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Interpretations of Enzyme Reaction Stereospecificity

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Enzymes vary in the degree of their substrate specificity.[1-4] Some are highly exacting, and others tolerate major changes in the substrate with little change in reaction rate. For a given substrate, however, the stereoselectivity with respect to the reacting centers is generally observed to be as complete as the methods of examination can reveal.[5]

It is apparent, therefore, that the study of enzyme reaction stereochemistry differs profoundly from the study of reactions in free solution. In chemical terms, an enzyme is both a chiral reagent and a chiral solvent; the formation and breaking of bonds take place under conditions where accessibility and motion of reactants are severly constrained in ways that cannot be deduced from their chemical structures. In the context of an active site, water behaves as a stereospecific reagent and carbonium ions, carbanions, and perhaps radicals seem to possess configurational stability. It follows that the generalizations derived from the study of free solution chemistry are not readily applicable—one must be circumspect in ruling out mechanisms on stereochemical grounds.

To arrive at mechanistic conculsions one needs additional principles of interpretation. In this essay we wish to show that such conclusions may be reached by comparing the stereochemistry of analogous enzyme reactions, if one views reaction stereochemistry in the context of natural selection.

Enzyme Stereochemistry as the Product of Natural Selection

We can think of three major ways in which the stereochemical characteristics of present-day enzymes could have evolved.

By Selection for Metabolic Coupling. Given the stereoregularity and chiral sense of nucleic acids[6] and proteins, much of metabolism must be directed toward the production of intermediates for the synthesis of these polymers. Other aspects of metabolism, including the formation of "secondary metabolites" and hormones, must be integrated with the metabolism of the D sugars and L amino acids. Each step in a pathway is coupled to the next so that a given product has the correct configuration to be a substrate for the following reaction or act on a physiological target. Mutant organisms with enzymes that produce the wrong stereoisomer will be eliminated.

By Divergent Evolution with Conservation of Active Site Structure. The high degree of organization associated with an active site is indicated by two findings. Firstly, many present-day enzymes have mechanisms that appear to approach an optimal level of physiochemical efficiency.[7] Secondly, the organization of the active sites of many enzymes (*e.g.*, glyceraldehyde-3-phosphate dehydrogenase[8]),

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Irwin A. Rose has been studying the mechanisms of enzymes involved in proton abstraction since the late 1950's using stereochemistry, tracer kinetics, and isotope effects, first at Yale University (1954–1963) and since then at the Institute for Cancer Research, where he is now Associate Director for Research (during part of the day).

(1) We apologize to the many authors whose work has been cited indirectly. To conserve space, review articles and papers citing earlier studies have often been noted instead of first reports.

(2) For comprehensive reviews of some 20 years of study see (a) W. L. Alworth, "Stereochemistry and its Application in Biochemistry," Wiley-Interscience, New York, N.Y., 1972; (b) D. Arigoni and E. L. Eliel, *Top. Stereochem.*, **4**, 127 (1969); (c) R. Bentley, "Molecular Asymmetry in Biochemistry," Academic Press, New York, N.Y., Vol. 1, 1969, Vol. 2, 1970; (d) T. W. Goodwin, *Essays Biochem.*, **9**, 103 (1973); (e) G. Popják, *Enzymes 3rd Ed.*, **2**, 115 (1970).

(3) For reviews covering some of the topics here discussed see (a) K. R. Hanson, *Annu. Rev. Plant Physiol.*, **23**, 335 (1972); (b) I. A. Rose, *Enzymes, 3rd Ed.*, **2**, 281 (1970); (c) I. A. Rose, *Crit. Rev. Biochem.*, **1**, 33 (1972).

(4) For stereochemical concepts and nomenclature see (a) IUPAC Tentative Rules, Section E, "Fundamental Stereochemistry," *J. Org. Chem.*, **35**, 2849 (1970). (b) Topic relationships: H. Hirschmann and K. R. Hanson, *Eur. J. Biochem.*, **22**, 301 (1971); K. Mislow and M. Raban, *Top. Stereochem.*, **1**, 1 (1967). (c) The *pro-R/pro-S* and *re/si* systems: K. R. Hanson, *J. Amer. Chem. Soc.*, **88**, 2731 (1966).

(5) Nonstereospecificity in the formation of products can be taken as evidence for a non-enzyme-directed step, *e.g.*, an enzyme may release a metastable product such as an enol which is randomly converted to the final metabolic product, a ketone. Also, the formation of an intermediate with a torsiosymmetric group[4b] may lead to an apparent lack of stereospecificity.

(6) It seems likely that the choice of the chiral sense of metabolism was fixed during the primitive evolution of the nucleic acids: H. Kuhn, *Angew. Chem., Int. Ed. Engl.*, **11**, 798 (1972); H. L. Segal, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **20**, 255 (1972); see also W. E. Elias, *J. Chem. Educ.*, **49**, 448 (1972).

(7) M. Eigen and G. G. Hammes, *Advan. Enzymol.*, **25**, 1 (1963); G. G. Hammes, *Accounts Chem. Res.*, **1**, 321 (1968).

(8) (a) The full sequence of the yeast and pig enzymes showed 68% homology: G. M. T. Jones and J. I. Harris, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **22**, 185 (1972). A hybrid 2 + 2 tetramer can be formed between subunits of the yeast and rabbit enzymes: G. M. L. Spotorno, and M. R. Holloway, *Nature (London)*, 226, 756 (1970). (b) M. Buehner, G. C. Ford, D. Moras, K. W. Olsen, and M. G. Rossmann, *Proc. Nat. Acad. Sci. U.S.*, **70**, 3052 (1973). (c) For similarities between yeast and horse liver alcohol

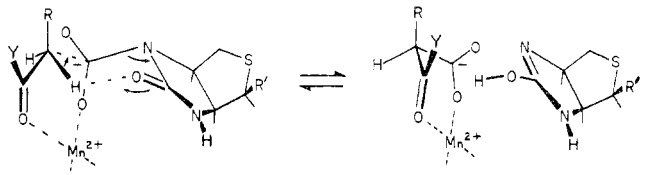


Figure 1. Cyclic mechanism accounting for retention of configuration: carboxylations by carboxybiotin. Pyruvate to oxaloacetate (Y = CO_2^-, R = H; ref 12a) and propionyl-CoA to (2*S*)-methylmalonyl-CoA (no metal, Y = S-CoA, R = CH_3; ref 12b). The biotin tautomer generated reverts to the urea form. The cyclic arrangement takes advantage of urea chemistry to ensure a tight kinetic coupling between H^+ removal and CO_2 transfer.

seem to have been fixed over 10^9 years ago prior to the divergence of the eukaryotes into separate kingdoms (animals, plants, and fungi).[9] It appears that, once an efficient active site has evolved for a given enzyme, its basic structure is conserved, although there may be many amino acid replacements in the protein sequence.

Because the active site is highly organized, enzymes with new substrate specificities are likely to arise through modification of the gene for an enzyme that had already evolved to interact with related substrates. (For example, the new β-galactosidase recently produced by multiple mutations of an *Escherichia coli* strain with a block deletion of the normal gene could have evolved from another β-glycosidase[10].) The stereochemistry of a parent enzyme will tend to be conserved (a) if the substrate binding region not associated with the reacting center is changed, (b) if the binding region for a key coenzyme or substrate is conserved (having once evolved by chance or for structural or mechanistic reasons), or (c) if a new enzyme evolves from one catalyzing a related function. The formation of new enzymes by such divergent evolution should be recognized to be part of the normal present-day competition between species, particularly in microorganisms and plants.

