SPECIFICATION OF THE STEREOSPECIFICITY OF SOME OXIDO-REDUCTASES BY DIAMOND LATTICE SECTIONS

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Our work on the stereospecificity of microbiological reductions of alicyclic ketones led us to take an active interest in the stereospecificity of the enzymic reduction of carbonyl compounds and the enzymic oxidation of alcohols. Some micro-organisms, *e.g. Curvularia falcata*, reduce a great number of mono-, bi- and tri-cyclic ketones, of widely varying structures, to the corresponding alcohols[1]. If such a microbiological reduction engenders a new asymmetric carbon atom, then often only one of the two possible epimeric products is formed. We call this type of stereospecificity "product stereospecificity", thus distinguishing it from "substrate stereospecificity", a term reserved for the selectivity of the enzyme for enantiomeric or diastereomeric substrates.

Such highly stereospecific microbiological reductions of racemic or meso carbonyl compounds lead to mixtures of optically active diastereomeric alcohols which can be separated by chromatography. *Figure 1* shows the

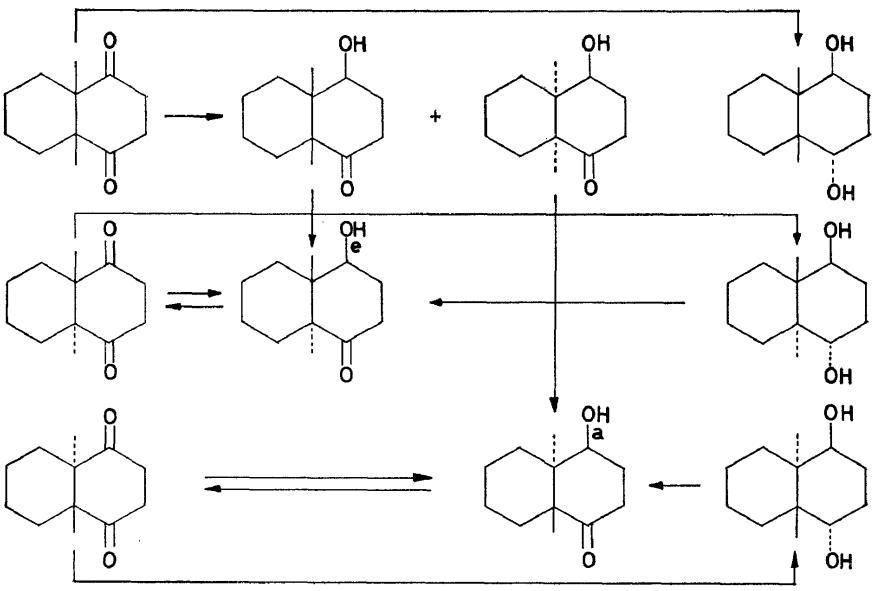

Figure 1. Absolute configuration of the products obtained by the microbial reduction of stereoisomeric decalin-1, 4-diones by *Curvularia falcata*

products obtained in the reduction of the stereoisomeric decalin-1, 4-diones by growing or resting cultures of *Curvularia falcata*[2]. The projection formulae in this figure represent the absolute configurations of the compounds in question, and these were determined unambiguously by several methods.

The optically active alcohols obtained by such microbiological reductions are exceedingly useful; they can be used, on the one hand, as substrates in the investigation of the stereospecificity of microbiological or enzymic oxidations, or, on the other hand, they can be oxidized chemically to the corresponding optically active ketones which in turn are important substrates in the study of the stereospecificity of microbiological or enzymic reductions.

Comparison of the absolute configuration of a great number of compounds obtained by reduction with cultures of *Curvularia falcata* showed that the "product stereospecificity" of the reduction can be represented by the very simple scheme shown in *Figure 2*[3, 4, 5]. In this L (large) and s (small) represent, respectively, a bulky and a small group adjacent to the carbonyl. That numerous facts can be rationalized in terms of such a simple scheme

Figure 2. Steric course of the microbial reductions of carbonyl compounds by *Curvularia falcata*

indicates that a simple mechanism of stereospecificity, worthy of more thorough investigation, must exist. We thought that such an investigation could give us a deeper insight not only into the mechanism of stereospecificity of the oxido-reductases but also into the mechanism of stereospecificity of enzymic reactions in general.

