ENZYME REACTION STEREOSPECIFICITY: A CRITICAL REVIEW

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INTRODUCTION

All enzyme-catalyzed reactions are stereospecific. Evidently the high catalytic rates of enzymes require the exact three-dimensional fit of substrates and intermediates that confers absolute specificity in the orientation of groups at the critical reaction center of the substrate. It is noteworthy that even in the interconversion of *D* and *L* amino acids, as with proline racemase,[1] every step of the reaction is spatially defined. One does not find that the proton is removed from C-2 and the resulting bound carbanion collapses randomly (nonstereospecifically) to racemic products. Rather, Cardinale and Abeles[1] have shown that protonation of the *bound* carbanion is stereospecific and makes use of a unique proton on the enzyme. This is shown schematically in Figure 1, where the likely possibility that the negative charge of the carbanion is delocalized by resonance is ignored. The absolute stereospecificity of enzymatic reaction steps, therefore, should not be confused with the matter of substrate specificity. An interesting example of this distinction between substrate specificity and reaction specificity comes from the work on propanediol

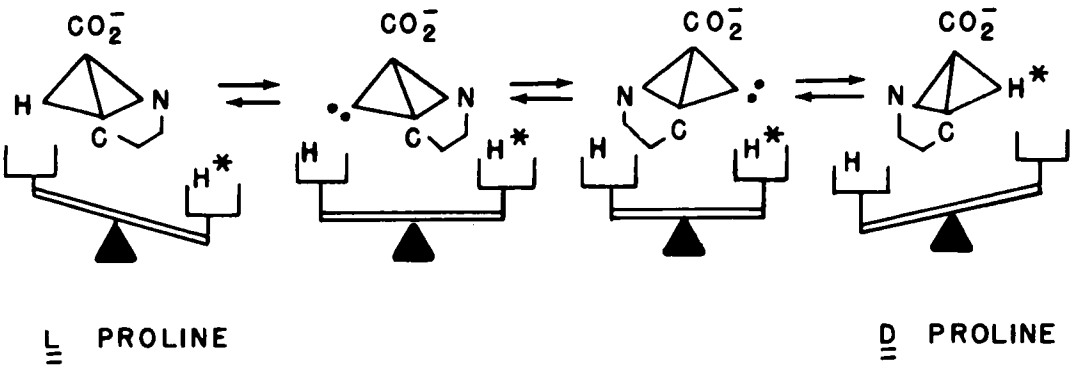
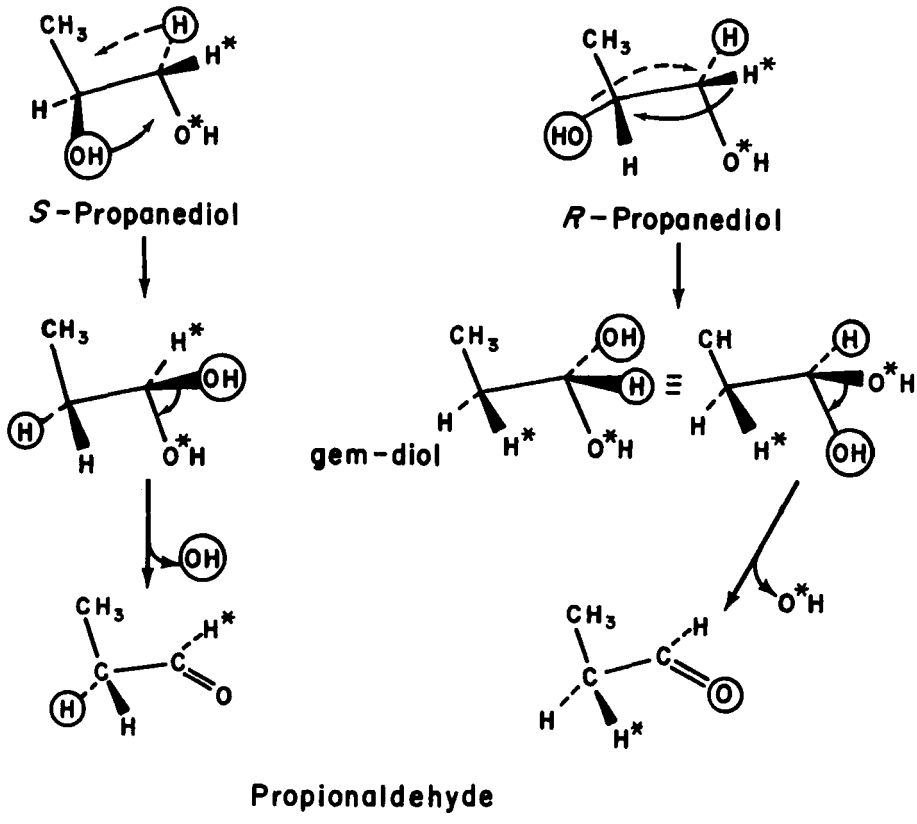


FIGURE 1.

dehydrase[2-5] in which both *R*- and *S*-propanediol are converted to propionaldehyde, but no step in the reaction, not even the dehydration of the presumed gem-diol intermediate, is left to chance, as shown by following isotopic hydrogen from the pro-*R* position of C-1 and ^{18}O from the primary hydroxyl of the two substrates. The enzyme specifically uses a different C-1 hydrogen depending on the configuration at C-2. Presumably the two propanediols fit the active site differently, thus orienting the alternate hydrogens for abstraction and transfer (Figure 2). The hydroxide migration from C-2 to C-1 that follows can be described as a stereospecific transfer since the gem-diol that is subsequently dehydrated gives rise to propionaldehyde that has either lost all or retained all the ^{18}O of the *R*- or *S*-substrates, respectively. The dehydration step must be enzymatic since it is stereospecific.

If, indeed, all enzyme catalyzed reactions are stereospecific, it follows that all reactions that are not stereospecific must have a critical step that is not enzyme-catalyzed. The first example of this was with another diol dehydrase, one that had evolved an entirely different mechanism of dehydration. It was shown[6] that the product of the 6-P gluconate dehydratase reaction that was carried out in the presence of D_2O contained one atom of deuterium that was randomly distributed between the two C-3 positions (Figure 3). That the production of the observed products was nonenzymatic was consistent with the finding that 2-keto-3-deoxy-6-P gluconate is neither a substrate nor an inhibitor of the reaction; i.e., it is not recognized by the enzyme. Evidently the enzymatic product is the free enol which randomly ketonizes.

An apparent exception to the statement that nonstereospecificity implies a nonenzymatic step occurs whenever positional randomization occurs by bond rotation in an intermediate. This is most often encountered as the result of transient methyl-group formation. An excellent example is seen in coenzyme B_{12}-dependent reactions. It is now supposed that in the course of catalysis the two C-5′ hydrogens of the deoxyadenosine residue of the coenzyme, which are distinguishable by NMR,[7] pass through a state in which randomization of position occurs before being regenerated in product formation. Thus a hydrogen from sub-



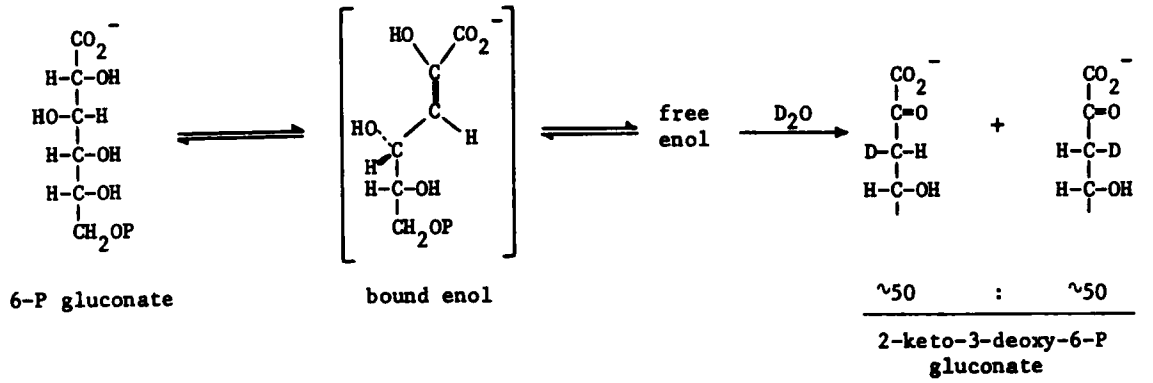


FIGURE 3.

strate becomes trapped in the C-5′ position,[8,9] and isotope in either C-5′ hydrogen position can be shown to react further in producing product.[10,11] Isotope partitioning is evident[12] through a state where three hydrogens can be made indistinguishable by torsion around the C-4′-C-5′ bond. Other examples of groups for which torsion leads to superposition of different members are: $-C\overset{O}{\underset{O}{<}}$ - and $-P\overset{O^-}{\underset{O^-}{=}}O$. The two hydrogens of $-C\overset{H}{\underset{H}{<}}$, the methylene carbanion, may become indistinguishable through inversion and torsion if there is no resonance stabilization. Protonation of a methylene carbanion makes the hydrogens indistinguishable by torsion alone. The whole molecule of squalene has a C_2 axis of symmetry. Thus, when it is released as an enzymatic product, the ends of the molecule are indistinguishable in further reactions; Etemadi et al.[13] have used this fact to examine the possibility that squalene synthase and squalene oxidase may be coupled through bound squalene. The randomization of radioactivity in positions of fumarate and mannitol, but not citrate or glycerol, in metabolism is well known.