By Selection for Mechanistic Efficiency. The relative efficiency of alternative mechanisms must be a major determinant in natural selection.[11] One can envision two limiting situations: (a) the chemistry of the reaction selected may be stereochemically precise and only depend upon the enzyme to provide a suitable matrix in which the reaction can take place, or (b) the mechanism may employ a uniquely efficient distribution of enzyme catalytic groups (as discussed below). In the former case one would expect the stereochemical precision to arise because the reaction has a multicentered transition state or occurs with a cyclic flow of electrons that does not involve catalytic groups on the enzyme (*e.g.*, Figure 1[12] shows the way in which the carbonyl of carboxybiotin could act as a base and promote carboxylation with retention).

Efficient mechanistic features will undoubtedly be conserved in the divergent evolution of new enzymes. Two enzymes that are unrelated structurally, however, may exhibit the same mechanistic features as a result of chance or because *convergent evolution* has taken place. Selection for the same uniquely efficient mechanism is analogous to the appearance of the same characteristics in whales and fishes under selective pressure for aquatic survival. [A striking example is provided by the serine proteases:[13] two families of enzyme differ in tertiary structure and sequence, yet contain the same "charge relay system" of amino acid side chains: in α-chymotrypsin and subtilisin (BPN′) the system residues Ser (reactive), His, Asp, Ser are ordered respectively 195, 57, 102, 214 and 221, 64, 32, 33.]

Importance of Prochirality and Reaction Classes

Only a detailed knowledge of primitive evolution would allow one to decide whether present day stereospecificity with respect to substrate configurations has been determined by metabolic, structural, or mechanistic imperatives, or by chance. To avoid arguments about the significance of metabolic selection, we shall limit our enquiry to aspects of enzyme stereochemistry that are not specified by establishing the structures of the substrates and products.

The approach may be illustrated by considering the four stereochemically distinct pathways for condensing acetyl-CoA and oxaloacetate to produce citrate and CoASH: inversion or retention at the acetyl methyl group and attack on one or the other stereoheterotopic[4b] face of the ketone carbon (*re* or *si* attack). Because the stereochemical course of these reactions is hidden to the experimenter and to the cell, we shall refer to them as being **stereochemically cryptic.** As the *same* substrates and products are linked in metabolism whatever the stereochemistry of the citrate synthase reaction, there can be no metabolic basis for selection between mechanisms of different stereochemistries. Only two of the four possible modes of citrate synthesis are realized in nature (inversion with *re* or *si* attack).[2a] In trying to explain this one must ask if the inversion mode is mechanistically more efficient and if the choice of carbonyl face is without mechanistic significance.

In the above example two prochiral centers[4c] are generated: a methylene carbon and the central carbon. In general, stereochemically cryptic reactions lead to the transformation or generation of centers which are not steric elements[3a,4b] (*e.g.*, gCH_3) or are

dehydrogenases see H. Jörnvall, *Proc. Nat. Acad. Sci. U.S.*, 70, 2295 (1973).

(9) For nonstereochemical aspects of enzyme evolution see (a) D. Boulter, *Current Advan. Plant Sci.*, 4, C. 8 (1974); (b) M. O. Dayhoff, C. M. Park, and P. J. McLaughlin, "Atlas of Protein Sequence and Structure," Vol. 5, National Biomedical Research Foundation, Washington, 1972, p 7; (c) G. H. Dixon, *Essays Biochem.*, 2, 149 (1966); (d) J. A. M. Ramshaw, D. L. Richardson, B. T. Meatyard, R. H. Brown, M. Richardson, E. W. Thompson, and D. Boulter, *New Phytol.*, 71, 773 (1972); (e) E. Smith, *Enzymes, 3rd Ed.*, 1, 267 (1970).

(10) J. H. Campbell, J. A. Lengyel, and J. Langridge, *Proc. Nat. Acad. Sci. U.S.*, 70, 1841 (1973); R. A. J. Warren, *Can. J. Microbiol.*, 18, 1439 (1972).

(11) As this is a key hypothesis in our arguments, it is, in principle, tested by its application to enzyme stereochemistry. However, it is a necessary hypothesis in order to explain the finding that many enzymes approach optimal physicochemical efficiency.[7] We assume that, when mechanistic efficiency can be achieved only at the expense of regulatory properties, natural selection results in a near optimal payoff. Attempts to transform inefficient to more efficient enzymes by selection of microorganisms have met with limited success (a) J. L. Betz, P. R. Brown, M. J. Smyth, and P. H. Clarke, *Nature (London)*, 247, 261 (1974); contrast (b) P. W. J. Rigby, B. D. Burleigh, Jr., and B. S. Hartley, *ibid.*, 251, 200 (1974)]. The ways in which selective pressures are exerted and the extent to which silent gene copies are important in the formation of new enzymes are of secondary interest in the present context.

(12) (a) A. S. Mildvan and M. C. Scrutton, *Biochemistry*, 6, 2978 (1967); I. A. Rose, *J. Biol. Chem.*, 245, 6052 (1970); (b) D. Arigoni, F. Lynen, and J. Rétey, *Helv. Chim. Acta*, 49, 311 (1966); D. J. Prescott and J. L. Rabinowitz, *J. Biol. Chem.*, 243, 1551 (1968); J. Rétey and F. Lynen, *Biochem. Z.*, 342, 256 (1965).

(13) Reviews: J. Kraut, *Enzymes, 3rd Ed.*, 3, 165, 547 (1971); F. S. Markland, Jr., and E. L. Smith, *ibid.*, 3, 561 (1971).

elements of prostereoisomerism[3a,4b] (*e.g.*, prochiral centers or the methylene of $H_2C{=}Cgh$, as in phosphoenolpyruvate). Most of the available information concerning stereochemically cryptic reactions pertains to prochiral carbon centers because it is relatively easy to employ substrates in which the equivalent center has isotopic chirality (*e.g.*, Cgh^1H^3H). Of great interest are the many stereochemically cryptic reactions, notably esterases and phosphatases, whose stereochemistry has yet to be determined because appropriate methods of investigation are lacking.

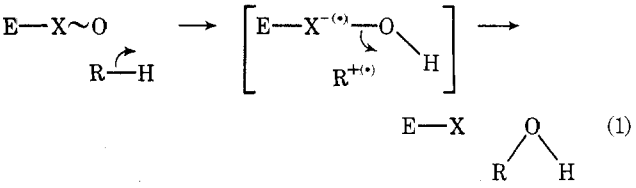
One cannot determine whether the observed course of a *single* stereochemically cryptic reaction is or is not the result of selection for a uniquely efficient mechanism. We propose to examine this critical question in terms of probabilities. We shall look for the occurrence or nonoccurrence of a common stereochemical mode among enzymes that catalyze cryptic reactions and can plausibly be grouped into a single reaction class.

A common stereochemistry may be attributed most firmly to mechanistic factors if the same catalytic device has independently arisen in unrelated proteins. Where a class of enzymes derive from a common ancestor, the greater the variation in substrate structure, the more likely that conservation of stereochemistry has mechanistic significance. We derive this proposition intuitively from a general reading of the literature on enzyme evolution[9] as follows: the greater the changes in amino acid sequence and chain folding to accommodate new substrates, the less likely that the original distribution of catalytic groups will be maintained or be restored by mutation[11b] without some mechanistic imperative.

The absence of a common stereochemistry may indicate that the alternative processes are of similar catalytic efficiency, so that it is a matter of chance which one occurs. It may also show that the reaction is divisible into steps which are mechanistically independent.