It was readily demonstrated that the cell-free extracts from the mycelia of *Curvularia falcata* contain an enzyme system which reduces alicyclic substrates with the same stereospecificity as both the growing and resting cultures. Application to such cell-free extracts of the procedures usually employed for the isolation, concentration, separation, and purification of enzymes, led to the detection of several enzymes. These enzymes required reduced nicotinamide-adenine dinucleotide phosphate (NADPH = TPNH) as coenzyme, and the activity of various preparations was easily measured photocolorimetrically using that coenzyme. In these activity measurements, cyclohexanone and the enantiomeric *trans*-decalin-1, 4-diones were employed as substrates.

By precipitation with ammonium sulphate, dialysis, and chromatography on diethylaminoethyl cellulose (DEAE) and "hydroxyl-apatite" it was possible to obtain a highly purified oxido-reductase, which we call "a-oxido-reductase". The purest fractions, which all had practically the same specific activity, were shown by disc electrophoresis to contain some foreign protein. However, in all probability, they contained only one enzyme. As a criterion of enzymic purity we used the ratio of the initial reaction rates obtained with the enantiomeric *trans*-decalin-1,4-diones. This ratio changes

considerably with different preparations obtained at the beginning of the isolation and purification procedure, but remains constant for those isolated in the latter stages, despite a ten-fold increase in the specific activity.

It is of interest to mention at this point that extracts from a large number of different micro-organisms, and homogenates from organs of higher animals, were investigated using cyclohexanone or the enantiomeric *trans*-decalin-1,4-diones as substrate, and NADPH or reduced nicotinamide-adenine dinucleotide (NADH) as coenzyme. In this way it was found that analogous oxido-reductases are widely distributed in Nature and that different micro-organisms, or even different strains of the same species, can differ markedly in their "substrate stereospecificity". This is illustrated by *Figure 3* which shows the ratios of the reduction rates of (+)- and (−)-*trans*-decalin-1,4-

	U_+/U_-
MUCOR sp.	14
CURVULARIA falcata	2–5
PSEUDOMONAS schuylkilliensis	2·7
schuylkilliensis	0·7
putida	0·1
sp.	0·05

Figure 3. Stereospecificity of cell-free extracts from different micro-organisms for (+)- and (−)-*trans*-decalin-1, 4-diones

diones for cell-free extracts from 5 different species, and for two strains of one of these. The observed differences in "substrate stereospecificity" are due partly to the presence of several different enzymes in the extracts, and partly to the fact that single enzymes react with enantiomeric substrates at different rates. We were able to isolate and purify from such extracts and homogenates several new oxido-reductases which we have used for our studies of enzyme stereospecificity. Using the enantiomeric *trans*-decalin-1,4-diones as substrates and NADPH as coenzyme it was possible to detect, *e.g.* in pig liver, an oxido-reductase which seems to differ from the numerous other oxido-reductases previously isolated from liver. The two most common alcohol dehydrogenases, namely the yeast alcohol dehydrogenase (YAD) and the horse liver alcohol dehydrogenase (LAD), do not reduce *trans*-decalin-1,4-diones, or oxidize the corresponding alcohols, though it has been known for some time that LAD reacts with cyclohexanone and some of its derivatives. Since LAD is one of the best known oxido-reductases we have included it in our investigation.

