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ENZYMES AND STEREOCHEMISTRY

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The connection of molecular asymmetry with the processes of life was noticed well before van't Hoff and Le Bel founded stereochemistry as a science. Biot[1] discovered and studied the power of various substances, as fluids or solutes, to rotate the plane of polarization of light: and it was not overlooked that all such substances (at that time) were of biological origin. Both Biot and Pasteur[2] recognized that optical activity in solution must be a reflection of dissymmetry in the solute "molecules", the *dextro* and *laevo* forms being non-identical mirror images of each other. The stereochemical *selectivity* of living processes was also noticed by Pasteur[3]: in 1858 he showed that the grey mould *Penicillium glaucum* destroyed the dextrorotatory form of ammonium tartrate and selected this form from a solution of the racemate.

Pasteur thought that fermentation ("life without oxygen") was a process inseparable from living organisms, and many years passed before this view was overthrown by Buchner's[4] demonstration of typical alcoholic fermentation in a cell-free filtrate from yeast. Long before this, however, "unorganized ferments" were known: preparations from seeds or saliva or gastric juice that showed no sign of organized life but were able to catalyse "simple" reactions such as hydrolysis. The term "enzyme" (meaning "in yeast") was certainly intended by its inventor, W. Kühne,[5] to include such ferments, as well as the elements of fermentation in a living cell.

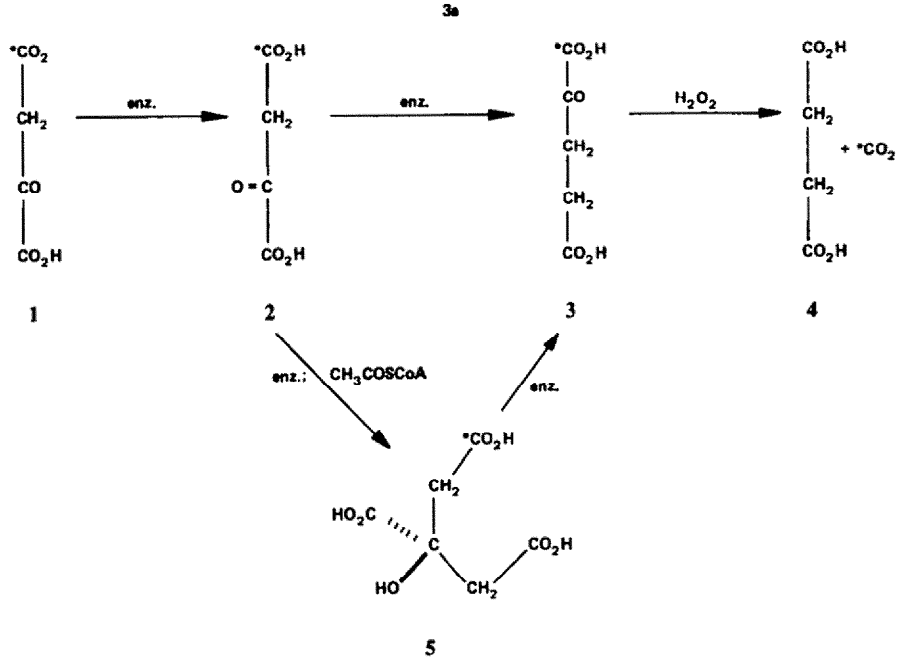
With the establishment of a satisfactory and fruitful theory of three-dimensional molecular structure, exploration of the stereochemistry of complex molecules produced by living organisms became a systematic part of organic chemistry. Emil Fischer, in his great work on sugar stereochemistry, became interested in the action of enzymes (mostly "unorganized ferments") on sugars and their derivatives; and he demonstrated clearly that the acceptability of a substrate by an enzyme depended typically on its stereochemistry and not just on its gross structure. He commented[6] "To use an illustration, I will say that enzyme and glucoside must fit each other like lock and key, if they are to exert a chemical action on each other." One wonders whether enzymology would have developed faster if Fischer had hit on a different comparison. The wards of a lock are effective in excluding the wrong kind of key, but they do nothing to accelerate the movement. "Like rifle and cartridge" would be closer to the modern view, though spatially less intricate than the reality. As it was, recognition of the enormous efficiency of enzymes as catalysts came slowly.

All of the early work on enzymes was done with extremely crude preparations and it was not until 1926 that Sumner[7] crystallized urease and provided convincing evidence that the catalytic activity was due to this chemically identifiable protein and not to some subtle impurity associated with the crystals. As enzyme after enzyme was subsequently purified it had to be recognized that all known enzymes are proteins and that many of them owe their catalytic activity to nothing except the manner in which their amino-acid components are connected.

The observed specificity of binding of a substrate (generally a small molecule) by an enzyme (generally large) led gradually to the belief that the reaction catalysed by an enzyme occurs at a definite and relatively small part of the protein molecule. This idea of an "active site" has been confirmed now for many enzymes.