The absolute specificity of enzymatic reaction steps tends to obscure the reaction mechanism. It severely limits the applicability of some important tools that physical organic chemists use in studying mechanism. For example, Bender and Heck[14] were able to conclude that general base-catalyzed ester hydrolysis proceeds through a tetrahedral intermediate (Figure 4) since ^{18}O-labeled ester lost isotope to the medium at a rate that could be predicted from the hydrolysis kinetics. On the other hand, the deacylation (hydrolysis) of the serine ester intermediate of chymotrypsin labeled with ^{18}O in the ester carbonyl group was not accompanied by oxygen exchange with solvent, whereas that of a suitable serine amide ester model did show exchange. The failure to show exchange in an esterase, even if it were required by kinetic arguments, would not exclude a tetrahedral intermediate since one would expect the reverse of step 1 to be stereospecific on the enzyme. In addition, the occurrence of carbanion or carbonium ion intermediates in organic chemistry is often established by the occurrence of racemic products. The failure to show racemization in enzyme-catalyzed steps is the rule and cannot be used to exclude these forms as intermediates. It is an example of unheralded sophistication that whereas at one time we considered the retention of configuration in the glycosidases to signify a double displacement mechanism (Figure 5), little use of the stereochemical information was made in considering the lysozyme mechanism which is believed to involve an enzyme-carbonium ion intermediate.[15]

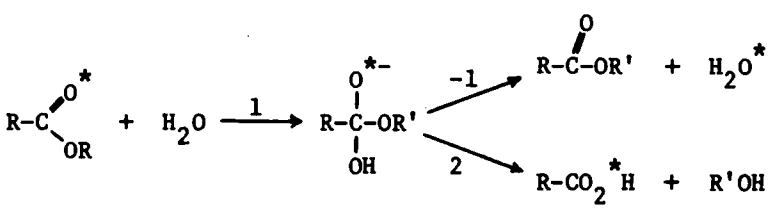


FIGURE 4.

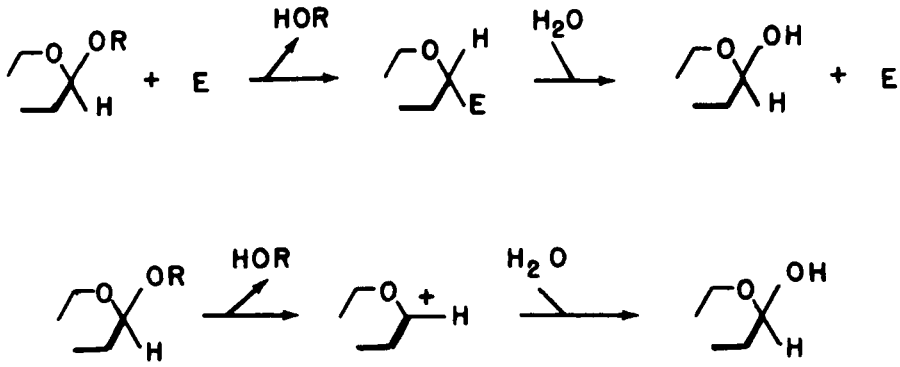
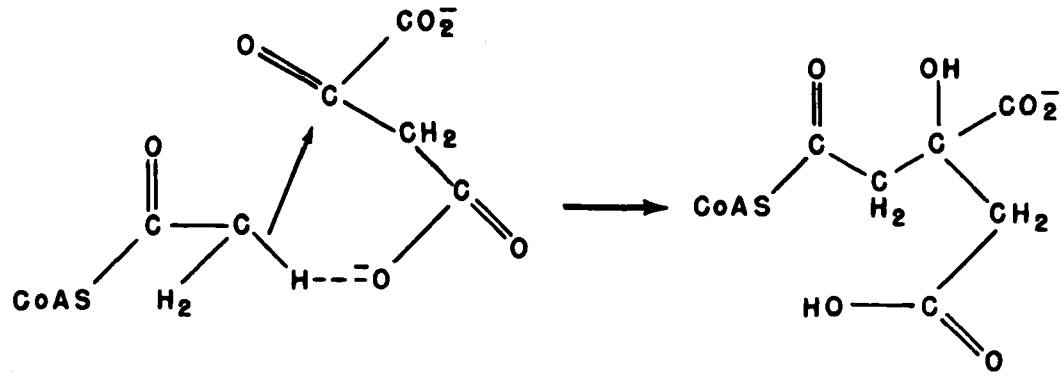


FIGURE 5.

We now know the absolute stereochemical course of a large number of the recognized biochemical reactions. Several important papers and reviews, new and old, can be consulted which deal with the symmetry properties of molecules,[16-18] approaches used to establish stereochemistry of important reference compounds,[18,19] systems for naming positions and aspects of molecules,[18,20] and compilations of results of 20 years of investigation of the absolute stereochemistry of numerous reactions.[19,21,22] Given the imposed stereospecificity of all enzymatic reactions, one may well ask what the binary choice system that is reaction stereochemistry can contribute to our understanding of the mechanism on natural history of enzymes. We hope to show that by extending our view to include the spectrum of reactions of a given type we may be able to infer both mechanistic and structural features.

EXCLUSION OF MECHANISMS BY STEREOCHEMICAL COURSE

In favorable cases the stereochemical course of fully determined enzyme-catalyzed reaction can be used to exclude mechanisms. Two cases in which stereochemical studies served to disprove a currently presumed mechanism are illustrated in Figures 6 and 7. In the first, which concerns citrate synthase, the dependence of oxalacetate, or an analog such as malate, for the proton activation of acetyl-CoA, was attributed to participation of the β-carboxylate group of the oxalacetate as a specific base.[23] The course of the reaction was subsequently shown to be that of inversion, which clearly is impossible to achieve with the proposed mechanism. With models it can be shown that six or seven carbon-carbon bond distances must intervene between the proton abstracting base and



ACETYL CoA + OXALACETATE ⟶ CITRYL CoA

FIGURE 6.

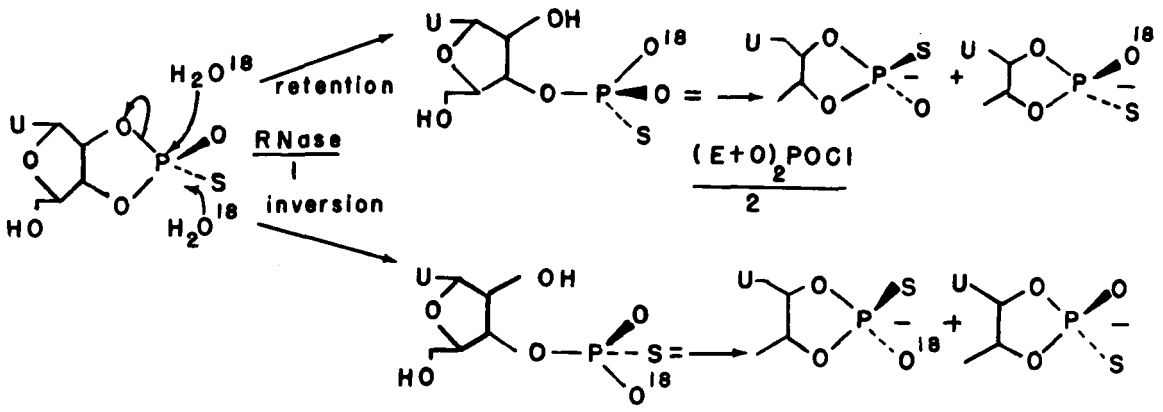


FIGURE 7.

the replacing electrophile in order to achieve such an intramolecular catalysis with inversion. A second study has served to clarify the ribonuclease A reaction mechanism by showing that the hydrolysis of the pyrimidine riboside-2′,3′ cyclic phosphodiester proceeds with inversion of configuration at the phosphorus.[24] In this work a uridine-2′,3′ cyclic phosphorothioate isomer was used for hydrolysis in $H_2{}^{18}O$ by ribonuclease, step 1. Subsequent chemical ring closure, step 2, was known to proceed with inversion; therefore, the stereochemistry of the first step could be determined by reisolation of the cyclic isomers and determination of their ^{18}O content. ^{18}O was not present in the isomer used in the enzymatic step, but rather in the other cyclic isomer. To lose the ^{18}O of the nucleotide by a reaction that involves inversion requires that the introduction of ^{18}O be with inversion. Hence, the authors exclude all mechanisms which require a single group to act first as a general base toward the attacking water molecule and then as a general acid toward the 2′ oxygen, as in the upper path shown in Figure 7. These data preclude pseudorotation as a step in bond cleavage and help to identify the position that H_2O must occupy in models derived from crystallographic studies of ribonuclease.