In the biogenesis of natural products the reduction of a carbonyl group is one of the most common steps, and so practically every organism must have enzymic systems which can perform this function. A multipurpose enzyme would be very economical in such a repeatedly occurring reaction. However, from experience, even such multipurpose enzymes as our oxido-reductases often react with a high degree of stereospecificity. It was imperative, therefore, to ascertain what features in a given molecule are responsible for the stereospecificity. To do this we used a large number of optically active substrates, which we either had at our disposal, or which we

prepared by biological reactions. The increasing number of oxido-reductases of different origin, and the variety of substrates used for such studies, naturally made it more and more difficult to keep a record of all the experimental facts and to draw any general conclusions from them. In order to overcome these difficulties we developed the following procedure. Initial reaction rates of enzymic reduction or oxidation were determined under comparable conditions (*i.e.* concentration of enzyme, coenzyme and substrate, pH *etc.*) with a standard series of cyclohexane and decalin derivatives of known absolute configuration. The carbon skeletons of these substrates, which are built from chair forms of cyclohexane, can be regarded as portions cut out of diamond lattice. By superposing the skeletons of all the substrates which react at a measurable rate with the enzyme in question, and by always using the same position for the reacting carbon and oxygen atoms, a diamond lattice section, characteristic of the enzyme, is obtained. In our opinion this section defines the space in the transition state of the reacting complex which is not occupied by the enzyme, coenzyme, inhibitors, or tightly bound solvent. A consideration of the characteristic diamond lattice section, and the skeletons of substrates which do not react, then furnishes important information concerning steric hindrance in the reacting complex.

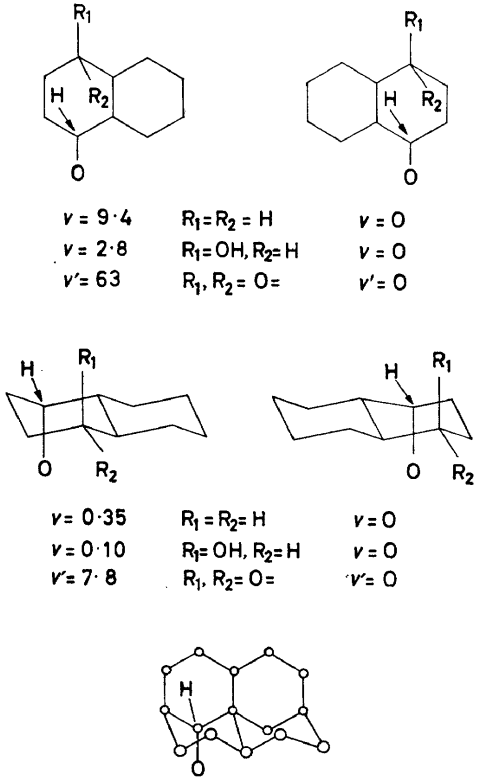

Figure 4. Derivation of the diamond lattice section for a-oxido-reductase from *Curvularia falcata*: relative reduction rates of *trans*-decal-1-ones

An example of our procedure is given in the following figures. The enzyme used is the a-oxido-reductase from *Curvularia falcata*. *Figure 4* shows the relative rates of reduction of several *trans*-decal-1-ones (to the corresponding *trans*-decal-1-ols), together with projection formulae; the latter are drawn in such a way that they can be considered as part of the diamond lattice. The location of the reacting carbon and oxygen atom corresponds to that in the alcohols and not in the ketones, since the rates of enzymic reductions reflect the building up of steric strains in the corresponding alcohols. Thus the reduction of a carbonyl group may be completely prevented if there are strong steric strains in the corresponding alcohol *i.e.* strong 1,3-interactions between bulky axial groups (*e.g.* methyl) and the axial hydroxyl group. Similarly the reduction can be prevented if the presence of a bulky axial group in position 3, with respect to the carbonyl, hinders the axial attack of the hydrogen from the dihydro-pyridine nucleus of the coenzyme. We therefore conclude that the geometry of the transition state resembles more that of the alcohol than that of the carbonyl compound, and postulate that the axial positions on C–3 and C–5, with respect to the reacting carbonyl group, in the substrates should be occupied by small atoms (for instance hydrogens). The superposition of the two skeletons of reacting compounds gives us the first