How far the study of enzymic catalysis would by now have progressed if, in the 1930's and 1940's, isotopic labelling had not become possible, it is hard to say. Certainly the techniques of physical chemistry (such as X-ray crystallography, reaction kinetics, and spectroscopy of many kinds), together with the use of specific inhibitors and unnatural substrates, would still have gleaned much information about the interactions between enzyme and substrate. As it was, stable or radioactive isotopes of all the chemical elements from which enzymes or substrates are made became available, along with techniques for detecting and measuring them. It became possible to label a normal substrate and to trace the label in the product. The effect on the study of enzymic reactions and (less predictably) of the stereochemical course of these reactions was immense and immediate.

In 1941, two groups of workers,[8,9] using the carbon isotopes ^{11}C and ^{13}C, reported on the formation of oxaloacetate (2) from labelled carbon dioxide and unlabelled pyruvate (1) (Scheme 1) in preparations of pigeon liver. In these conditions some of the oxaloacetate was converted further into 2-oxogutarate (3), and this was found to lose all the isotopic label as carbon dioxide when oxidized to succinic acid (4).



SCHEME 1. Citric acid synthesis and degradation.

This was not in itself a startling observation, but it was made about the time when Krebs[10] was establishing the operation and importance of what is now known as the tricarboxylic acid cycle, in pigeon liver and in other tissues. According to this view, 2-oxoglutarate was formed from oxaloacetate *via* citric acid (5). The results with isotopic carbon were at first regarded as positive proof that citric acid, or at any rate free citric acid, could not possibly be an intermediate in the formation of this 2-oxoglutarate. Citric acid, it was asserted, is a symmetrical molecule in which the two carboxymethyl groups are chemically identical, so that if it were an intermediate the two carboxyl groups of 2-oxoglutarate should be (in the absence of an isotope effect) equally labelled.

Yet no alternative pathway presented itself, and the labelling experiments remained without an explanation until Ogston,[11] in a short classic note, exposed the fallacy in the earlier reasoning by showing how an enzyme might distinguish between two chemically identical groups attached to the same carbon atom. Enzymes are asymmetric in themselves, and they characteristically form dissociable complexes with substrates before chemical reaction occurs. It happens that the initial reaction of citrate on the way to 2-oxoglutarate is a dehydration, the water being formed from the tertiary hydroxyl and one of the hydrogens on one carboxymethyl group. Assume that on the enzyme catalysing this reaction there are three specific binding sites, in a definite spatial relation to each other, for three parts of a citric acid molecule: (i) for the tertiary carboxyl, (ii) for the tertiary hydroxyl, and (iii) for a carboxymethyl group; and assume that sites (ii) and (iii) co-operate in catalysing the removal of water. A citric acid molecule thus bound is shown schematically in Fig 1a. If one distinguishes the carboxymethyl group bound at (iii) by some private mark (here an asterisk on the carboxyl group!) and removes the citric acid molecule from the enzyme surface, it can easily be verified that reattachment of citric acid to these three binding sites can *never* involve the unmarked carboxymethyl group: one always gets associations (such as in Fig 1b) where at least one of the binding sites is occupied by a group for which it is not specific.

This is one way of saying that two chemically identical *groups* may be stereochemically in different environments when the molecule as a whole is considered. A simple test to decide whether an enzyme can discriminate between two groups (not necessarily on the same carbon atom) in a molecule is to make a model of the molecule, pick it up by

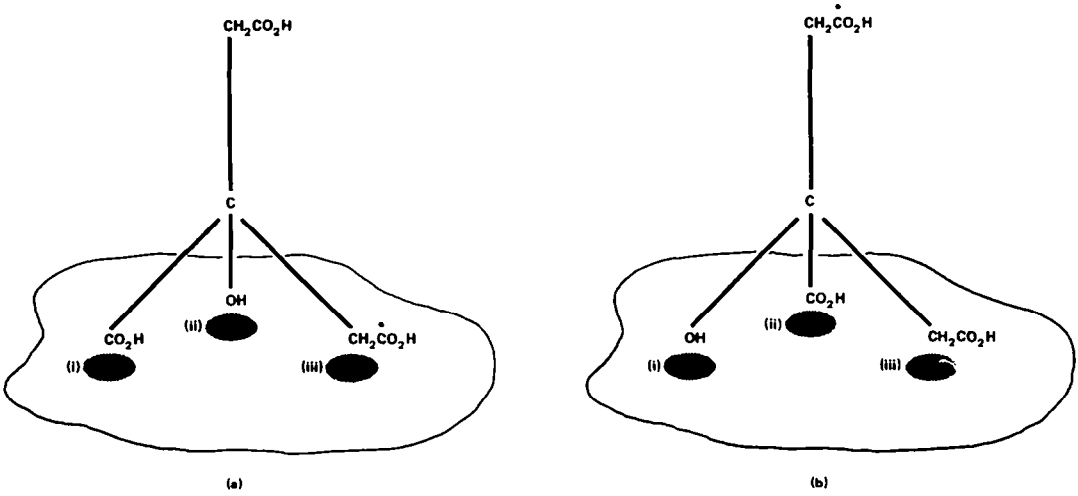


Fig 1. Demonstration of the stereochemical non-equivalence of the two carboxymethyl groups of citric acid.