Selective Pressures and Stereochemical Course

The stereochemical course of an enzymatic reaction is undoubtedly fixed by the orientation of binding and catalytic elements of the protein catalyst. It is not possible *a priori* to decide whether the detailed stereochemical relation between reactant and product has any mechanistic significance beyond the three-dimensional structural organization of the groups on the protein. A way to approach the question of the mechanistic significance of stereochemical course is to compare the stereochemistry of a wide range of enzymes that catalyze the same reaction or reactions of the same chemical type. The principle implied is that the stereochemical course is expected to be widely conserved if it provides a critical aspect of the mechanism.

There are two forces, not related to mechanism, that could lead to a wide-ranging conservation of stereochemical course in a reaction type. The first of these depends on selective pressures in evolution based on the chemical nature of the reactants and products. For example, all the transaminases that are known operate between substrates of the same stereochemical series, *L*-amino acids. It is clear from the evidence for a ping-pong mechanism in the transaminases that the two amino acids are not represented in a common transition state. The conservation of the *L*-specificity, however, is easily explained by the function of intermediary metabolism to deal with the amino acids to be made and degraded in relation to protein synthesis. Thus, the selective pressure of the substrate specificity of protein synthesis related to stereospecific elements in mRNA, ribosomes, and the enzymes concerned could have led to the disappearance of mixed specificity transaminases. Selective pressure based on metabolic use and economy will be a potential factor in the evolution of stereochemical reaction course whenever optically active metabolites are concerned.

In spite of this restriction, one may cite examples in which the course of a reaction leading to a chiral product seems to be clearly contrary to an economy-based natural selection. In these cases one suspects that other factors, perhaps mechanism, may determine the stereochemical

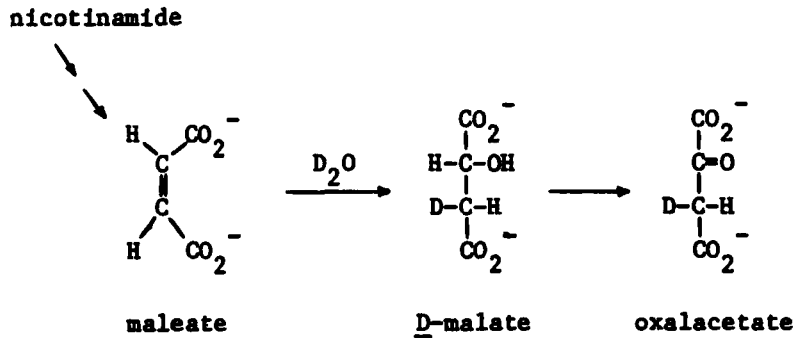


FIGURE 8.

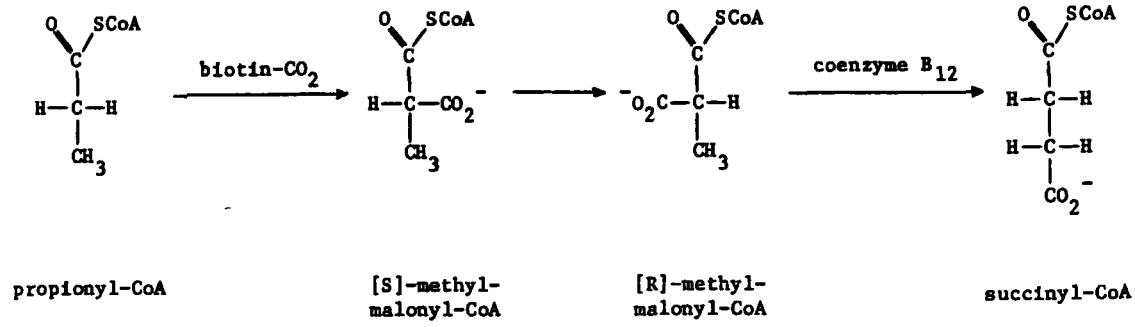


FIGURE 9.

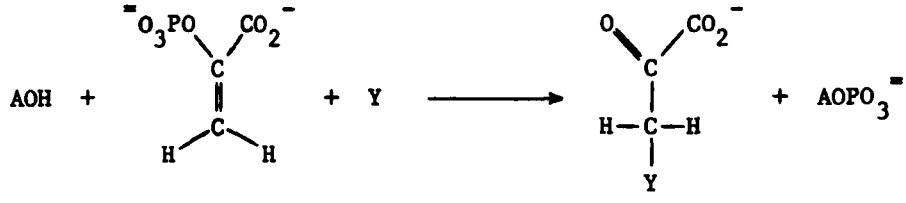


FIGURE 10.

course. Consider the peculiar path converting maleate to oxalacetate found in kidney (Figure 8).[25] The hydratase produces a "new" form of malate and hence seems to require a "new dehydrogenase," *D*-2 hydroxy acid dehydrogenase, rather than the regular malate dehydrogenase of long evolutionary history. Another example of inefficiency is seen in the main path of lipid degradation (Figure 9). The stereochemical course of the carboxylation step[26,27] imposes the need for an additional racemase that seems to function *only* to correct an apparently unnecessary choice at the prochiral methylene carbon.

A second factor that would lead to a conservation of stereochemical course in evolution is a high degree of permanence in the structure of the affinity site in relation to a second (donor or acceptor) site. An example of this seems to be given by the enzymes that catalyze allylic replacement of phosphate in reactions of phosphoenolpyruvate (Figure 10), where Y = CO_2, and AOH = H_2O, P_i, or GDP in the PEP carboxylases from peanut, propionobacter, and liver;[28] Y = H and AOH = ADP in pyruvate kinase;[29,30] and Y = erythrose-4-P in heptulonic-7-P synthase.[31] In all cases the addition occurs from below the plane, as written, from the 2 *si* face.*[20] There is no reason to suppose that the face of PEP that is oriented

*In the *re/si* system of Hanson,[20] the face of a trigonal carbon such as C-2 of PEP is designated by determining whether the substituents of that carbon, when ordered according to the Cahn, Ingold, Prelog sequence rules,[32] are seen in a clockwise, *re*, or counterclockwise, *si*, sequence. When identical ligands prevent the application of this rule, as for C_3 of PEP, the faces take the designation given of the adjoining trigonal carbon[20] – C-2 in this case.

toward the electrophilic component should be conserved in such a range of cell types for either mechanistic or utilitarian reasons. The products that would be formed from the alternative approach of the adduct would be the same. Rather, this finding suggests that these PEP enzymes diverged from a common source and that a common affinity site was retained much as is found for residues 70-80 of all cytochrome *c*'s, which are thought to represent a critical contact area for the heme and perhaps cytochrome oxidase.[33] If such studies do not inform us about mechanism, they have obvious relevance to the analysis of the natural history of the evolution of structure and function in the enzymes. This subject of the PEP enzymes will be considered further below.

The Special Importance of Achirality

There can be no selective pressure on the basis of metabolic use if alternate stereochemical courses produce the same molecule. For example, the condensation of acetyl-CoA and oxalacetate by citrate synthase is stereospecific. The alternate routes give rise to citrates which, except for the distribution of isotope from the substrates, are indistinguishable. Representatives of both routes of addition to the carbonyl group of oxalacetate have been reported (Figure 11).[34] The finding that both types of citrate synthase catalyze the displacement reaction with inversion of configuration[35,36] may be relevant to the reaction mechanism since it is found in many other condensation reactions of this general kind, as will be discussed further. In another example, the aldol cleavages of fructose diphosphate by muscle aldolase and of *L*-rhamnulose-1-P by a bacterial enzyme produce dihydroxyacetone-P, which would have different protons labeled from water (Figure 12a).[37,38] Both forms will be equally effective substrates for the other aldolase and for triose-P isomerase. However, the fact that both

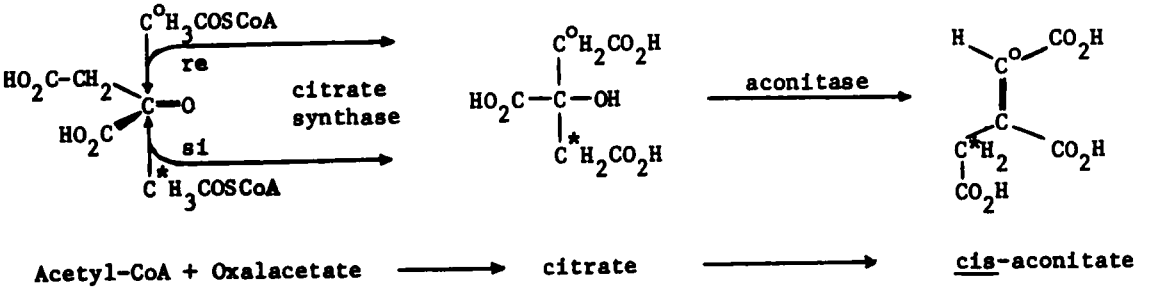


FIGURE 11.