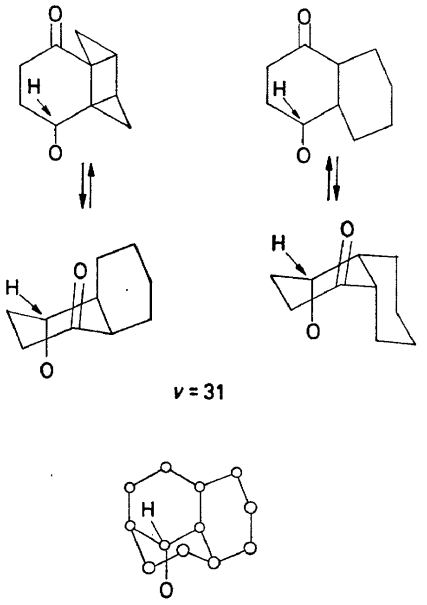

Figure 5. Derivation of the diamond lattice section for a-oxido-reductase from *Curvularia falcata*: relative reduction rates of *cis*-decal-1-one; the enantiomeric transition states for reactions with $v = 0$ are not represented

part of the characteristic diamond lattice section. Further information, summarized in *Figure 5*, has been furnished by the enzymic reduction of the *cis*-decalin-1,4-diones. The interpretation of the results obtained with this compound is complicated by the fact that *cis*-decalin derivatives are conformationally unstable, and two conformations have to be considered for

every transition state. One of these can, however, always be neglected since in it one of the axial positions (on C–3 with respect to the reacting carbonyl) is occupied by a bulky group (fragment of the ring). The results obtained by the reduction of two enantiomeric *trans*-decal-2-ones are summarized in *Figure 6*. In this case it would appear that the reduction is

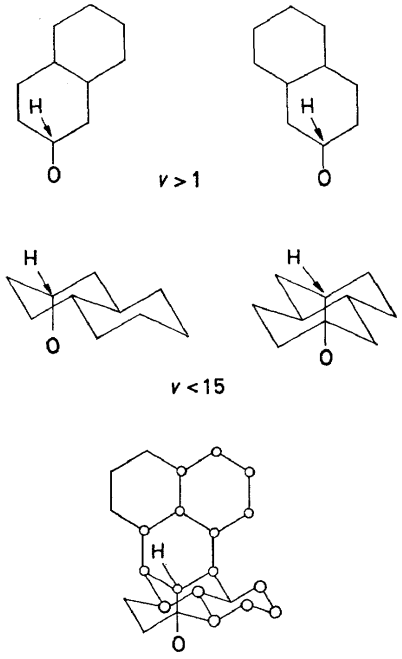

Figure 6. Derivation of the diamond lattice section for a-oxido-reductase from *Curvularia falcata*: relative reduction rates of *trans*-decal-2-ones

not so highly stereospecific as it is for the decal-1-ones, however the transition states, represented on the left hand of the figure, are none the less strongly preferred.

The characteristic diamond lattice section of the a-oxido-reductase from *Curvularia falcata* obtained by the superposition of the three partial sections is shown in *Figure 7*. The most interesting feature is its direction of development. Thus, starting at the reacting carbon it expands mainly in the three directions corresponding to the positive sides of the three conventional x, y and z coordinate axes and therefore there exist boundaries parallel to the xy, yz, and xz planes.

As a second illustration of the derivation of the characteristic diamond lattice section, we will consider briefly the case of the horse liver alcohol dehydrogenase. This well known, extensively investigated, crystalline enzyme requires nicotinamide-adenine dinucleotide (NAD = DPN or NADH = DPNH) as coenzyme. It does not react with the *trans*-decal-1-one derivatives, used in the present work. However, as shown in *Figure 8*, it does

react with cyclohexanone and cyclohexanol and some of their derivatives. Based on the information obtained with these substrates, suitable decalin derivatives could be selected (*cf. Figure 9*) which enabled us to extend the

