any assumptions about the mechanisms of the enzymes used, we had a convenient meth-



Scheme 11. Actual distribution of tritium in *S* malate made from synthetic chiral acetates and glyoxylate on malate synthase

od for determining whether a given specimen of chiral acetate was *R* or *S*. At ETH. Zürich, Duilio Arigoni and Janos Rety independently produced a very similar solution of the same problem. With this analytical method it became possible to solve not only the problem of the addition of hydrogen to isopentenyl pyrophosphate but also to deduce the stereochemistry of a large and still growing number of enzymic reactions in which a methyl group is either generated or transformed. When at last we knew the stereochemical origin of all fifty hydrogens in squalene biosynthesized from mevalonic acid, I had a three-dimensional model made to illustrate this. The last scheme summarizes the information conveyed (Scheme 12).



Scheme 12. Complete stereochemistry of squalene biosynthesis from 3*R*-mevalonic acid

Our adventure with the chiral methyl group reinforced the conviction that stereospecificity is something not just incidental, but essential, to enzymic catalysis. Life does depend on accurate replication of molecules and its complexity often requires that an enzyme shall accept one molecular species or type and transform it to equally specific products. But the hidden specificity that we have helped to reveal goes much further than this: an enzyme must, it seems, catalyse strictly stereospecific reactions even when this specificity is not required by the structural relation of substrate to product. Indeed, many examples are now available in which an enzyme can accept more than one molecular species as substrate but still transforms each of them with absolute, though hidden, control of the stereochemistry of reaction.

By combining chemical, biochemical and physical techniques it has thus become possible to investigate the nature of enzymic catalysis in a novel manner, complementary to the other approaches which have developed over the same period. The work required concentrated effort, and I owe much to the skill and dedication of many collaborators; but I call your attention to three. George Popják sustained the biochemical side of these investigations with exceptional insight, ability and resource until 1967. I was fortunate enough to be associated with him as colleague and partner for over twenty years and, after him, to enjoy collaboration with another great biochemist, Hermann Eggerer, whose co-operative spirit made light the difficulties of concerting experiments in laboratories a thousand kilometers apart. Thirdly, my wife Rita Cornforth, with patience and great experimental skill, executed much of the chemical synthesis on which the success of the work was founded. To her, in this as in other ways, I owe more than I can well express.

To my teacher and friend, Robert Robinson, whose death early this year sadly forestalled his presence on this occasion, I remain especially grateful, and could hope for nothing better than to retain, as he did to the end of a long and creative life, fresh curiosity and wonder at the chemistry of Nature.