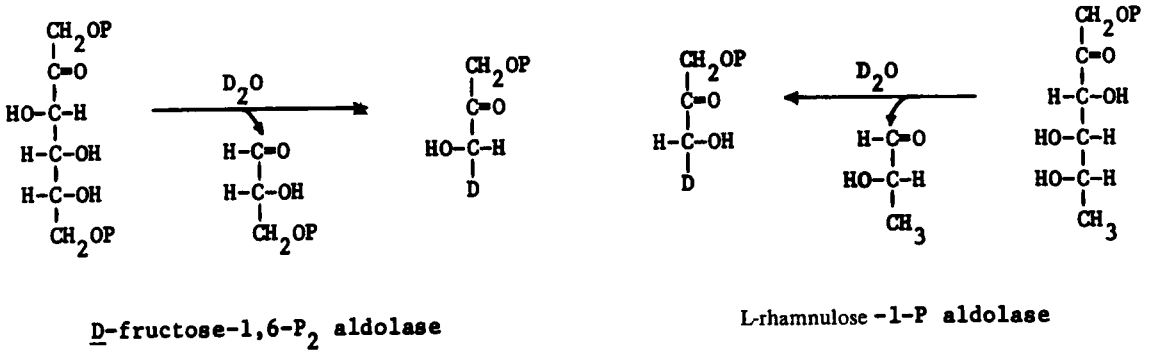


FIGURE 12a.

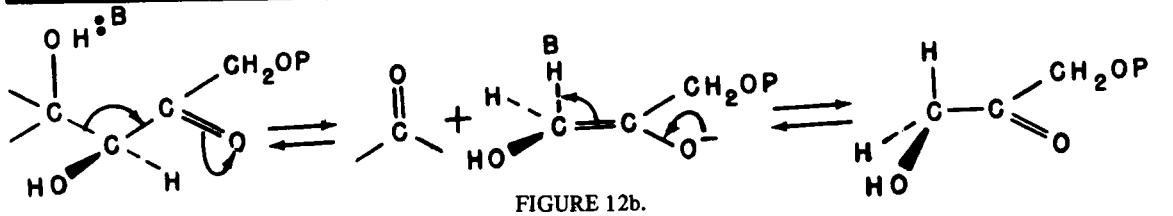


FIGURE 12b.

reactions lead to retention of configuration at the reactive carbon would suggest a mechanistic correlation (Figure 12b) if, indeed, other reactions of this type did the same. The correlation becomes the more interesting as the variation in substrate structure is great.

It is worth remembering that the reactions as we find them today probably evolved from simpler reactions. Thus, a strictly achiral reaction for which no selective pressure is expected may have evolved from a reaction of chiral reagents and hence may have been subject to selection pressures based on chirality. The evolution by gene duplication and mutation to produce an enzyme specific for achiral substrates could then carry through the stereochemical course of the antecedent. For example, today we recognize the citrate synthase reaction as a first step in the 1 carbon lengthening of an α-keto acid. The simplest example of this type, malate synthesis, involves the conversion of achiral reactants to *S*-malate by the addition of acetyl CoA to the *si* face of glyoxalate. This stereochemical course is subject to selective pressure since existing reactions may be expected to distinguish between *S*- and *R*- malates. The fact that the major form of citrate synthase uses the *si* face of oxalacetate for acetyl CoA approach to make achiral citrate may be dictated by the evolution of the citrate synthase-gene from a malate synthase-gene. In this case the reaction involving achiral reagents was subject to a selective pressure that was imposed on a cell containing an antecedent chiral reaction.

Eventually it may be possible to infer from their amino acid sequences whether enzymes that catalyze a particular type reaction diverged from a common antecedent. Whether the variations of substrate specificities within the group represent divergent or convergent evolution, it is postulated that the finding of a common stereochemical course implies either a common mechanism or a common disposition of interacting groups.

The Degree to which Stereochemical Course is Conserved in Evolution

It is clear that living forms of today represent but a short span of evolutionary development. For example, it has been shown that the subunits of glyceraldehyde-P dehydrogenases of yeast and rabbit will interact to form active hybrids.[39] Thus it should be no surprise that the reactions of the same substrate specificity will usually be shown to have the same stereospecificities. For example, the alcohol dehydrogenases of yeast and liver transfer the hydrogen from the 4′ pro-*R* position of NADH[40] to the *re* face of acetaldehyde.*[41] Clearly any genetic change that altered stereochemistry of either reactant would have no effect on the nature of the reaction.

The case of the two citrate synthases with different stereochemistries represents one of the rare examples in which a compound is formed by reactions of different stereochemical course.

A. Nucleophilic Substitution

A compilation of enzyme reactions involving nucleophilic substitution at tetrahedral-carbon deals mainly with reactions at a glycosidic bond. Other reactions such as alkyl transfer have not yet been clarified in terms of the stereochemistry of the transfer. Among the reactions at glycosidic bonds, there are no examples of random product stereochemistry; reactions go with either inversion or retention. The mutarotase reaction is not one of nucleophilic substitution since there is no exchange with $H_2{}^{18}O$.[42] For the enzymes that cleave disaccharides and polysaccharides, either hydrolytically or phosphorolytically, there is no obvious pattern of correlation. Retention and inversion occur with about equal frequency. For example, the phosphorylases for sucrose, maltose, and cellobiose, acting at α, α, and β linkages, respectively, produce glucose-1-P with α, β, and α stereochemistry.[43-45] On the other hand, all of the phosphorylases and pyrophosphorylases that are specific for N-riboside bonds invariably give inversion of configuration in the reaction. This is true since all of the purine, pyrimidine, and pyridine nucleoside bonds are β linkages, whereas the phospho- or pyrophosphoglycoside bonds to ribose or deoxyribose are invariably α. There are two simple mechanisms that might account for inversion in these reactions: direct nucleophilic displacement (the so-called single displacement mode),[46] or a carbonium ion intermediate in which the approaches of the base and phosphate occur from opposite faces, thus giving inversion. Clearly for the latter different specificity is required for sites accommodating P_i or PP_i on the

*That the same hydrogen of ethanol is transferred by both dehydrogenases is cited as unpublished results of A. Umani-Ronchi, H. Weber, and D. Arigoni, page 156, reference 19.

one hand, and an organic base on the other, so that inversion seems a reasonable consequence providing adequate space for the two sites in close proximity to the flattened pyranose ring. A distinction between the alternative mechanisms is suggested by the occurrence of base exchange in the absence of phosphate or P_i (PP_i) exchange in the absence of base. The single displacement mechanism can be considered unlikely where such exchanges are found. Although such exchanges have not always been sought, there are several examples in which one of the exchanges or arsenolysis of ribose-1-P has been demonstrated.[47-50] The cases where such partial reactions have been observed far exceed the failures.[51]

A critical criterion of a carbonium ion mechanism seems to be that applied by Dahlquist et al. to lysozyme,[15] namely, the occurrence of reaction retardation by substitution of deuterium at the glycosidic carbon. In order to see such an effect it is necessary that the glycosidic bond be broken in the rate-determining step (kinetic secondary isotope effect) or that the formation of the new bond be rate-determining (equilibrium secondary effect superimposed on an inverse kinetic secondary effect). The failure to observe an isotope effect, like the failure to show glycosyl transfer, is not sufficient evidence for discarding the carbonium ion mechanism, whereas the occurrence of either one is probably inconsistent with the so-called single displacement (S_N2) mechanism.

Sucrose phosphorylase is an enzyme which shows exchange of both the fructose portion and the phosphate portion of the sucrose and α-glucosyl-1-P, respectively,[43] implying an intermediate glucosyl group either covalently fixed or simply complexed as a carbonium ion to the enzyme. The overall stereochemistry, retention, does not by itself distinguish between these two mechanisms. However, the evidence for a covalent glucosyl intermediate strongly points to a double displacement reaction[52] which would provide retention.

There are few other examples of nucleophilic displacement for which stereochemical questions have been resolved. The recent study by Usher, Richardson, and Eckstein,[24] indicating inversion in the ribonuclease-catalyzed hydrolysis of a uridine-2′, 3′ cyclic phosphodiester analog, has been discussed earlier. Whether this general approach will be applicable to the many other phosphorous-centered reactions is of great interest.