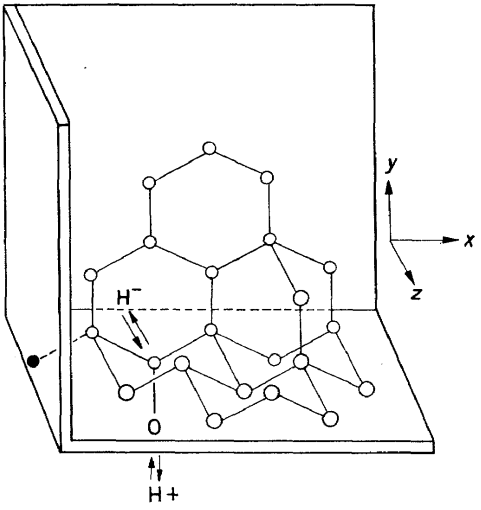

Figure 7. Characteristic diamond lattice section for a-oxido-reductase from *Curvularia falcata*: ● = "forbidden position"

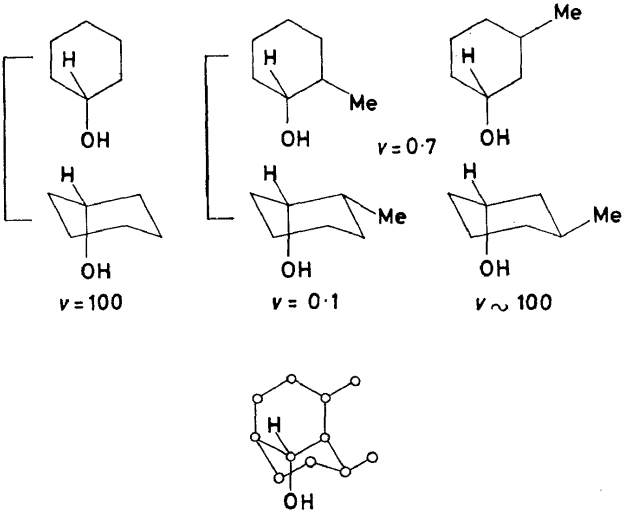

Figure 8. Derivation of the diamond lattice section for horse liver alcohol dehydrogenase: relative oxidation rates of cyclohexanols

characteristic diamond section depicted in *Figure 10* so far that the difference in stereospecificity between the horse liver alcohol dehydrogenase and the a-oxido-reductase from *Curvularia falcata* was clearly noticeable: the horse liver alcohol dehydrogenase reacts only with substrates in which the equatorial

position on C–2 on the right hand side of cyclohexanone or cyclohexanol (marked with ●) is unsubstituted. It is also noteworthy that the a-oxidoreductase transfers, preferentially, the axial hydrogen of the substrate (and is therefore called an a-enzyme), whereas in the case of liver alcohol

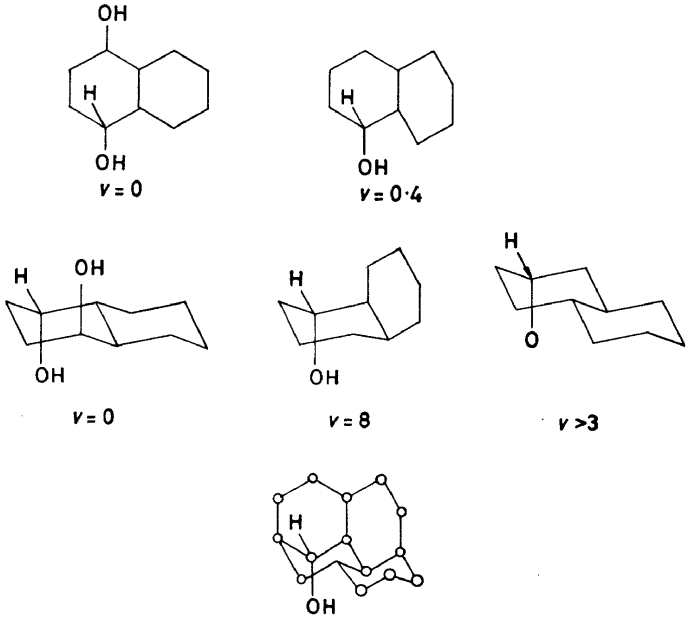

Figure 9. Derivation of the diamond lattice section for horse liver alcohol dehydrogenase: relative oxidation and reduction rates for decalin derivatives