Examples of Classes Showing Consistency. The aliphatic hydroxylases catalyze the replacement of H by OH, with the oxygen derived from O_2 and electrons from a suitable donor.[14] Hydroxylations proceed with retention whether the substrates are steroids,[15a] fatty acids or their esters,[15b] proline,[15c] dopamine and α-amphetamine,[15d] ethylbenzene,[15e] *p*-hydroxyphenylacetonitrile,[15f] β-carotene[15g] or a precursor of the alkaloid haemanthamine.[15h] The list includes di- and monooxygenases with a variety of prosthetic groups, metal ions and cosubstrates.[14a] Evolution from a common ancestor can, therefore, be excluded, and one must look for a common mechanistic feature.

Attention has been drawn[14b] to analogies with carbene and nitrene chemistry, and on the basis of model reaction studies it has been proposed that all have an "oxenoid" step in which insertion of the equivalent of a free oxygen atom with its six outer-shell electrons occurs. This is formally represented in eq 1.[14c] The oxenoid reagent E–X∼O is generated



(1)

from O_2 and a group on the enzyme by reduction. In some, but not all, cases this reagent could be a peracid ion, $RC(=O)O{\sim}O^-$, formed from a carboxyl group and the superoxide ion, $O_2{\cdot}^-$, or Fe^{III}–O_2^-.

The hypothesis may also apply to olefin formation.[14b] In ten examples of the oxidative formation of nonactivated double bonds (conjugated, at the most, to a second double bond) the removal of the two hydrogens is apparently syn. The list includes double bonds in steroid rings[16a,b] (one H eliminated is axial, the other equatorial), steroid side chains,[16c] and fatty acids.[16d] As olefin formation by carbenes, nitrenes, and an oxenoid reagent has been observed, the enzymic reactions could proceed in a stepwise manner with E–$X^{-(\cdot)}$–O–H (as in eq 1) picking up an adjacent proton to give olefin + H_2O + E–X. (It was earlier proposed that "active O_2" and substrate yield H_2O_2 and olefin by way of a six-membered cyclic transition state.[16a])

A different conclusion is suggested by the steric consistency of the enzymic reactions of pyridoxal phosphate and pyridoxal. The coenzyme forms a Schiff base with the amino acid substrate by transimination from the ϵ-amino group of a lysyl side chain. Loss of an α substituent (H^+, CO_2, or R^+) then gives an aza-allylic system (Figure 2).[17a] In the seven enzymes studied protonation of this at C-4′ occurs by *si* attack. The list[17b] includes enzymes that labilize each of the substituents: four normal transaminases [glutamate-aspartate, glutamate-alanine, pyridoxamine-pyruvate, and "dialkylamino acid transaminase" (on L-alanine; with decarboxylation on 2-aminoisobutyrate)] and three enzymes that can be inactivated by transamination of the coenzyme [tryptophan synthase B protein, L-glutamate decarboxylase, and serine hydroxymethylase].

Although *a priori* an enzyme, being chiral, must

(14) "Molecular Mechanisms of Oxygen Activation," O. Hayaishi, Ed., Academic Press, New York, N.Y., 1974: (a) O. Hayaishi, p 1; (b) G. A. Hamilton, p 405; (c) see ref 14b, p 429; M. Hamberg, B. Samuelsson, I. Björkhem, and H. Danielsson, p 29.

(15) (a) At 7α, 11α, 11β, and 12β: M. Hayano, M. Gut, R. I. Dorfman, A. Schubert, and R. Seibert, *Biochim. Biophys. Acta*, **32**, 269 (1959), and references therein. (b) At C-2, C-12, and C-(ω-1); Review: L. J. Morris, *Biochem. J.*, **118**, 681 (1970); also ref 14d. (c) Y. Fujita, A. Gottlieb, B. Peterkofsky, S. Udenfriend, and B. Witkop, *J. Amer. Chem. Soc.*, **86**, 4709 (1964). (d) By dopamine β-hydroxylase: A. R. Battersby, P. W. Sheldrake, J. Staunton, and D. C. Williams, *J. Chem. Soc. Chem. Commun.*, 566 (1974); K. B. Taylor, *J. Biol. Chem.*, **249**, 454 (1974). (e) R. E. McMahon, H. R. Sullivan, J. C. Craig, and W. E. Pereira, Jr., *Arch. Biochem. Biophys.*, **132**, 575 (1969). (f) Biosynthesis of cyanogenic glycosides: (*R*)- and (*S*)-*p*-hydroxymandelonitrile β-D-glucopyranoside, M. R. Rosen, K. J. F. Farnden, E. E. Conn, and K. R. Hanson, in preparation. (g) Reference 2d, p 155. (h) A. R. Battersby, J. E. Kelsey, J. Staunton, and K. E. Suckling, *J. Chem. Soc., Perkin Trans. 1*, 1609 (1973); G. W. Kirby and J. Micheal, *ibid.*, 115 (1973). The hydroxylation of the alkaloid caranine to give lycorine, which takes place with *inversion*, is thought to be a complex process: I. T. Bruce and G. W. Kirby, *Chem. Commun.*, 207 (1968); W. C. Wildman and N. E. Heimer, *J. Amer. Chem. Soc.*, **89**, 5265 (1967).

(16) (a) S. M. Dewhurst and M. Akhtar, *Biochem. J.*, **105**, 1187 (1967). (b) Ring B, Δ^5 and Δ^7: Ref 16a; R. B. Clayton and A. M. Edwards, *J. Biol. Chem.*, **238**, 1966 (1963); D. C. Wilton and M. Akhtar, *Biochem. J.*, **116**, 337 (1970), and references therein; ref 2d, p 150. (c) Δ^{22} (analogous trans double bonds by alternative syn eliminations): M. Akhtar, M. A. Parvez, and P. F. Hunt, *Biochem. J.*, **106**, 623 (1968); T. Bimpson, L. J. Goad, and T. W. Goodwin, *Chem. Commun.*, 297 (1969), and references therein; ref 2d, p 152. (d) Δ^9, Δ^{12}, and Δ^{15}: (reviews) ref 14d, 15b; L. J. Morris, K. V. Harris, W. Kelly, and A. T. James, *Biochem. J.*, **109**, 673 (1968), and references therein.

(17) (a) H. C. Dunathan, *Advan. Enzymol.*, **35**, 79 (1971); (b) H. C. Dunathan and J. G. Voet, *Proc. Nat. Acad. Sci. U.S.*, **71**, 3888 (1974).

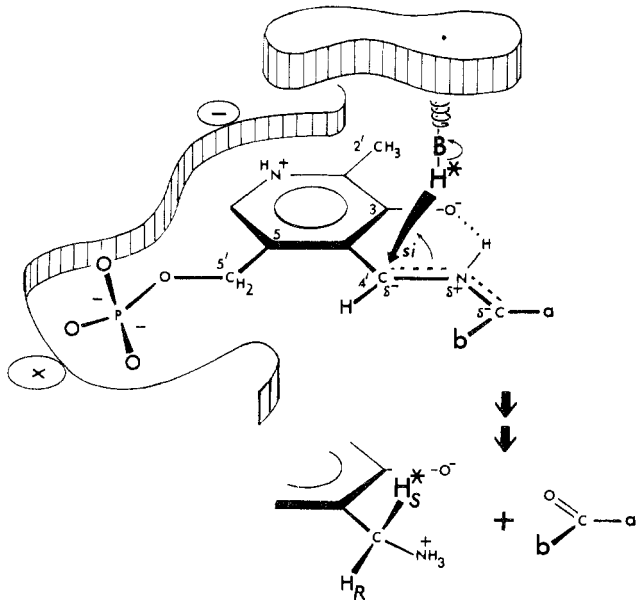
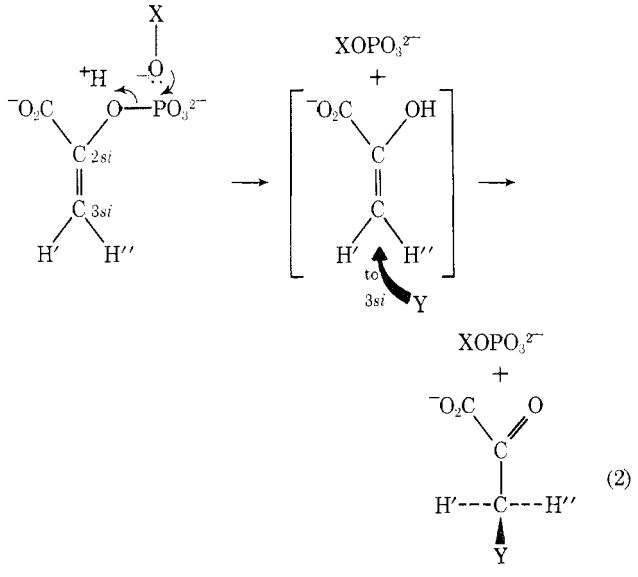