each group in turn, and look at the rest of the molecule. If this looks the same (or can be made to look the same by permissible rotations of single bonds) whichever group is held, then an enzyme cannot discriminate between these two groups. If the two views are different, then the enzyme can and probably will discriminate between the two groups. This judgment of identity or non-identity can be made by anyone who can tell left from right, up from down, near from far. In effect the enzyme, because its active site has no element of symmetry, makes the same distinctions. Ogston's concept of "three-point attachment" is only one way in which this discrimination might be exercised. For instance, one could revert to Fischer's "lock and key" image and suppose that the "keyhole" is of a shape that will obstruct the substrate molecule unless it is presented in a particular orientation.

In considering Ogston's reasoning, we distinguish one carboxymethyl group of citric acid by a private mark. Citric acid synthesized enzymically from pyruvate *via* oxaloacetate must, on the experimental evidence, bear the experimenter's private mark—the carbon isotope—on just one of its two stereochemically distinguishable carboxymethyl groups. It follows that the synthesis of citrate from oxaloacetate, which is a condensation with acetyl-coenzyme A, must also be a stereospecific process. The reaction occurs at the ketonic carbon of oxaloacetate which has three different groups (oxygen, carboxyl, carboxymethyl) in a planar arrangement around it. Again, simple inspection shows that this arrangement is not the same when viewed from above and from below the plane: therefore an enzyme can discriminate between these two directions. In fact, on the citric acid synthase of the tricarboxylic acid cycle the addition of the second carboxymethyl group (as acetyl-coenzyme A) is to the *si*-face (the upper side in (2)) of the carbonyl group in oxaloacetate. In a rarer enzyme, found so far only in some *Clostridia*, addition (though still stereospecific) is to the opposite, *re* face.

The convention for distinguishing between paired groups such as the two carboxymethyl groups of citric acid, and non-equivalent faces such as the two faces of the ketonic carboxyl group of oxaloacetic acid, was developed by Hanson.[12] For example, in citric acid the central carbon atom is called prochiral: if either carboxymethyl group is modified in any way (for example by replacing one of its atoms by an isotope of higher atomic number) that gives it priority over the other in the Cahn-Ingold-Prelog convention,[13] then the central carbon becomes an asymmetric centre which application of the convention designates at *R* or *S*; if *R*, the altered group was pro-*R*, if *S*, pro-*S*. Experimentally, the isotopic lable may be in either group: the nomenclature enables us to say (see Fig 1a) that the pro-*R* carboxymethyl group is the one participating in the enzymic dehydration of citric acid. The enzyme responds to the different stereochemical environments of the two paired groups; not to the presence in one of them of an isotope, which is necessary only because it enables the experimenter to find out what is happening.

Exploration of enzyme stereospecificity

From a drop of water, Sherlock Holmes said once, a logician could predict an Atlantic or a Niagara. Given the asymmetry of enzymes and their faculty of binding substrates for reaction, our logician might have predicted the stereospecific synthesis and stereospecific transformation of citric acid even before the availability of isotopes forced them on the scientific world's attention. Once that attention was gained, it quickly discerned

a powerful probe for studying the events at an enzyme's active site. Asymmetric synthesis and degradation may have been predictable, but their precise orientation was not, and solution of this problem gave information about the spatial relation between substrate and substrate, or between substrate and coenzyme, or between substrate and enzymic functional group, on the active site at the instant of reaction. Sometimes, the product has asymmetry of the ordinary kind and a knowledge of its absolute configuration gives the information required; much more often, the asymmetry of the enzymic process does not reveal itself in the normal product, and stereospecific labelling with an isotope is still the only known way to detect it. After 25 years of development it is possible to survey the technique used in such investigations. An enzyme is chosen for study. It does not have to be available in pure form or to be unaccompanied by other enzymes, so long as any side-reactions of substrate or product are understood and can be allowed for, but naturally the cruder the system the greater is the possibility of unexpected interference. To obtain the necessary stereochemical information, either a substrate (or coenzyme) or the product must be stereospecifically labelled with an isotope.

When substrate or coenzyme is to be stereospecifically labelled, so that a prochiral centre in the normal substrate or coenzyme becomes a chiral centre of known absolute configuration in the labelled substrate, the possibilities are:

(1) The substrate or coenzyme is produced by stereospecific chemical synthesis.

(2) The substrate or coenzyme is produced by use of enzymic processes of known stereochemistry.

(3) Methods (1) and (2) are combined.

When the stereospecific labelling is to be in the product (or coenzyme product) one has, in addition to generation from a stereospecifically labelled substrate, the further possibilities:

(4) The aqueous medium is labelled (with ^{2}H, ^{3}H, or ^{18}O).

(5) The substrate or coenzyme is labelled but without stereospecificity; the asymmetry is introduced by the enzymic reaction.

(6) The enzyme is labelled.