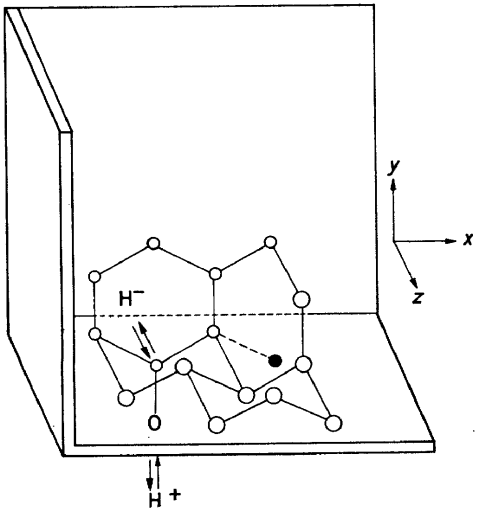

Figure 10. Characteristic diamond lattice section for horse liver alcohol dehydrogenase: ● = "forbidden position"

dehydrogenase it is the equatorial hydrogen which is transferred the faster (this enzyme is therefore classed as an e-enzyme).

Having considered the way in which the characteristic diamond lattice sections are derived, we will now consider their applications. It was mentioned earlier that they should represent the space available to the substrates in the transition state of the hydrogen transfer, that is the space which is free (or can be made free) from enzyme, coenzyme, inhibitors, or tightly bound solvent. From this it is possible to deduce that substrates which are not built up from chair forms of cyclohexane (*e.g.* cyclohexene or cyclopentane derivatives), and which therefore cannot be cut out of the diamond lattice, should react with the enzyme provided they fit into the space occupied by the characteristic diamond lattice section, and that they should not react if they cover prohibited areas. Up to now all the experimental evidence supports this deduction, and therefore the characteristic diamond lattice sections can, with caution, be used to predict whether or not a particular substrate will react with the enzyme in question.

The most important field of application of characteristic diamond lattice sections, however, is in the elucidation of the mechanism of enzyme action which, in turn, is so intimately bound up with stereospecificity. Obviously any proposed mechanism, or model, of enzyme action must not conflict with the spatial demands of the characteristic diamond lattice section.

At this stage it would be profitable to review briefly the known facts and the relevant theories concerning the mechanism of the action of oxido-reductases which require pyridine nucleotides as coenzymes. From kinetic and other evidence it would appear that the ternary complexes, enzyme-coenzyme-substrate, are formed in the so called "compulsory order"[6]. First of all the enzyme and the coenzyme (NAD, NADH, NADP, or NADPH) form a "binary complex", and this then reacts with the substrate to give the "ternary complex". The hydrogen transfer now takes place, and a second "ternary complex" is formed, which in turn dissociates into the product and a new "binary complex": enzyme-reacted coenzyme. Finally, the latter dissociates into its components.

From the extensive studies of Westheimer, Vennesland, and others[7] with labelled coenzymes and substrates it is evident that the hydrogen transfer takes place from the position 4 of the dihydro-pyridine portion of the reduced coenzyme to the substrate, or from the substrate to the 4-position of the pyridine nucleus of the oxidized coenzyme. This hydrogen transfer is stereospecific not only with respect to substrate but also with respect to coenzyme: the two hydrogens in the position 4 of the dihydro-pyridine portion of the reduced coenzyme are enzymically not equivalent, nor are the two sides of the pyridine nucleus in oxidized coenzyme. Enzymes activate exclusively either one of the hydrogens of the reduced coenzyme, and the corresponding side of the oxidized coenzyme, or the other.