Figure 2. Consistency in stereospecificity shown by pyridoxal phosphate enzymes: 4′*si* protonation of the aza-allylic intermediate to yield the Schiff base between pyridoxamine phosphate and a ketone or aldehyde (ref 17b). If the substrate is alanine, a = CO_2^- and b = CH_3.

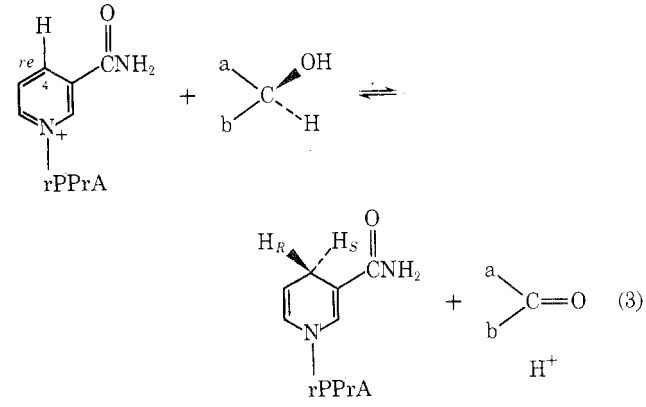
react differently toward the stereoheterotopic faces of a trigonal atom (whether enantiotopic or diastereotopic), there is nothing known about the mechanism of these enzymes that would suggest a mechanistic imperative for the independent evolution of enzymes catalyzing 4′*si* attack. It has therefore been suggested[17b] that divergent evolution from a single ancestral protein is responsible for the uniformity. Once an efficient enzyme capable of binding Schiff bases between coenzyme and substrate and between coenzyme and enzyme emerged, it could have displaced any less efficient enzymes that had independently evolved, whatever their stereochemistry. Later this could have given rise to enzymes with related functions that displaced existing enzymes with less satisfactory pyridoxal phosphate binding structures. Extensive X-ray and sequence data may be required before one can begin to test this hypothesis.



(2)

Analogous conclusions are suggested by the finding that several reactions of phophoenolpyruvate occur by 3*si* electrophilic attack, possibly on enolpyruvate, an "active" intermediate (eq 2: Y = H^+, CO_2, or erythrose 4-phosphate).[3c] A search for active site sequence homologies in these and other enolpyruvate enzymes would be pertinent.

Examples of Classes Lacking Consistency. The nicotinamide-nucleotide dehydrogenases comprise more than 150 enzymes that transfer hydrogen from substrates with a wide range of structures to one or the other[18] stereoheterotopic positions at C-4 of reduced NAD^+ (eq 3) or $NADP^+$. If the enzymes are



(3)

grouped by substrate specificity, no correlation is observed between the nature of the substrate and the choice of 4 position. A common evolutionary origin of the liver alcohol, L-malate, and lactate dehydrogenases (all *pro*-4*R* stereospecific) and glyceraldehyde-3-phosphate dehydrogenase (*pro*-4*S*) is implied by crystallographic and sequence data.[8b] They have similar coenzyme binding regions, but only minor structural changes are required to allow the region complementary to one face of nicotinamide to bind the alternate face. (Pyridoxal phosphate appears to offer a more distinctive set of binding features.) A mechanistic imperative that could lead to the conservation of stereospecificity with respect to the nicotinamide ring must also be lacking.

The challenging problem of coenzyme B_{12} action has also been approached through stereochemistry. In three-interchange reactions Y-C^a-C^b-H → H-C^a-C^b-Y, both retention and inversion at C^a and C^b are observed [retention-retention: methylmalonyl-CoA mutase, Y = -COSCoA; inversion (C^b)-unknown (C^a): methylaspartate mutase, Y = -CH-$NH_3^+CO_2^-$; inversion (C^a)-unknown (C^b): propanediol dehydratase, Y = -OH].[19] Apparently the transfers of hydrogen to and from the coenzyme with breaking of the RCH_2-Co^{2+} bond are independent of each other and of the rearrangement process.

Importance of the Disposition of Catalytic Groups and the Orientation of Substrates

As noted above, some cases of stereochemical consistency can be explained by mechanisms in which the electron flow, whether concerted or stepwise, is cyclic. For most enzymes the flow is acyclic (from source to sink) with one or more acidic and basic groups on the enzyme participating. As there are

(18) See ref 2c, Vol. 2, p 6.
(19) J. Rétey and B. Zagalak, *Angew. Chem., Int. Ed. Engl.*, 12, 671 (1973), and references therein.

Table I
Stereochemical Course of Aldose–Ketose Isomerases[a]

(2*R*)-Aldose Isomerases

α-D-glucose-6-*P* ⇌ α-D-fructose-6-*P*

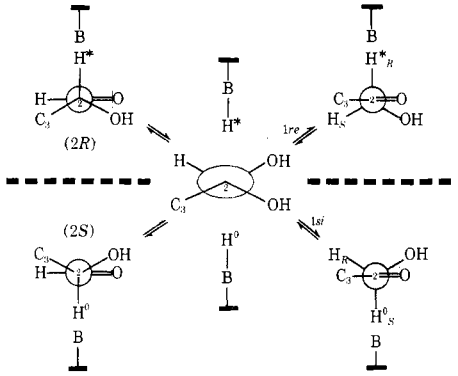
α-D-glucosamine-6-*P* ⇌ D-fructose-6-*P* + NH_3

D-ribose-5-*P* ⇌ D-ribulose-5-*P*

β-L-arabinose $\underset{Mn^{2+}}{\rightleftharpoons}$ L-ribulose

α-D-xylose $\underset{Mn^{2+}}{\rightleftharpoons}$ L-ribulose

D-glyceraldehyde-3-*P* ⇌ dihydroxyacetone-*P*



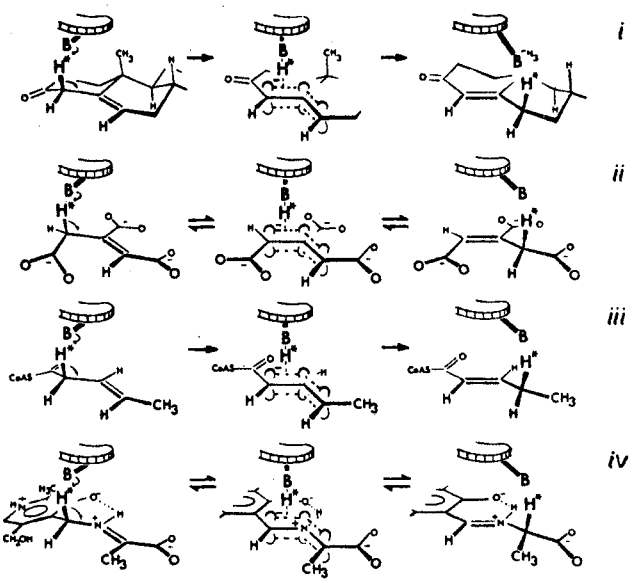
(2*S*)-Aldose Isomerases

β-D-mannose-6-*P* $\underset{Zn^{2+}}{\rightleftharpoons}$ D-fructose-6-*P*

D-arabinose $\underset{Mn^{2+}}{\rightleftharpoons}$ D-ribulose

[a] For references see ref 3b, also C. F. Midelfort and I. A. Rose, in preparation. The preferred anomer is indicated where known. *P* = phosphate.