B. Electrophilic Substitution

Most enzyme-catalyzed electrophilic substitutions on a tetrahedral carbon involve replacement of a proton. Of these reactions the great majority concern carbon centers that are adjacent to an electron withdrawing group, such as a carbonyl, carbimine, thioester, or carboxyl group. The inference that a carbanion intermediate is formed, in which the electron pair is delocalized onto the more electronegative element, seems secure for many of these reactions in that proton exchange has been shown to occur prior to the introduction of the entering electrophile. Thus, the reactions in this class should be viewed as either definitely involving the carbanion intermediate, if exchange has been established, or if exchange requires the complete reaction mixture, the SE1 pathway may still be operative with the second substrate serving a structural role rather than being involved in the transition state for proton abstraction. In Table 1 the enzymes have been arranged according to reaction type and the occurrence or absence of proton exchange in the partial reaction noted, where this information is available. In most of these cases a prochiral center is created or altered in the reaction.

Of these groups, the only one that fails to conform to a common stereochemical course within the group is the β oxidative decarboxylase series. Most of these enzymes have been shown to catalyze proton exchange from the decarboxylated product, indicating the production of an enol intermediate. This is shown for isocitrate dehydrogenase, where reduced pyridine nucleotide and Mg^{2+} are required, presumably for reasons of induced protein conformation (Figure 13). It is not yet known whether the enol has the *cis-* or the *trans-* structure (as written) – only that the proton and CO_2 approach from the same face.

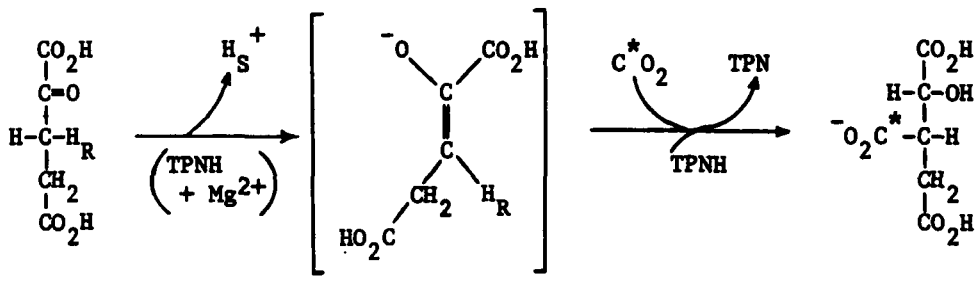
The aldolases and the amino acid decarboxylases have in common proton exchange in the absence of replacing groups and are characterized by retention of configuration in the substitution reaction. This latter, due to its invariance, may suggest that a single group serves both as a base to activate the -C-H bond and to add the proton subsequently to the carbonyl group during the condensation, Equation 12b. In opposition to this hypothesis is the observation of Rose et al.[73] that

TABLE 1

Stereochemistry of Proton Replacement

	Proton Exchange	Stereochemistry
Amino Acid Decarboxylases		
Glutamate	Yes[53]	–
Lysine	Yes[53]	–
Tyrosine	Yes[53]	Retention[54]
Histidine	–	Retention[55]
Serine (hydroxymethylase)	Yes[56]	Retention[57]
Aldolases*		
Fructose-1,6-diP (Schiff's base)	Yes[58]	Retention[37]
Fructose-1,6-diP (metal-dependent)	Yes[58]	Retention[37]
Rhamnose-1-P	Yes[38]	Retention[38]
Biotin-dependent Carboxylases		
Propionyl-CoA	No[27]	Retention[26,27]
Pyruvate	No[59]	Retention[29]
β Oxidative Decarboxylases		
Isocitrate (TPN-specific)	Yes[60]	Retention[61,62]
Isocitrate (DPN-specific)	No[63]	Retention[63]
6-P gluconate	Yes[64]	Inversion[64]
Malate	–	Retention[29]
UDP-glucuronate	–	Inversion[65]
Acetyl-CoA-like Condensations		
Malate Synthase	No[66]	Inversion[67,68]
Citrate Synthase (*R*)	No[23]	Inversion[35,36,69]
Citrate Synthase (*S*)	–	Inversion[35]
Citrate Lyase	–	Inversion[35,69]
ATP-Citrate Lyase	–	Inversion[35,69]
Isocitrate Lyase	No[70]	*Inversion*[71,72]

*Two aldolases, specific for pyruvate in condensation with glyoxalate in one case or a variety of aldehydes in the other, have been shown in unpublished work by Meloche, to involve proton replacement with retention.



α-ketoglutarate enolate (2R,3S)isocitrate

FIGURE 13.

the transaldolase and aldolase processes in the muscle fructose-1,6-P_2 aldolase reaction (the latter including protonation of the carbanion) are independently sensitive to alterations of the enzyme. Also, Meloche[74] found that bromopyruvate which inactivates 2-keto-3-deoxy-6-P gluconate aldolase seems to alkylate either one of two distinct nucleophiles, carboxyl or -SH, at the same binding site. This has been interpreted to show the presence of two functional bases within range of the β carbon of the substrates, presumably serving the two functions ascribed above to a single base. The stereochemistry of substitution with this aldolase remains to be determined. If, indeed, separate catalytic sites are involved in the aldolase reaction, further stereochemical studies should provide examples in which inversion is found.

The biotin-CO_2-dependent carboxylases represent a group in which the entering carboxy group of carboxybiotin must be present in order to obtain proton abstraction, but in which the substitution occurs with retention. Although it is possible that a two-step process occurs in which the dependence on carboxylation of the biotin is structural rather than functional, the retention mode should place the carboxyl group in close proximity to the leaving proton. Based primarily on these considerations, Retey and Lynen[26] and Mildvan and Scrutton[75] proposed a concerted displacement not involving an intermediate carbanion (Figure 14). The group of acetyl-CoA condensation reactions, of which only a few have been studied in sufficient detail to be listed in Table 1, is characterized by a requirement for the second substrate in order to obtain proton abstraction. It is unlikely that a concerted replacement is involved since electrophilic substitutions tend to give retention.[76] It is likely that the inversion mode is characteristic since it allows space for the proton-abstracting group and the replacing group on opposite faces of an enol intermediate at the same time.

A final group in the substitution class, which may not be properly classed as electrophilic replacements, are the enzymes that catalyze hydroxylation at an aliphatic carbon. These reactions take the retention mode exclusively. Several examples are proline hydroxylase[77,78] and at least four distinct steroid hydroxylases.[79-82] Whether the stereochemical correlation is based on structural or mechanistic factors is not clear. The ribonucleotide reductase reactions have some characteristics similar to the hydroxylases, perhaps not the least of which is the retention stereochemistry.[83,84]

Aldo-ketol Isomerases

The stereochemistries of the aldo-ketose isomerases have been analyzed by Rose and co-workers in terms of an enediol mechanism.[85-87] It has been observed that a relation between the aldehyde structure and the stereospecificity of proton labilized at C-1 of the corresponding ketose conforms to a *cis*-enediol intermediate, if one applies the constraint that proton approaches either carbon of the enediol from the same face. This constraint is derived from the observation of intramolecular proton transfer between C-1 and C-2 in many of the isomerases which requires a single base to act as carrier. The stereochemical course of seven of these enzymes is given in Table 2. The observation that only the *cis*-enediol fits the stereochemical data for enzymes from the whole range of cell types and for a variety of substrate specificities suggested

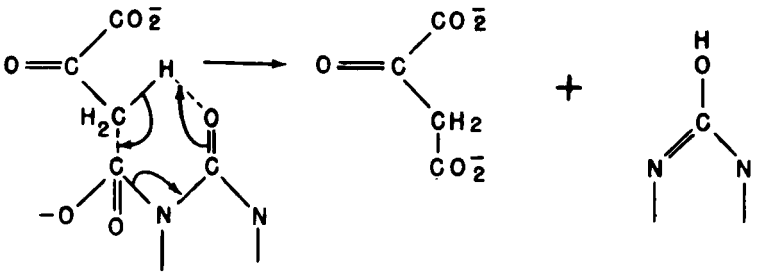
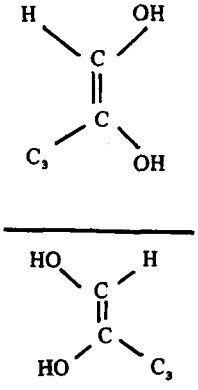


FIGURE 14.