Thanks to the ingenious work of Cornforth, Popják *et al.*[8], we know the absolute configuration of these two hydrogens or sides usually designated as A and B respectively (*cf. Figure 11*).

Now we can try to locate the substrate with respect to the coenzyme in the transition state for the hydrogen transfer. In order to explain the stereospecificity of the hydrogen transfer to or from the coenzyme we make two

postulates; firstly, that one side of the coenzyme is shielded stereospecifically by the protein; secondly, we assume that the transfer will be most favourable, energetically, when the overlap of the participating orbitals is strongest and the non-bonded repulsion least. Furthermore, in constructing a model for the transition state, it has to be remembered that both reductions and

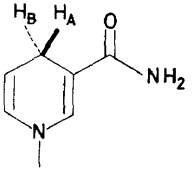

Figure 11. Absolute configuration of the C-4 of pyridine nucleotide coenzymes

oxidations go through the same transition state, but that the geometry of the latter resembles more closely that of the alcohol than that of the carbonyl compound (*cf.* p. 123).

In the transition state, appropriate to hydrogen transfer, there are, schematically two principal ways of locating a carbonyl compound, or an alcohol, with respect to the pyridine nucleus of the coenzyme. In the first,

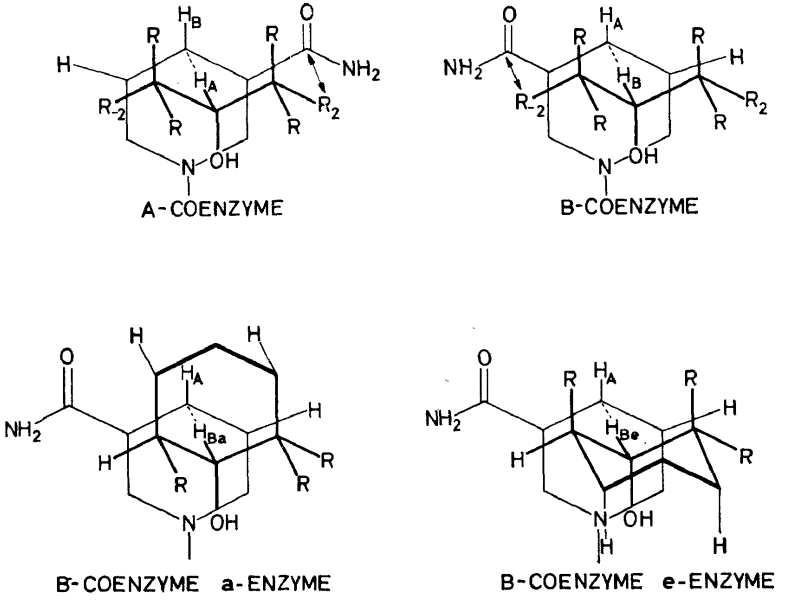

Figure 12. Transition states of hydrogen transfer

the oxygen of the substrate points towards the nitrogen of the pyridine in the coenzyme; in the second, it points away from it. The first, shown in *Figure 12*, seems to be more favourable because of the smaller non-bonded interactions. We do not postulate, that the second one is impossible, but

for the sake of simplicity we shall not discuss it here. Furthermore, we assume that in a favourable transition state, in order to minimize the non-bonded interactions, the bulky group (L), of the substrate, should be opposite to the hydrogen on C–5 of the pyridine nucleus in the coenzymes, whereas the small group (s) should be opposite to the bulky carboxyamide group of the coenzyme as shown in the figure for the transfer of H_A and of H_B. This in turn means that there is a "forbidden position" in the diamond lattice for larger groups, which of course is different for enzymes using H_A, *e.g.* horse liver alcohol dehydrogenase, from that for enzymes using H_B, *e.g.* a-oxido-reductase from *Curvularia falcata.* The location of the "forbidden positions" of these two enzymes, shown in *Figure 13*, is in agreement with the one which can be predicted from the absolute configuration of the