Table II
Suprafacial Allylic and Aza-allylic Proton Transfers



Enzyme	Transfer or incorporation	Eq
Steroid Δ-isomerase[a]	D transfer in H_2O, >95%	i
Aconitate Δ-isomerase[b]	T transfer in D_2O, 4%	ii
Vinylacetyl-CoA Δ-isomerase[c]	T incorporation in THO, 0%	iii
Pyridoxamine–pyruvate aminotransferase[d]	D transfer in H_2O, 4% H transfer in D_2O, 50%	iv

[a] S. F. Wang, F. S. Kawahora, and P. Talalay, *J. Biol. Chem.*, **238**, 576 (1963); S. K. Malhotra and H. J. Ringold, *J. Amer. Chem. Soc.*, **87**, 3228 (1965). [b] J. P. Klinman and I. A. Rose, *Biochemistry*, **10**, 2259 (1971). [c] Substrate studied: propenylacetyl-CoA: H. Hashimoto, H. Günther, and H. Simon, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **33**, 81 (1973); H. C. Rilling and M. J. Coon, *J. Biol. Chem.*, **235**, 3087 (1960). [d] J. E. Ayling, H. C. Dunathan, and E. E. Snell, *Biochemistry*, **7**, 4537 (1968); ref 17a, p 104.

limited possibilities for arranging these catalytically active amino acid side chains about a substrate molecule, the same constraints upon their use must have operated repeatedly in the course of evolution. We have, therefore, attempted to order enzyme classes that show consistency in terms of the roles of their catalytic groups. The observed patterns, expressed here as normative rules, should imply underlying structural or mechanistic factors in catalysis. We suggest that natural selection for catalytic efficiency has led to: (1) the use of the minimal number of acidic and basic catalytic groups; (2) the maximal separation of catalytic groups and/or replacing groups; (3) minimal motion of the substrate.

1. Minimal Number Rule. The following three sections discuss reaction classes that use, or may be thought to use, one catalytic group where two or more could have been employed.

Aldose–Ketose Isomerases. These reactions occur with hydrogen migration between C-2 of the aldose and C-1, the prochiral carbon, of the ketose in all eight cases examined (Table I): R-CH*OH-CHO → R-CO-CHH*OH. As partial exchange of this labile hydrogen with the medium occurs during the reaction, a base on the enzyme is thought to form an ionizable conjugate acid and the substrate an enediol intermediate (we use the term enediol to include the structure R—CHOH=CHOH and its ionized forms R—CHOH=CHO⁻ and R—CHO⁻=CHOH). Since transfer occurs between adjacent carbons of the same molecule, the same group is involved in H^+ abstraction and donation. Given this, and having determined which stereoheterotopic hydrogen is introduced at C-1, the configuration of the enediol intermediate follows: all eight cases are cis, but both steric possibilities for suprafacial transfer are realized (Table I).

The combination of a cis intermediate and a single base for transfer represents a dual economy in the use of catalytic groups: the cis arrangement allows hydrogen bonding, metal coordination, or the charge on an amino acid side chain to polarize either C=O group and thus assist H^+ abstraction from C-1 or C-2, whereas a trans intermediate would require two polarizing groups. An antarafacial process would require two bases, one to act on C-1 and the other on C-2.

1,3-Allylic and Aza-allylic Rearrangements. The stereochemistry of reactions i to iii in Table II shows that the processes are suprafacial and the findings (i and ii), and inference (iii), that hydrogen transfer occurs confirm this and show that each reaction employs a single base. Differences between the reactions in the efficiency of transfer may imply the use of different bases. As transfer in iv suggests an equivalent suprafacial process, the configuration of the N=C$^\alpha$ bond and the torsional angle about the N—C$^\alpha$ bond in the imine intermediates may be deduced.[17a] In

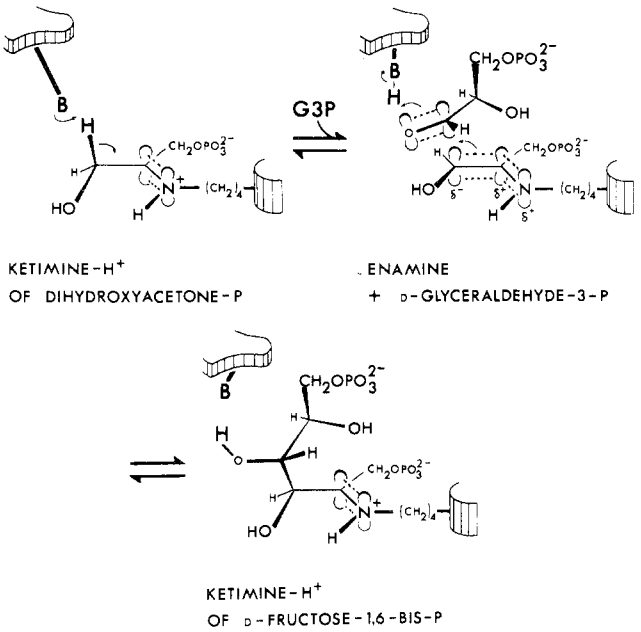


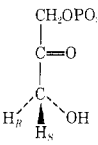
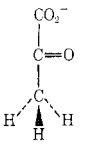
Figure 3. Postulated reuse of a single catalytic group resulting in condensation with retention: steps in the reaction sequence for class 1 fructose-bisphosphate aldolase (Table III). The ketone substrate forms a Schiff base with the amino group of a lysyl side chain. Configurations of the C=N and C—N—C systems have been chosen arbitrarily and the orientation of the carbonyl group is conjectural. Hydrolysis of the ketimine completes the reaction.

each case the allylic carbanion intermediate is stabilized by conjugation. The diversity of these enzymes implies that the same economy in the use of basic groups has evolved in at least four independent contexts.