TABLE 2

Stereochemical Course of the Isomerases

Reaction	Face Approached by H^+ at C_1 Carbonyl	Face Approached by H^+ at C_2 Carbonyl	Allowed Enediol for Front Side Approach
D-glucose-6-P → *fructose-6-P*	*re*	*re*	
D-ribose-5-P → ribulose-5-P	*re*	*re*	
D-xylose → xylulose	*re*	*re*	
L-arabinose → L-ribulose	*re*	*re*	
D-glyceraldehyde-3-P → dihydroxyacetone-P	*re*	*re*	
D-arabinose → D-ribulose	*si*	*si*	
D-mannose-6-P → fructose-6-P	*si*	*si*	

that the *cis*- relation between the oxygens at C-1 and C-2 might be of mechanistic significance. It would allow a single electrophilic center to polarize each carbonyl group in turn and hence facilitate the proton abstraction step, as in Figure 15. The anomeric specificities for the aldose substrates of three isomerases have been established.[88-90] The results were shown to be consistent with a *cis*-enediol intermediate in a single base mechanism[90] in the following sense, as in Figure 16. The approach of a proton toward C-2 from the *re* face of the enediol postulated in the *D*-xylose isomerase mechanism will orient the C_2-C_3 bond in the opposite direction. If rotation around C_1-C_2 is prevented in the bound aldehyde, ring closure from the C_5 hydroxyl can only occur from the *si* face of the C_1 carbonyl group. This structure is a simple rotational isomer, skew boat form, of the most stable chair form of the α-anomer, which is probably the true substrate.[89,90] The mechanism also predicts the anomeric specificity of the furanose form of the ketose substrate. However, such studies must await the availability of pure ketose anomers of known structure.

1,3 Allylic Rearrangement

The stereochemistries of two enzymatic reac-

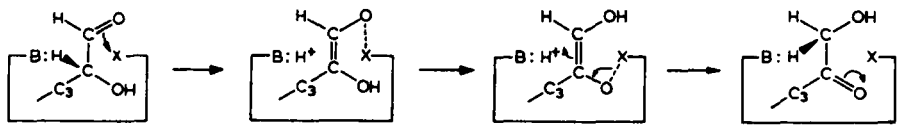


FIGURE 15.

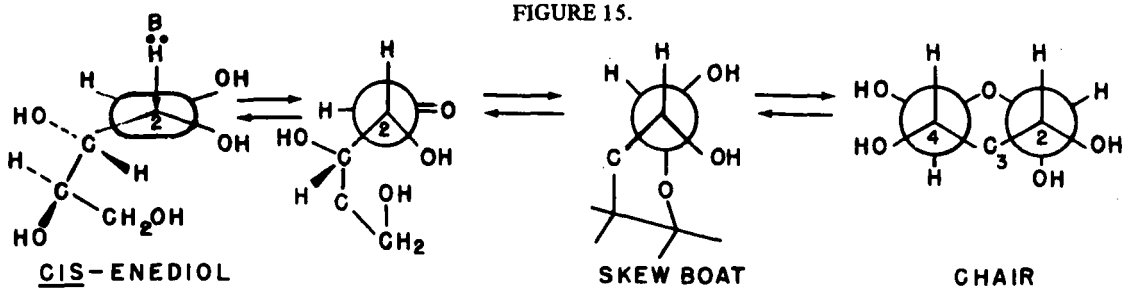


FIGURE 16.

tions in this class have been fully established, the 3 keto steroid isomerase[91] and *cis-trans*-aconitate isomerase,[92] both from Pseudomonas. These reactions (Figures 17 and 18) have been shown to proceed with proton retention that is complete in the former case and partial in the latter. Thus, the stereochemistries should be consistent with a single group on the protein acting as a proton carrier between carbanionic centers of the planar intermediate. Since maximal orbital overlap is expected in the direction perpendicular to the resonance plane, the axial position is predicted for the labilized protons of the steroid isomerase substrates and was observed.[91] In the case of *cis-trans*-aconitate isomerase, the stereochemistry of the allylic carbons agrees with the mechanism in which the carbon skeleton of the intermediate is a planar hybrid of the carbanion forms of both substrates. It is not clear whether the resonance delocalization in the intermediate extends into carboxylate groups. Since the aconitate isomerase is not dependent on metal ions, it is likely that the three carboxylate groups are neutralized by fixed positive centers at the substrate binding sites. These may contribute inductively to proton release.

An important example of an allylic rearrangement occurs in the aldimine to ketimine conversion that leads to transamination between α-amino acids and pyridoxal-P (Figure 19). It has been established with alanine-pyridoxal transaminases, in which pyridoxamine is a product instead of an intermediate, that proton transfer occurs between the α-carbon of the *L*-alanine and the pro-*S* hydrogen of the amino carbon of pyridoxamine.[93] This result is readily interpretable in terms of the generally accepted mechanism for transaminase reactions if it is assumed that a single base is concerned in the 1,3 proton shift. As a consequence of the stereochemical course, one may exclude two of the four structures for the proposed carbanion (Figure 20b

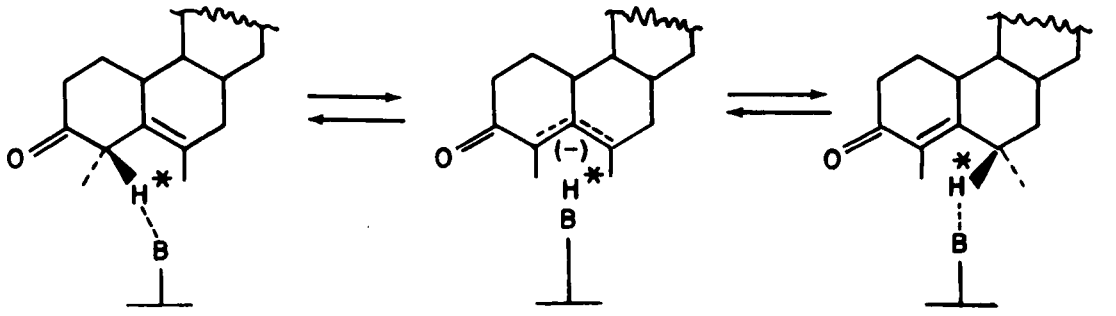


FIGURE 17.

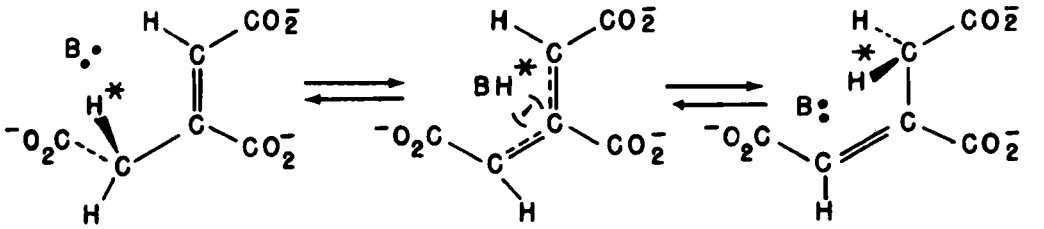


FIGURE 18.

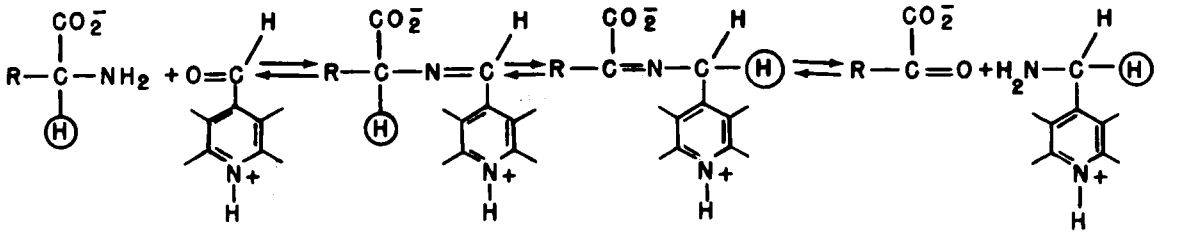


FIGURE 19.

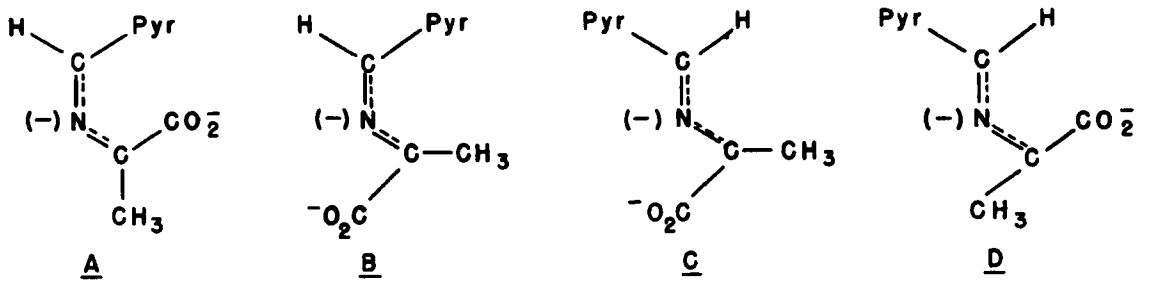


FIGURE 20.

and d) since it is not possible to approach the allylic carbons from the same face and produce the proper alternate products. The distinction between A and C, the *cis-* and *trans-* structures, requires additional information. Dunathan et al.[94] prefer the *trans-* structure, C, for steric reasons.