When an experiment is set up with one or more of the foregoing provisions, the stereochemistry of the enzymic process can be deduced in a number of ways:

(a) Substrate or coenzyme stereospecifically labelled by methods (1)→(3) suffers loss (or, if the alternate prochiral group is labelled, retention) of label in conversion to product. This can be shown by radioactive counting, mass spectrometry, or any other way in which the presence or absence of abnormal proportions of an isotope can be detected. The determination may be on the product itself or on a derivative.

(b) Transfer (or absence of transfer) of isotope between stereospecifically labelled coenzyme and substrate, or between stereospecifically labelled substrate and coenzyme, is shown as in (a).

(c) The product (or coenzyme product) stereospecifically labelled according to any of the procedures (1)→(6) is suitable for determination of absolute stereochemistry by one or more of the following techniques:

(i) Neutron diffraction crystallography (so far, only one example).[14]

(ii) Spectropolarimetry (deuterium labelling only).

(iii) Loss or retention of isotope in stereospecific (or unambiguous) chemical reaction.

(iv) Loss or retention of isotope in stereospecific enzymic reactions.

(v) NMR comparison with spectra of unlabelled products or with labelled products of known stereochemistry.

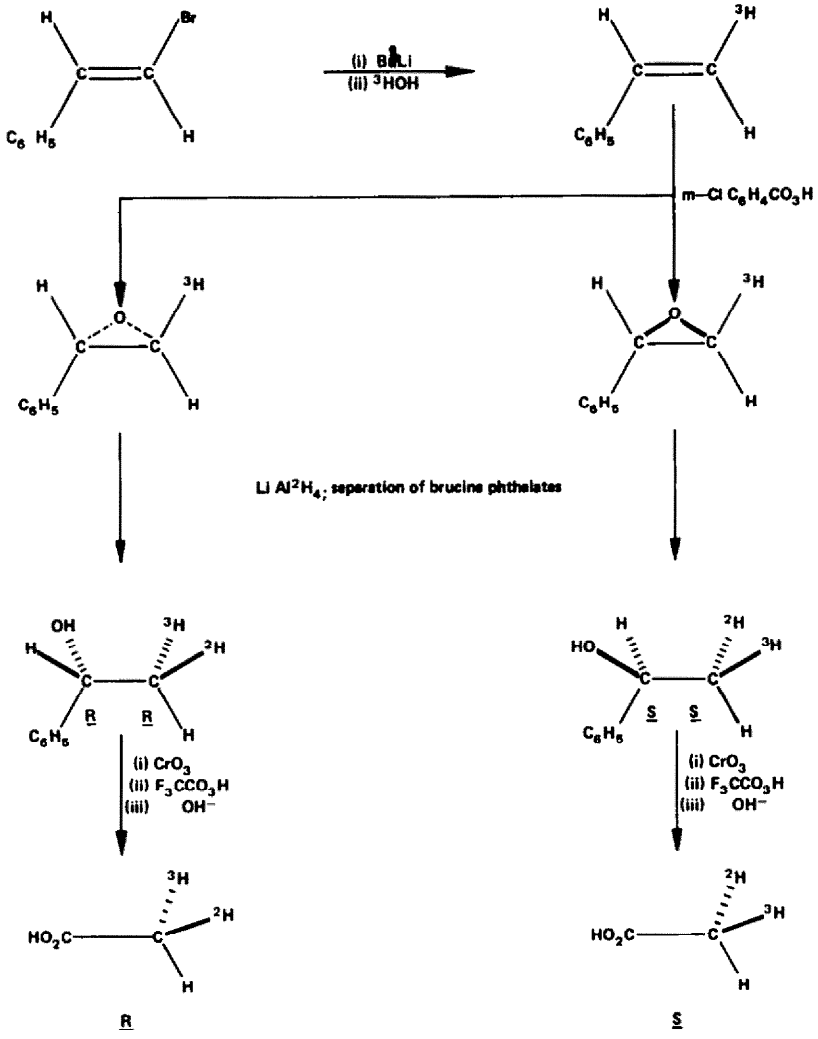
(vi) Infra-red comparison with labelled products of known stereochemistry (deuterium labelling only).

(d) The product (or coenzyme product) is submitted to chemical transformation before application of techniques (i)→(vi).

(e) The product (or coenzyme product) is submitted to enzymic transformation before application of techniques (i)→(vi).

A special case is presented by the investigation of enzymic reactions in which methyl groups are converted into methylene, or methylene into methyl. It is obvious that so long as free rotation is possible about the bond joining a methyl group to the rest of a molecule, no discrimination is possible between its three hydrogens. But this is not to say that enzymic reactions at a methyl group have no stereospecificity: there may be a perfectly definite spatial relationship between the group that becomes attached in the enzymic reaction and the hydrogen atom which it displaces. In other words, the substitution may have the spatial character of an inversion or of a retention of configuration, and the same applies to the opposite type of reaction in which a methyl group is generated from methylene. The problem of demonstrating stereospecificity of this type was solved independently by two groups.[15,16]