The basic group responsible for transamination may be reused in further reaction steps. This has been demonstrated for reactions catalyzed by cystathionine γ-synthase.[20] The use of catalytic groups alternately in the acidic and basic mode in the β and γ replacement reactions catalyzed by pyridoxal enzymes is both kinetically and sterically plausible (loss of the β or γ substituent could be acid assisted and nucleophilic attack by the replacing group base assisted).[21]

Aldolases. The aldolases are a diverse group of some 15 enzymes in which electrophilic attack by an aldehyde group takes place upon an enamine (derived from a substrate carbonyl group and a lysyl side chain of the enzyme: six examples established) or on a metal coordinated enol (six examples). As, in general, enamine or enol formation occurs readily in the absence of cosubstrate, H^+ donation to the aldehyde in the full reaction sequence appears to be kinetically independent of the prior H^+ abstraction from C^α. It is, therefore, surprising that, in all the six reactions studied (Table III), the aldehyde approach-

Table III
Aldolases for Which the Stereochemistry (Retention) Is Known

H^+ donor (synthesis)	Product[a]	Source (and type[b])
 dihydroxyacetone phosphate	D-Fructose-1,6-P_2 [(*pro*-3*R*)-H substituted]	Muscle[c] (SB)
		Yeast[c] (M)
	L-Rhamnulose-1-*P* [(*pro*-3*S*)-H substituted]	*E. coli*[d] (M)
 pyruvate	3-Deoxy-D-*erythro*-2-hexulonate-6-*P*	*P. putida*[e] (SB)
	(4*R*)- or (4*S*)-4-Hydroxy-2-ketoglutarate	Liver[f] (SB)
		E. coli[f] (M)

[a] *P* = phosphate. [b] SB = Schiff base forming (class 1), M = metal requiring (class 2). [c] Cosubstrate D-glyceraldehyde-3-*P* (see Figure 3). I. A. Rose, *J. Amer. Chem. Soc.*, **80**, 5835 (1958). [d] Cosubstrate L-lactaldehyde. The reaction may be represented as the mirror image of Figure 3 with $-CH_3$ in place of $-CH_2OPO_3^{2-}$. In both cases the 3- and 4-hydroxyls generated are threo. (Contrast L-fuculose-1-*P* aldolase). T. H. Chiu and D. S. Feingold, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **26**, 835 (1967). [e] Cosubstrate D-glyceraldehyde-3-*P*; product usually known as 2-keto-3-deoxy-6-phosphogluconate (KDPG): H. P. Meloche, J. M. Wurster, and L. Mehler, submitted for publication. [f] Cosubstrate glyoxylate: H. P. Meloche and L. Mehler, *J. Biol. Chem.*, **248**, 6333 (1973).

es the same face of the enol or enamine from which the proton was abstracted. There are significant differences in the specificity of these enzymes, thus it is unlikely that the retention mode would have been conserved if the enzymes derived from a common ancestor, or that it would have repeatedly evolved if retention were less efficient than inversion. The uniformity may be explained[3b,22] if the base that catalyzes enol (or enamine) formation acts as a conjugate acid in the condensation[23] (Figure 3).

A contrasting example is provided by the five enzymes catalyzing β oxidative decarboxylation in which a β-hydroxy acid is first oxidized to a β-keto acid which undergoes immediate decarboxylation [R^1-C^βHOH-$C^\alpha R^2R^3CO_2^-$ → R^1-C(=O)-$CR^2R^3CO_2^-$ → R^1-C(OH)=CR^2R^3 (+CO_2) → R^1-C(=O)-CHR^2R^3]. The nonoxidative steps are formally analogous to an aldol cleavage, yet these reactions are about equally distributed between retention and inversion.[3c] Lack of uniformity could imply that there is no selective pressure for either mode because a base is not used in the CO_2 elimination step, only in enolization.

Selection for a Minimal Number of Catalytic Groups. Parsimony in the use of catalytic groups is consistent with natural selection for catalytic efficiency. Selection should lead to the reuse of groups (a) to permit one site to catalyze multiple steps and (b) to permit **intrinsic proton recycling.**

(20) B. I. Posner and M. Flavin, *J. Biol. Chem.*, 247, 6412 (1972).

(21) Proton transfer from C^α to a β leaving group has not yet been demonstrated. Recently, however, retention of configuration at C^β has been observed: for tryptophan synthetase [the OH of serine replaced by indole: G. E. Skye, R. Potts, and H. G. Floss, *J. Amer. Chem. Soc.*, 96, 1593 (1974); C. Fuganti, D. Ghiringhelli, D. Giangrasso, P. Grasselli, and A. Santopietro Amisano, *Chim. Ind. (Milan)*, 56, 424 (1974)]; and for tyrosine phenol-lyase [phenol replaced by resorcinol: R. K. Hill, S. Sawada, H. Kumagai, and H. Yamada, 9th IUPAC Symposium on the Chemistry of Natural Products, Ottawa, Canada, June, 1974, Abstracts, p 12C; and with the OH of serine replaced by phenol: C. Fuganti, D. Ghiringhelli, D. Giangrasso, and P. Grasselli, *J. Chem. Soc., Chem. Commun.*, 726 (1974)]. Retention is consistent with a single base mechanism. For a review of β replacement reactions see L. Davis and D. E. Metzler, *Enzymes, 3rd Ed.*, 7, 33 (1972).

(22) H. P. Meloche and J. P. Glusker, *Science*, 181, 350 (1973).

(23) Model studies do not give evidence of general base participation in aldol cleavage reactions [F. H. Westheimer and H. Cohen, *J. Amer. Chem. Soc.*, 60, 90 (1938)], but it seems reasonable that protonation of the aldehyde oxygen, or conversely the loss of the carbinol proton, would facilitate the reaction either by raising the population of the ground state through preequilibrium proton transfer or by lowering the free energy of activation by concerted proton transfer.

(a) Enzyme catalysis functions by replacing steps with high free energies of activation by multiple steps linked by low energy barriers. To accommodate multiple steps at one site, reuse of binding regions and catalytic groups is essential. It is commonly observed that the steps in catalysis tend to recur in inverted sequence: *e.g.*, in many group-transfer reactions alternative acceptor substrates occupy a single site on the enzyme to receive the transferred group (transaminases, transacylases, transglycosidases, transaldolases, and many others). Reuse of the same site is often accompanied by the formation of covalent enzyme–substrate intermediates.[24] For example, crystallographic studies of chymotrypsin suggest that in the serine proteases the histidine of the charge relay system is reused within each half of a shuttle.[25] In the forward shuttle the base, E(Im), abstracts H^+ from the Ser-195 hydroxyl to assist attack on the amide carbon of the substrate, then E(ImH^+) protonates the nitrogen of the tetrahedral intermediate. In the reverse shuttle the acyl enzyme produced is hydrolyzed (H_2O in place of $-NH_2$). The spatial limitations which lead to these simplifications must also operate in the isomerases and aldolases.

(b) The alternate use of catalytic groups in their acidic and basic modes has kinetic advantages. In sequential catalysis by two groups B_1 and B_2 on the enzyme such that overall $E(B_1H + B_2^+) \rightarrow E(B_1^+ + B_2H)$ the initial state must be regenerated. The greatest expected[7] rate for regeneration, achieved when B_2 has a pK of ~ 7, is $\sim 10^3$ sec^{-1}. This rate-limiting factor in overall catalysis is dependent upon protonation by the solvent and does not apply if proton recycling occurs within the catalytic sequence so that the original acid or base is regenerated. The rate of hydrogen transfer within a reaction complex[26a] can be $\sim 10^{13}$ sec^{-1}; thus factors other than hydrogen transfer become rate limiting: side-chain flexibility may determine the choice of basic group rather than pK values. It appears that Δ-steroid isomerase (Table II, i) has been selected to take a 100-fold advantage of the intrinsic proton recycling (turnover number, 2.8×10^5 sec^{-1}). Triosephosphate isomerase, which shows only a few per cent hydrogen transfer, is only marginally better than expected for an equivalent two-base enzyme ($\sim 5 \times 10^3$ sec^{-1}).[27]

2. Maximal Separation Rule. The following two sections discuss reaction classes whose stereochemistries imply that a pair of catalytic groups attack a substrate molecule in an opposed rather than adjacent relationship.

1,2 Eliminations. The steric mode of overall elimination of HX from H–C^β–C^α–X, where either C^α or C^β is a prochiral center, has been found to be anti for some 11 enzymes.[28] For eight of these, the trans or cis double bond formed is between two carboxylate ions[28a] (*e.g.*, fumarate); in two, between an α-carboxylate ion and a β-aromatic system;[28b] and in one, the activating substituents are both at C^β (phosphoenolpyruvate).[28c] The leaving groups include –OH, $-NH_3^+$, substituted guanidinium, an adenylo group or amide group, and[28b,29] a group formed between a prosthetic group on the enzyme and $-NH_3^+$. Although some of these enzymes may be related in evolution, the diversity of reaction types rules out an explanation for uniformity in terms of a common ancestor.[28d] The anti mode implies that the groups catalyzing the elimination are on opposite sides of the plane defined by the double bond produced.