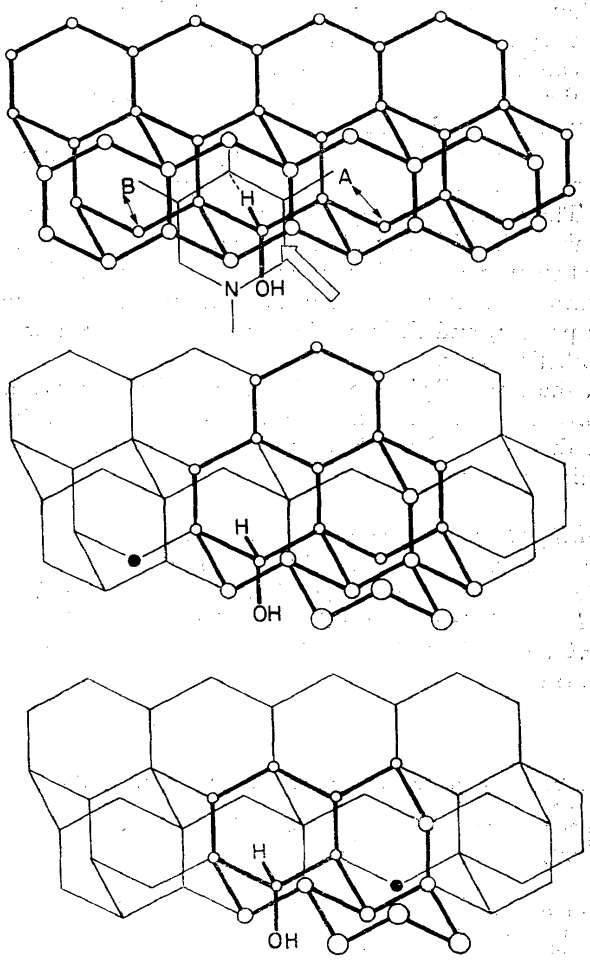

Figure 13. Position of the pyridine nucleus with respect to the diamond lattice and positions of the characteristic diamond lattice sections of a-oxido-reductase and liver alcohol dehydrogenase in the diamond lattice: ● = "forbidden position"

substrates and of C–4 of the dihydropyridine nucleus in coenzymes as determined by Cornforth, Popják *et al.*[8]. Thus an important part of stereospecificity seems to be determined by non-bonded interactions of substrate and coenzyme in the hydrogen transfer transition state.

On the other hand, the characteristic diamond lattice sections of the two enzymes clearly indicate that these interactions cannot be the sole factors determining the stereospecificity; evidently the structure of the enzyme protein plays a decisive rôle. There is little known about the primary, secondary and tertiary structures of oxido-reductases, and it is therefore not possible at present to discuss in any detail the influence of these structures on stereospecificity. One should not forget however, that the active site of the coenzyme-enzyme complex must in some way be the "negative" of the characteristic diamond lattice section. If the positions of the reacting carbon and oxygen are defined with respect to the coenzyme by the geometry of the transition state of the hydrogen transfer, then this "negative" can be described as a chiral corner. In this corner the ring skeletons of the substrate can be either parallel to the plane of the pyridine nucleus (*xy*-plane), which is the case for axial hydrogen transfer (preferred in a-enzymes); or perpendicular to it (*i.e.* parallel to the *xz*-plane), which is the case for equatorial hydrogen transfer (preferred in e-enzymes) (*cf Figure 12*). The position of the *yz*-plane left or right of the reacting carbon then determines the chirality of the "negative" corner and consequently the "product stereospecificity" in the reduction of the carbonyl compound. All these features of stereospecificity depend on the unknown structure of the enzyme protein, and their origin can at present be discussed only in a highly hypothetical way.

Although the over-all picture is therefore still vague, we hope that our work will have helped to rationalize at least some aspects of stereospecificity, and it is for this reason that I have presented the results of our incomplete studies to this conference.

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