Glutamate-aspartate transaminase[94,95] and α dialkylamino acid transaminase[96] also activate the pro-*S* position of pyridoxamine. In the latter case the same position is labeled from tritiated water, whether the substrate is *L*-alanine or α-aminoisobutyrate, that is whether the α proton or α carboxyl are abstracted. With *L*-[α-^{3}H]alanine, significant amounts of tritium transfer to the pyridoxamine-P were observed. The results for the three enzymes examined, therefore, indicate a correlation between the *L*-amino acid series and pro-*S* activation of the pyridoxamine, suggesting that a common geometry pertains, perhaps 20C.

Double Bond Addition Reactions

Most enzymatic additions to a double bond occur in the *trans-* (*anti-*) manner. Within this group the proton is often added to an ethylene carbon that bears a carboxyl and a hydrogen such that a prochiral carbon is formed in the final product (Figure 21). Of the enzymes that can be classified in this way, all such proton additions occur from the *re* face, as shown in Table 3. The possibility is thus raised that there is a structural

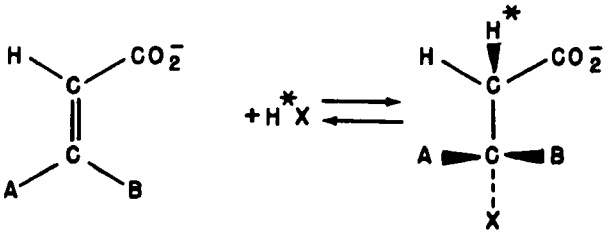


FIGURE 21.

TABLE 3

Addition of HX to α,β Unsaturated Acids as in Figure 21

Enzyme	A	B	X	Face of H* Approach to C_2	Stereochemistry	Ref.
Fumarase	CO_2^-	H^+	OH	*re*	*anti-*	97-99
Malease	H^+	CO_2^-	OH	*re*	*anti-*	25
Aspartase	CO_2^-	H^+	$-NH_2$	*re*	*anti-*	97,100
Arginine Succinase	CO_2^-	H^+	-arginine	*re*	*anti-*	101
Adenyl Succinase	CO_2^-	H^+	-adenyl	*re*	*anti-*	102
Aconitase (*cis*-Aconitate → Citrate)	$-CH_2CO_2^-$	Co_2^-	-OH	*re*	*anti-*	103
Mesaconote Hydratase	CO_2^-	CH_3	-OH	*re*	*anti-*	104

feature of these enzymes that was conserved in their evolution that relates the direction of proton approach to the site of binding of the substrate. Such a constraint might explain the fact that *D*-malate is formed in the maleate hydratase reaction, whereas a requirement for *anti*-addition alone would also be satisfied by *si* face addition of the proton with the formation of *L*-malate, as discussed earlier for reaction sequence 8.

The *anti*- mode addition is by far the dominant one for the other enzyme reactions of this class. These reactions cannot be compared with respect to the absolute direction of proton approach since the substrates do not have structural homology. The reactions that show *anti*-addition are aconitase (*cis*-aconitate to isocitrate),[105,106] dimethyl citraconate (→ β isopropyl malate) hydratase,[107] β-methyl aspartase,[108] enolase,[109] histidine deaminase,[110,111] phenylalanine deaminase,[112] oleic acid hydratase,[113] and pyrophosphomevalonate decarboxylase.[114] The reactions that show *syn*- (or *cis*-) elimination are 3 hydroquinate dehydratase[103,115] and *cis-cis*-muconate cycloisomerase.[116]

Since the reactions of this class are almost invariably characterized as *anti*-additions, in spite of a large variation in substrate nature, biological source, and other aspects of mechanism such as requirements for metal in some cases and/or for specific protein functional groups,[110,117,118] one is compelled to consider seriously the implications of the *anti*- mode. There is a mechanistic approach and a structural way to rationalize the stereochemistry. Both of these seem rather compelling. In terms of mechanism, one knows from model systems,[119] as well as from molecular orbital interaction rules,[120,121] that *anti*-additions to double bonds are favored energetically when the species H^+ and X^- are added in a concerted or near-concerted manner. There is really very little information directly bearing on the timing of the two additions. Nevertheless, the orbital interaction evaluations suggest that if the nucleophile, X^-, is added first, the paired electrons on the neighboring carbon will be oriented *anti*-periplanar to the approaching X^- group (Figure 22). To achieve a *syn*-addition, the new carbanionic center must invert. This will be energetically costly in the ordinary sense of going from a staggered to an eclipsed conformation, but also to the extent that binding groups on the enzyme will be dragged along rather than promote the inversion. If the proton is added first, the resulting carbonium is planar (sp^2) so that no inversion barrier is imposed on the direction of net addition. Thus, from the electronic view, the almost exclusive preference for *anti*-addition would favor either a concerted or carbanion mechanism.

A structural factor relating to the relative disposition of the catalytic sites may be of importance. The groups of the enzyme that donate X^- and H^+ will be in the plane perpendicular to the plane of the alkene and passing through the two unsaturated carbons (Figure 23). In the case that the two oppositely charged groups are on the same face of the molecule, there would be a strong tendency for neutralization by bonding – AX···HB, AH···B, or A···HB – which would interfere with the reaction. The *anti*-disposition of A and B may also be favored for steric reasons since the "horizontal" separation of their centers will be only a bond's distance.

There are several examples of enzymes in which

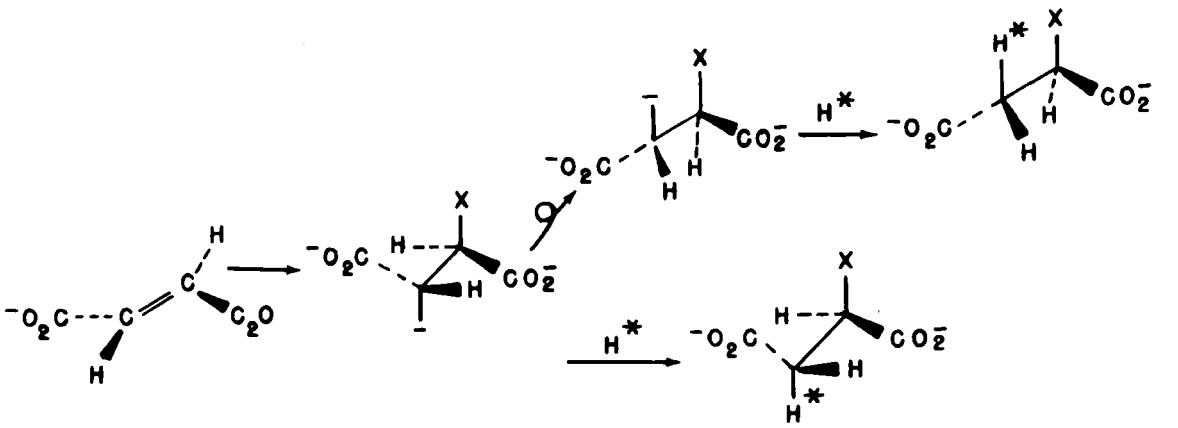


FIGURE 22.

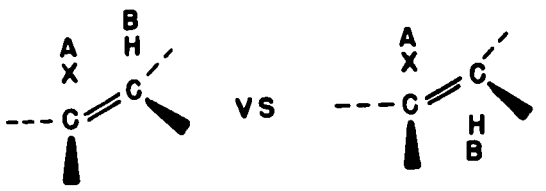


FIGURE 23.

the substrate resides in a crevice between surfaces that contribute both contact points that define substrate specificity and catalytic groups. Advantages of this kind of topology are that it increases the surface available and permits catalytic groups that function at a given bond of the substrate to be separated in space. Two examples taken from x-ray diffraction studies show this kind of "*anti-*" arrangement very clearly: (1) In lysozyme carboxyl and carboxylate groups, (asp 52) and (glu 35) are thought to promote glycosidic bond cleavage from their locations 3Å away from the C_1 and glycosidic oxygen, respectively.[122] The two groups lie opposite to each other across the crevice holding the substrate. (2) In the carboxypeptidase-catalyzed cleavage of an amide bond, the side chain oxygen of tyrosine 245 forms a hydrogen bond with the amide N, and a Zn atom complexes with the carbonyl oxygen. The tyrosine is known to swing down after the substrate has become bound, covering the zinc. Thus, the two centers are at opposite faces of the plane of the NH and CO groups of the sensitive peptide bond.[123]

Since both the molecular orbital interaction rules and the steric arguments favor the *anti-*addition to neighboring carbons, it would be of great interest to examine those few enzymes that catalyze *syn*-reactions for clues to relate them and contrast them with the others. It should also be of interest to examine examples of 1,4 elimination, such as chorismate synthase (Figure 24). For these reactions, orbital interaction rules predict *syn-*addition,[121] whereas steric factors would favor *anti-*addition. The latter is the case,[124,125] as seen in Figure 24.