Of the known examples of syn eliminations,[30] the conversion of 3-dehydroquinate to 3-dehydroshikimate[30a] differs from the above in that the double bond produced is conjugated to a carbonyl as well as a carboxylate group. A Schiff base formed with a group on the enzyme probably yields an enamine intermediate.[30b] A single-base stepwise mechanism could account for the stereochemistry. The enzymic dehydration of 3-dehydroshikimate to protocatechuate is formally a syn elimination followed by an enolization.[30c] Alternative stepwise sequences involving the repeated use of a single catalytic group can be written as the 5-OH group and the (*pro*-6*S*)-H and 4-H are all removed from the same side of the ring and the C-3 carbonyl group activates both hydrogens (one indirectly, *via* the 1,2 double bond). In the dehydration of (3*S*)-3-hydroxy-3-methylglutaryl-CoA only a thio ester group is activating.[30d] If this grouping stabilizes an intermediate carbanion, *i.e.*, if an aci thio ester intermediate is formed, a single base mechanism could also apply. The alternative syn eliminations of water that occur when mevalonate is supplied to *Fusarium cubense* and another Fusarium species may take place at the thio ester level.[30e] Lastly, because *cis,cis*-muconate is planar, its conversion to (4*S*)-(+)-muconolactone[30f] involves unique steric constraints not present in the above examples of anti eliminations.

Claisen-Type Condensations. In contrast to the enzymic aldol condensations, the seven reactions studied[3c,31] occur with inversion at C^α. For (2*R*,3*S*)-isocitrate lyase, attack on succinate by the carbonyl carbon of glyoxylate results in loss of the (*pro*-2*S*)-H. For the others, attack by a carbonyl carbon occurs at the methyl carbon of an acetyl group: L-malate synthase (Figure 4), citrate (*si*)-synthase and the rare (*re*)-synthase, ATP citrate (*pro*-3*S*)-lyase, citrate (*pro*-3*S*)-lyase, and (3*S*)-3-hydroxy-3-methylglutaryl-CoA synthase.[31] Unlike the aldolases, the α-H exchange observed in the absence of cosubstrate is,

(24) R. M. Bell and D. E. Koshland, Jr., *Science*, **172**, 1253 (1971).

(25) (a) W. N. Lipscomb, *Chem. Soc. Rev.*, **1**, 319 (1972); (b) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, **46**, 337 (1969); G. P. Hess, *Enzymes, 3rd Ed.*, **3**, 213 (1971).

(26) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N.Y., 1969: (a) p 232; (b) Chapter 1.

(27) B. Plaut and J. R. Knowles, *Biochem. J.*, **129**, 311 (1972).

(28) (a) Hydro-lyases acting on L-malate, (2*S*)-citramalate, D-malate, (2*R*)-citramalate, and citrate. Carbon–nitrogen lyases: L-aspartate ammonia-lyase, L-argininosuccinate arginine-lyase, adenylosuccinate adenylate-lyase (also acts stereospecifically on AICAR-succinate). For references see ref 2b, p 186–199; 2c, p 127–158; and 29, p 98. (b) L-Histidine ammonia-lyase and L-phenylalanine ammonia-lyase (also acts stereospecifically on L-tyrosine) both give trans double bonds. Review: ref 29, pp 94–106. (c) 2-Phospho-D-glycerate hydro-lyase, *i.e.*, enolase: M. Cohn, J. E. Pearson, E. L. O'Connell and I. A. Rose, *J. Amer. Chem. Soc.*, **92**, 4095 (1970). (d) The microbial conversion of oleate to D-10-hydroxystearate [G. J. Schroepfer, Jr., *J. Biol. Chem.*, **241**, 5441 (1966)] clearly falls into a different class as the double bond hydrated has only alkyl substituents.

(29) K. R. Hanson and E. A. Havir, *Enzymes, 3rd Ed.*, **7**, 75 (1972).

(30) (a) K. R. Hanson and I. A. Rose, *Proc. Nat. Acad. Sci. U.S.*, **50**, 981 (1963); (b) J. R. Butler, W. L. Alworth, and M. J. Nugent, *J. Amer. Chem. Soc.*, **96**, 1617 (1974); (c) K. H. Scharf, M. H. Zenk, D. K. Onderka, M. Carroll, and H. G. Floss, *Chem. Commun.*, 765 (1971); (d) B. Messner, H. Eggerer, J. W. Cornforth, and R. Mallaby, *Eur. J. Biochem.*, in press; (e) H. Anke and H. Diekmann, *FEBS (Fed. Eur. Biochem. Soc.), Lett.*, **17**, 115 (1971); (f) G. Avigad and S. Englard, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **28**, 345 (1969).

(31) J. W. Cornforth, G. T. Phillips, B. Messner, and H. Eggerer, *Eur. J. Biochem.*, **42**, 591 (1974).

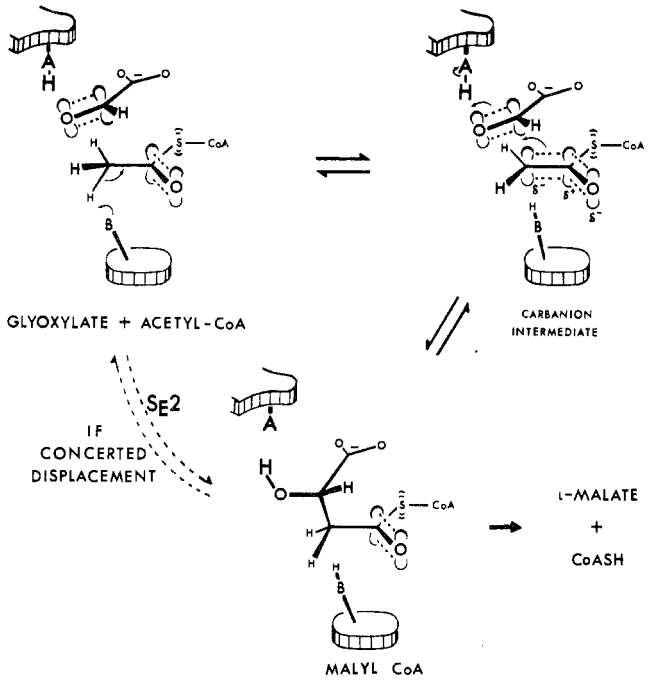


Figure 4. Opposed catalytic groups resulting in condensation with inversion: stereochemistry of L-malate synthase. The orientation of the carbonyl group is conjectural.

at best, slow[31] and may be unobservable unless the enzyme is brought into the correct conformation by a cosubstrate analog.[32] Figure 4 shows the opposed distribution of acidic and basic groups implied by the inversion mode.

Some examples of the use of opposed groups do not fit into a larger class. For proline racemase,[33] H^+ is removed from one face of the $N\text{-}C^{\alpha}\text{-}CO_2^-$ plane and H^+ added to the other. A single base mechanism may be excluded for isopentenyldiphosphate Δ-isomerase as the process is antarafacial and occurs without 1,3 hydrogen transfer.[34] An acid (push)–base (pull) process catalyzed by opposed groups, and perhaps influenced by the orientation of the C(1)-O bond,[35] seems probable.