First, asymmetric methyl groups of known absolute configuration were prepared. Asymmetry is only possible in a methyl group if all three known isotopes of hydrogen are present in it: if they are, the group has either *R* or *S* chirality according to their arrangement. Enantiomeric specimens of acetic containing, respectively, *R* and *S* methyl groups were prepared by chemical[15] or enzymic-chemical[16] methods. An improved version[17] of the chemical synthesis is shown in Scheme 2. It will be noticed that the conversion of *trans*-bromostyrene ((E)-1-bromo-2-phenylethylene) to acetic acid used three stereospecific reactions (retention of geometry in



SCHEME 2. Preparation of *R* and *S* acetic acid.

conversion of a vinyl bromide to an ethylene *via* a vinyl-lithium; *cis*-expoxidation by a peroxy-acid; reduction of an epoxide by lithium borohydride with inversion at the centre of substitution) which "tied" the absolute configuration of the asymmetric methyl groups generated by the synthesis to that of an adjacent carbinol group which was asymmetric in the ordinary way. Optical resolution of this carbinol (1-phenylethanol) then automatically separated the enantiomeric methyl groups; and since the absolute configurations of the enantiomeric 1-phenylethanols were already known, the absolute configuration of the methyl groups in each enantiomer was also known. The remainder of the synthesis is simply a transformation of 1-phenylethanol to acetic acid without disturbance of the methyl groups.

The acetic acids thus prepared were not homogeneous specimens: in particular, tritium was present in relatively few molecules. However, the synthesis had been planned so as to ensure that substantially all tritium was present in asymmetric methyl groups; and since subsequent operations were based on measurements of radioactivity, such groups were the only significant components.

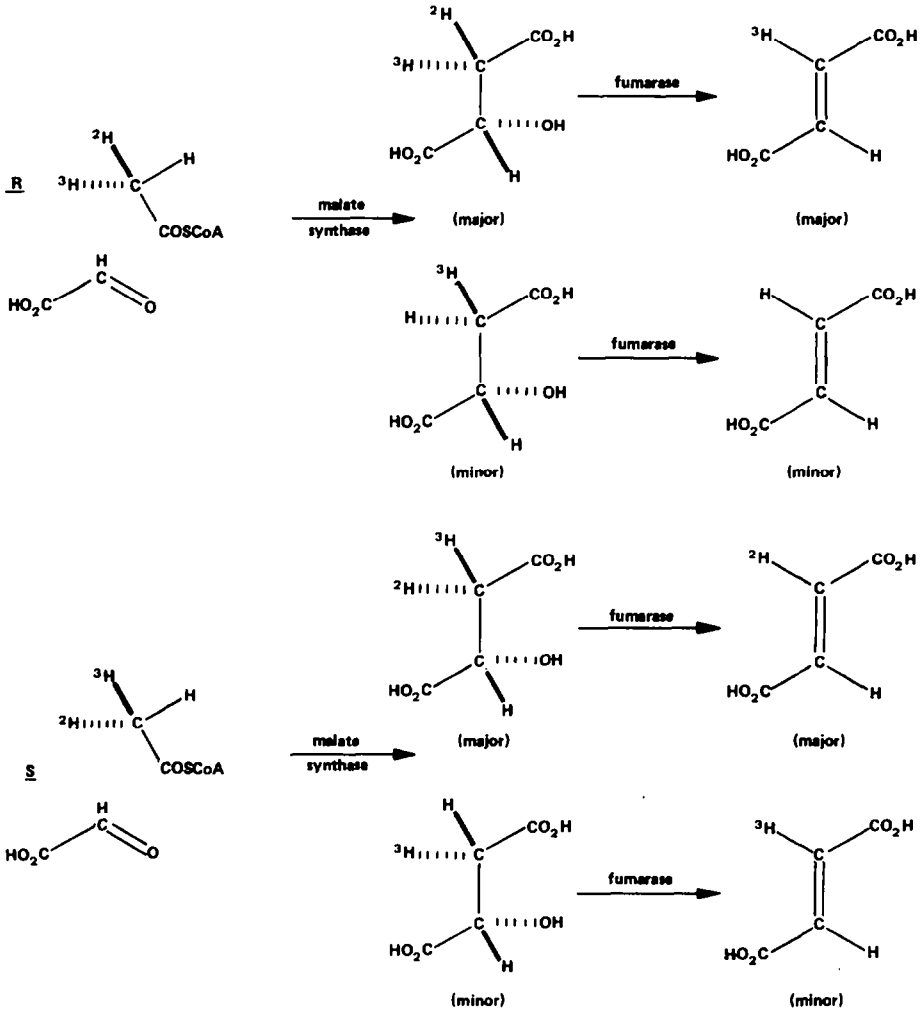
The next step was to find a system which would discriminate between these two specimens. Both groups of workers chose the enzyme malate synthase, on which acetyl-coenzyme A and glyoxylate are condensed, irreversibly, to *S* malate. It is now known (though it was not at the time!) that this enzymic reaction proceeds with inversion of configuration at the methyl group and that deuterium is displaced three or four times less easily than protium when both are present on the same methyl group. Thus the product of this reaction with *R* acetyl-coenzyme A contains two tritiated species in unequal amounts, the ratio of their abundance

being equal to the kinetic isotope effect. From *S* acetyl-coenzyme A, two other tritiated species are formed (Scheme 3).