In the elimination of H: and H^+ (oxidation) to produce double bonds, *syn-* and *anti-* stereochemistries are found with about equal frequency. Thus, although *anti-*elimination is found with succinate dehydrogenase,[126,127] *meso*-tartrate dehydrogenase,[128] 3-keto steroid Δ1 oxidoreductase,[129] and acyl-CoA dehydrogenase,[130-133] one finds *syn*-elimination in the oxidation of stearate to oleiate[134] and in the oxidation of steroids, both in the ring system and side chain.[135,136]

Enolpyruvate – a Reaction Intermediate

Enzyme-bound enolpyruvate seems to have evolved as a high-energy form, protected from the medium by its bound state, but available for a variety of synthetic purposes through nucleophilic addition of C-3 to sugars and CO_2. It is interchanged between enzymes in metabolism through the agency of a stabilized form – phosphoenolpyruvate. The latter can act as a phosphoryl donor, to ADP or to a protein of the PEP:sugar phosphotransferase system of Roseman,[137] presumably through bound enolpyruvate, which by collapsing to the more stable keto form, pyruvate, displaces the equilibrium further towards phosphoryl transfer. The evidence for bound enolpyruvate as an entity in these reactions is rather incomplete, but the hypothesis is worth further attention. The first evidence of this entity was the observation that pyruvate kinase catalyzes the hydrogen exchange of the methyl group of pyruvate in the absence of ATP.[138] These studies imply that pyruvate is

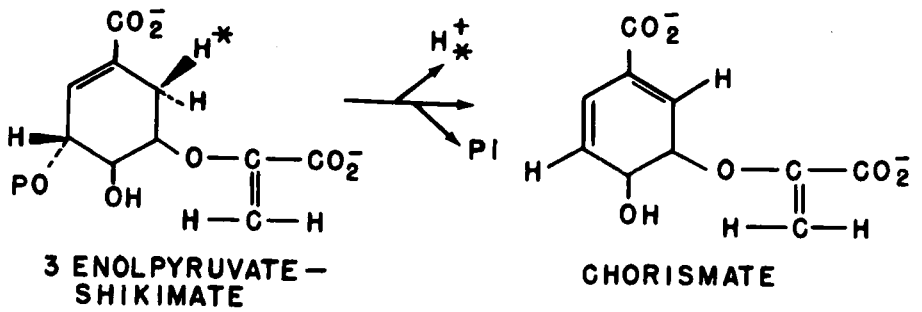


FIGURE 24.

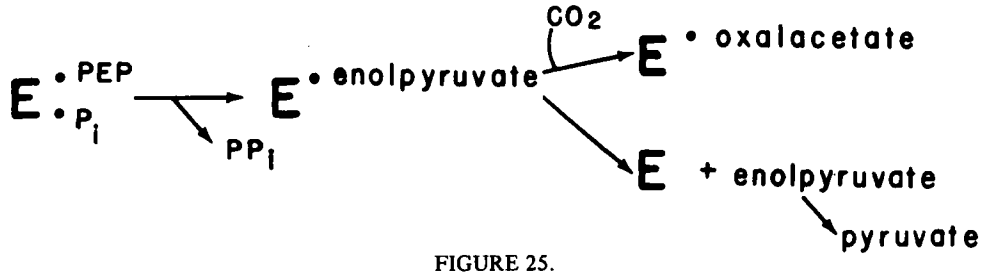


FIGURE 25.

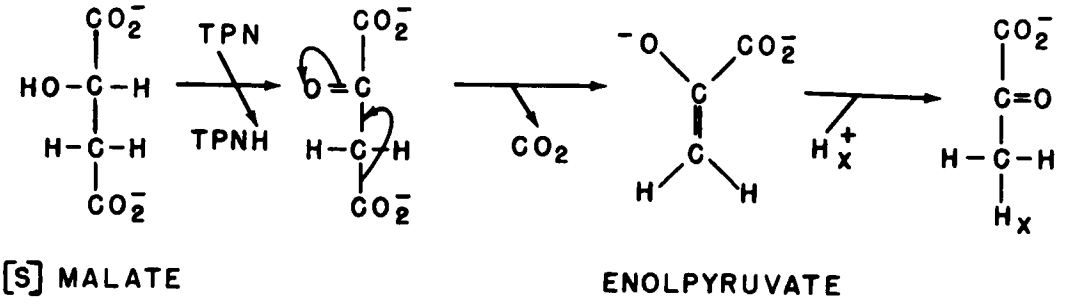


FIGURE 26.

converted to enzyme-bound enolpyruvate. More recently it has been observed by Davis et al.[139] that pyruvate is a side product of the action of phosphoenolpyruvate carboxyphosphotransferase on phosphoenolpyruvate, and that the proton addition to C-3 to form pyruvate is not stereospecific.* This result clearly suggests that enolpyruvate is protonated after leaving the enzyme. Since carboxylation is stereospecific,[28] the proton is not simply replacing CO_2 in a reaction concerted with phosphoryl transfer. The simplest interpretation would correspond to the sequence given in Figure 25.

As was discussed earlier (Figure 10), all of the enzymes that catalyze the addition of an electrophile to C-3 of phosphoenolpyruvate do so from the *si* face of the molecule. With the single exception in which erythrose-4-P is the adding species, in which the expulsion of phosphate has been shown to involve C-O bond cleavage,[140] all of these reactions are likely to use enzyme-bound enolpyruvate as an intermediate.

This class of enzymes should also be enlarged to include enzymes that do not produce phosphoenolpyruvate, but probably also involve an intermediate of bound enolpyruvate. Kosicki[141] has shown that the metal ion-dependent oxalacetate decarboxylase of cod catalyzes proton exchange of pyruvate in the absence of CO_2. The reductive carboxylation of pyruvate by malic enzyme is most reasonably formulated to occur on the enzyme as shown in Figure 26. Although the absolute stereochemistry of proton and CO_2 addition to bound enolpyruvate cannot be determined at present for either enzyme, it has been shown for the malic enzyme that the overall reaction proceeds with retention.[29] This is consistent with the possibility that malic enzyme may have a structural relationship to both pyruvate kinase and phosphoenolpyruvate carboxylases which add proton and CO_2, respectively, from the same direction to C-3 of a presumed bound enolpyruvate.

Furthermore, one might consider the pyruvate carboxylase reaction in this light instead of interpreting the stereochemistry of retention in terms of a concerted reaction, as in Equation 14. Thus, the retention mode (Figure 27) would be interpreted in terms of an evolutionarily stable protein structure specific for enolpyruvate binding such that all additions occur from the same, *si*, face. Phillips and Kosicki[142] have shown that pyruvate kinase catalyzes the reduction of pyruvate by borohydride to *D*-lactate. The approach of BH_4^- is then preferentially to the *si* face of the C-2 of the bound pyruvate.

*Unpublished results of I.A. Rose, H.G. Wood, and J.M. Willard.

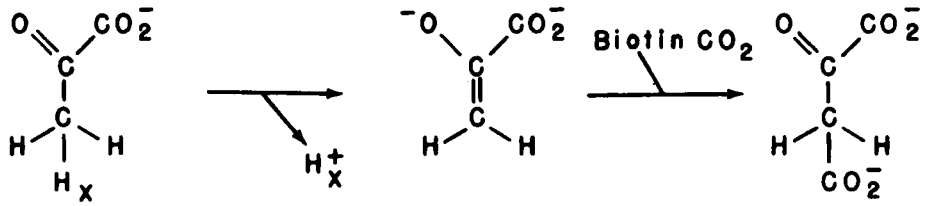


FIGURE 27.

CONCLUSION

The purpose of this review has been to organize the literature on enzymatic reaction stereochemistry to determine whether a particular stereochemical course dominates in the reactions of a given type. Finding that this is the case for several kinds of reactions suggests that there is some stabilizing factor to counterbalance random mutational events in evolution. Very often reactions that may be considered stereochemically degenerate are involved; that is, the same product would result from either stereochemical course. The basis for stereochemical conservation may be embedded in the reaction mechanism or in the perpetuation of a segment of protein structure that defines certain stereochemical imperatives in the active site. The former interpretation is strengthened by including enzymes within the reaction type that differ greatly in substrate specificity. Further work seems justified to test the extent of stereochemical conservatism in enzymatic reactions as a tool for the study of reaction mechanism, enzyme structure, and evolution.

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