When each of these two specimens of *S* malate was incubated with the enzyme fumarase, a reversible dehydration of known stereospecificity occurred. It had been demonstrated[18,19] that the enzymic formation of fumarate from *S* malate is an *anti* elimination of the elements of water: this means that the pro-3*R* hydrogen is eliminated and the pro-3*S* hydrogen is retained (Scheme 3). In the event, the malate from *R* acetate lost less than one-quarter of its tritium on treatment with fumarase whereas the malate from *S* acetate lost more than three-quarters of its tritium.

This enzymic procedure, therefore, constitutes an assay for asymmetric methyl groups which is quite independent of the mechanism of the enzymes used: one obtains one result from *R* and a quantitatively different result from *S* acetate. Thus if a stereospecific procedure (enzymic or chemical) of unknown stereochemistry generates an asymmetric methyl group one can determine whether this is *R* or *S*, and one can thence deduce without ambiguity the stereochemistry of its generation. On the other hand, it is not possible to conclude from the above experiments that the condensation on malate synthase proceeds with inversion of configuration unless the assumption is made that the deuterium isotope effect is *normal* (deuterium displaced less easily than protium). This is the same thing as saying that the major tritiated species of malate also contains deuterium, and the minor species does not. This has been established for malate synthase,[20] and no inverse isotope effect has yet been noted in reactions of this type; but the logical proviso remains.

The procedures classified above have succeeded

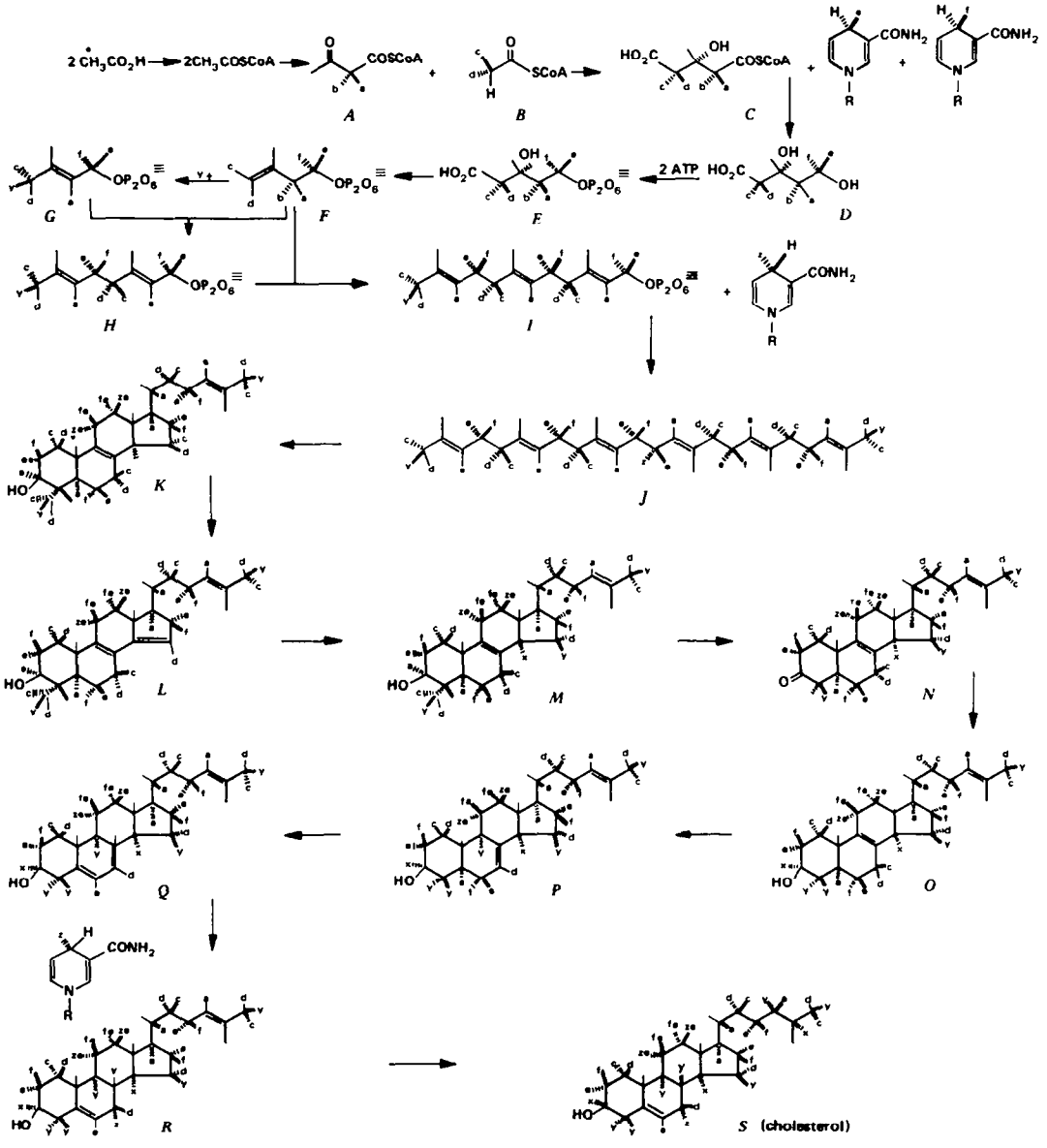


SCHEME 3. Analysis of asymmetric methyl groups.

in assigning absolute stereochemical meaning to a considerable variety of enzymic processes. Many might be singled out for mention—for example, the work on alcohol dehydrogenases which is treated by A. R. Battersby in this Volume; I. A. Rose's studies on the enzymes of carbohydrate metabolism and the tricarboxylic acid cycle; or the work of R. H. Abeles and of D. Arigoni on propanediol dehydratase. A monograph is now available.[21] The example selected—cholesterol biosynthesis—reflects the writer's own interest.

Stereochemistry of cholesterol biosynthesis

During the last quarter of the century that we celebrate in this volume, knowledge of the pathway of steroid biosynthesis, and of the stereochemistry of its steps, has progressed from total ignorance to extensive comprehension. To the mapping of the biosynthetic pathway the major contributions were from Bloch and from Lynen. Acetate was the first efficient precursor to be discovered; next were the intermediates squalene (J, Scheme 4) and lanosterol (K). The discovery of 3*R* mevalonic acid (D) was



SCHEME 4. Hidden stereospecificity in the biosynthesis of cholesterol from acetate. x = hydrogen from reduced nicotinamide nucleotide (chirality uncertain), y = hydrogen introduced from aqueous medium.

most important of all: in liver preparations, it may be transformed nearly quantitatively into cholesterol and it is not utilized for any metabolic process other than that of isoprenoid biosynthesis. This means that it can be used as a labelled precursor of later intermediates, even in crude enzyme preparations or intact organisms, without the confusion of labelling which could attend breakdown and resynthesis of the precursor.

This biosynthetic sequence is, so far as has been ascertained, completely stereospecific. The specificity is sometimes obvious (for example, the absolute configuration of the intermediate squalene epoxide shows clearly which face of a terminal double bond in squalene (J) has been attacked by oxygen); but more often it is hidden in the sense that the normal product gives no indication of the stereochemistry of the process, which can be found out only by isotopic labelling. In the overall biosynthesis there are around forty changes in bonding which are ambiguous in the absence of isotopic labelling but which suitable labelling could show to be stereospecific. Evidence is now available for the majority of these changes; and reviews are available.[21,22]

In Scheme 4, a somewhat condensed version of cholesterol biosynthesis in animal cells is presented to show the *hidden* specificities that have been brought to light: that is, specificities that do not show themselves in the structure of unlabelled intermediates or products. Hydrogen atoms are replaced by lower-case letters so that their origin and stereochemical progress can more easily be traced.

In Table 1, a concise description is attempted of the methods by which each hidden aspect of asymmetry was revealed. The classification of methods is that given in the previous section (p. 1518). A supplement to Table 1 is the elucidation of the hidden chirality in nicotinamide nucleotide coenzymes, which are co-factors at several stages of cholesterol biosynthesis. This determination would be classified in the Table as (5) (d) (ii): that is, the asymmetrically labelled coenzymes were generated from substrates or coenzymes without stereospecific labelling; the product was chemically degraded; and the final determination of absolute configuration was made by spectropolarimetry. The procedure is mentioned elsewhere,* but the correlation of absolute configuration with sign of rotation in the deuteriosuccinic acids is worth detailing (Scheme 5) since these acids have been used as reference substances in many other determinations.

It has already been mentioned that the enzyme fumarase catalyses a reversible *anti* elimination-addition of water, thus interconverting *S* malate and fumarate. It follows that if fumarate is incubated with the enzyme in deuterium oxide the product will be $2S, 3R[3-{}^2H_1]$ malate. Simple chemistry to reduce the hydroxyl group without disturbing the adjacent centre of asymmetry then leads to $2R[2-{}^2H^1]$ succinic acid, which turns out to be the laevorotatory form in aqueous solutions.[23]

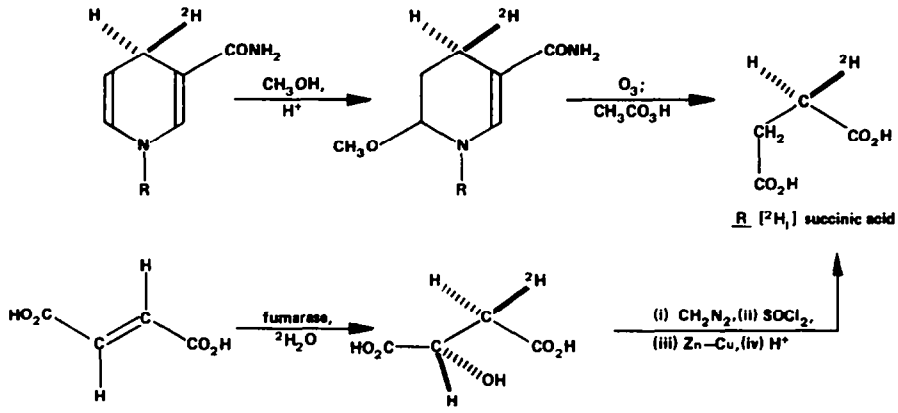
CONCLUSION

The examples collected above, and many others not mentioned, provide overwhelming evidence for the stereospecificity of enzymic *reactions*. It is not difficult to understand why enzymes should be

*A. R. Battersby and J. Staunton, *Tetrahedron* **30**, 1707 (1974)

Table 1. Methods used to obtain the information on hidden stereospecificity shown in Scheme 4. The letters and figures in the "Experimental method" column refer to the classification on p. 1518

Transformation	Stereospecific element	Experimental method	Ref
A + B → C	Inversion at methyl group	(1)(d)(iv)	23
C → D	Reduction by nicotinamide coenzyme	(2)(b)	24, 25
E → F	*Anti* elimination of H_2CO_3	(3)(e)(d)(iii)	26
F → G	(i) Loss of prochiral H	(3)(a)	27
	(ii) Stereospecific addition of H^+	(3)(4)(e)(d)(iv)	28
	(iii) Geometry of methyl groups	(4)(e)(v)	29
G → H,	(i) Inversion at allylic C	(3)(e)(d)(ii)	27, 30
H → I	(ii) Stereospecific addition to C=C	(3)(e)(d)(ii)	26
	(iii) Loss of prochiral H	(3)(a)	27
I → J	(i) Inversion at allylic C	(3)(d)(vi)	27, 30
	(ii) Reduction by nicotinamide coenzyme	(2)(b)	27
	(iii) Prochirality of H inserted from coenzyme	(3)(d)(ii)	27
K → L	Loss of prochiral H	(3)(a)	31, 32
L → M	Prochirality and origin of inserted H	(3)(4)(c)(iii)	33, 34
M → N	Loss of prochiral methyl	Non-isotopic	35, 36
O → P	Loss of prochiral H	(3)(c)(iii)	37, 38
P → Q	Loss of prochiral H	(1)(a)	39, 40
Q → R	Prochirality and origin of inserted H	(2)(5)(b)(iii)	41
R → S	(i) Prochirality of inserted H	(5)(e)(d)(iv)	42
	(ii) Prochirality of gem-dimethyl	(2)(e)(iii)	43



SCHEME 5. Absolute configuration of [$^2H_1$] succinic acids.

stereospecific with respect to the substrates that they will accept and to the products that they form, in so far as these substrates or products are particular stereoisomers of the ordinary kind (e.g. *cis* rather than *trans*; *R* rather than *S*); for after all the processes of life, which enzymes mediate, depend on exact replication of particular molecules. But the stereospecificity revealed by isotopic labelling goes much further than this: generally, *enzymic reactions occur by stereospecific mechanisms* regardless of whether this specificity determines the structure of the product. We are thus forced to conclude that stereospecificity is inherent in the catalytic action of enzymes, and that enzymes would not be equally efficient as catalysts of non-stereospecific reactions.

Stereochemical studies of the type described in this article do not, and by themselves cannot, prove particular reaction mechanisms. They do, however, demonstrate unique spatial relations between substrate and substrate; substrate and coenzyme; entering and leaving group. We must therefore regard the precise spatial orientation of a substrate, or at least of the reactive portion of its molecule, at the active site of an enzyme as a part of the catalysis.

A chemist seeking to accelerate (without raising the temperature!) a reaction in solution has a choice of several methods. He may, for example, increase the concentration of the reactants; he may tamper with the the polarity of the solvent and with its capacity to form hydrogen bonds or to solvate ionic species; he may introduce catalytic amounts of reagents which form reactive intermediates. What he cannot change is his dependence on random collision of molecules; and reactions which require the *simultaneous* collision, in correct spatial orientation, or more than two molecular species become too improbable to consider.

The active site of an enzyme can combine all the devices mentioned above and at the same time provide a matrix for the *simultaneous* presence, in correct orientation, of a relatively large number of molecules or groups that can undergo or facilitate reaction. It is easy to see that catalysis of this type is dependent upon the reaction following a specific stereochemical pathway: if it did not, some at least of the groups would be misplaced. One might compare the enzymic reaction to the progress of a racing eight, with each man in his proper position and all pulling together, and contrast this with reactions in solution, where the rowers are continually moving in and out of the boat and may sit facing in any direction. Thus it would not be surprising to find that some enzymic reactions proceed by mechanisms which, in solution, would be too improbable to concert. On this view the spatial precision of an enzyme is the basis of its catalytic activity, and in studying enzymic reactions we are moving from van't Hoff's and Le Bel's stereochemistry of molecules to the dynamic stereochemistry of reactive aggregations.

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