Selection for Maximal Separation. In many nonenzymic eliminations studied the syn/anti ratio can be varied by changing the medium, base, and substituents.[36] Likewise, changing the dielectric constant and proton-donating ability of the solvent can vary the retention/inversion ratio in electrophilic displacements.[37] In the enzymic reactions, therefore, syn eliminations and replacement with retention are significant options. One may discern one general and several specific factors that could lead to opposed rather than adjacent catalytic groups.

(a) The cleft effect: X-ray crystallography has shown that the active sites of many enzymes lie in a deep cleft within the protein. Critical catalytic groups are often placed on opposite walls of the cleft:[38,39] in ribonuclease,[39a,40] His-12 and His-119.

(32) P. A. Srere, *Curr. Top. Cell. Regul.*, 5, 229 (1972).
(33) G. J. Cardinale and R. H. Abeles, *Biochemistry*, 7, 3970 (1968).
(34) J. W. Cornforth, *Chem. Soc. Rev.*, 2, 1 (1973); J. W. Cornforth, K. Clifford, R. Mallaby, and G. T. Phillips, *Proc. Roy. Soc., Ser. B*, 182, 277 (1972).
(35) P. Ahlberg, *Chemica Scr.*, 3, 183 (1973).
(36) J. F. Bunnett, *Surv. Progr. Chem.*, 5, 53 (1969); J. Sicher, *Angew. Chem., Int. Ed. Engl.*, 11, 200 (1972).
(37) D. J. Cram, "Fundamentals of Carbanion Chemistry," Academic Press, New York, N.Y., 1965, p 152.

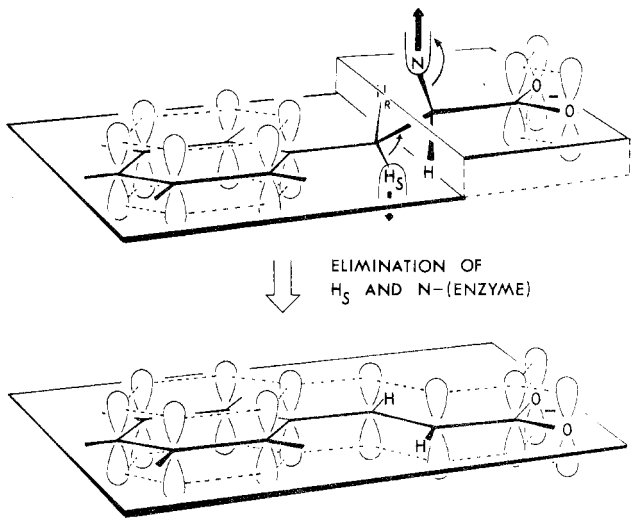


Figure 5. Opposed catalytic groups catalyzing an anti elimination with minimal motion: possible formulation of the elimination step in the conversion of L-phenylalanine to *trans*-cinnamate and NH_3 by L-phenylalanine ammonia-lyase. A concerted process with maximal π–p and p–p overlap is assumed. The N atom is attached to the prosthetic group of the enzyme. Viewed along the $C^{\alpha}\text{–}C^{\beta}$ bond, C^{α}–N and C^{β}–H_s are antiperiplanar (ref 42).

In lysozyme,[39b] Glu-35 acts as an acid and Asp-52 on the other wall acts as a counterion to an oxicarbonium ion intermediate. In carboxypeptidase,[39c] the OH of Tyr-248 is thought to protonate the substrate amide nitrogen while Zn, and perhaps Glu-270, on the other wall interact with the amide C=O. The chance occurrence of an acid–base pair spanning a cleft could have provided the starting point for the evolution of many active sites.

(b) A pair of adjacent catalytic groups forming an ion pair would be less effective in their reversible roles as acid and base than opposed groups with an interposed substrate because electrostatic interactions would modify their acidities and limit their flexibiliy.

(c) Anti eliminations (catalyzed by opposed groups) involve less motion than syn (see Rule 3 below). This principle was first enunciated for simple compounds[41] but becomes more significant as the sizes of the α and β substituents increase. The collapse of two planes into one as shown in Figure 5 for a concerted anti process is much more compatible with substrate and product binding than a syn process.[42]

(d) The influence of stereoelectronic factors in determining the choice between anti and syn is a matter for debate.[43] Eliminations now stepwise could have passed through a concerted stage in the course of evolution. It is pertinent that the E2C elimination process implies anti stereochemistry.[44] A weak base

(38) For drawings illustrating the active sites of the enzymes discussed here, see ref 25a.
(39) (a) F. M. Richards and H. W. Wyckoff, *Enzymes, 3rd Ed.*, 4, 647 (1971); (b) T. Imoto, L. N. Johnson, A. C. T. North, D. C. Phillips, and J. A. Rupley, *ibid.*, 7, 665 (1972); (c) J. A. Hartsuck and W. N. Lipscomb, *ibid.*, 3, 1 (1971); W. N. Lipscomb, *Accounts Chem. Res.*, 3, 81 (1970).
(40) D. Findley, D. G. Herries, A. P. Mathias, B. R. Rubin, and C. A. Ross, *Nature (London)*, 190, 781 (1961).
(41) J. Hine, *J. Amer. Chem. Soc.*, 88, 5525 (1966).
(42) Reference 29a, p 112.
(43) Reference 29a, pp 79–86; 3a, p 356.
(44) (a) Differing views on the mechanism of eliminations catalyzed by weak bases have been summarized: W. T. Ford, *Accounts Chem. Res.*, 6, 410 (1973), and ref 36; (b) D. Cook, R. E. J. Hutchinson, and A. J. Parker, *J. Org. Chem.*, 39, 3029 (1974), and references therein.

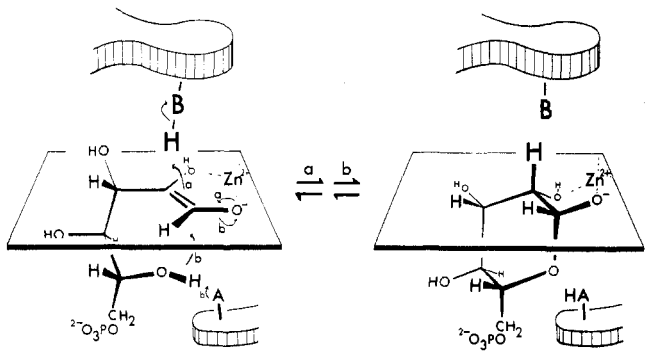


Figure 6. Minimal motion process in the action of mannose phosphate isomerase (Table I): postulated steps converting the enediol intermediate into β-D-mannopyranose 6-phosphate. Protonation at C-2 generates a tetrahedral center and thus promotes 1*re* attack on the transiently formed aldehyde group (a, then b). The resulting 1,2-hydroxyls are cis, the stepwise addition to the enediol is anti, and in going to the cyclic hexose from the ketose there is a net inversion at C-1.

(enzyme groups would qualify) is thought to back-assist C^{α}–X fission but, instead of completing an SN2 displacement, it picks up the β-H.[44b]

(e) In Claisen-type condensations a tightly coupled[45] process with retention (*cf.* Figure 1) could be of higher energy than one in which the polarizable π electrons of the attacking C=O back-solvate a carbanion or participate in a transition state for a concerted displacement.

These suggestions do not explain why two opposed catalytic groups are used when a single base might suffice. The formation of a stable carbanion, enol, or enamine intermediate may be a factor in favor of syn eliminations. If, however, the syn dehydration of (3*S*)-3-hydroxy-3-methyl-glutaryl-CoA takes place by a single-base stepwise mechanism with the formation of an aci thio ester intermediate, it is difficult to see why some Claisen-type condensations do not proceed with retention using a single base.[46] Conversely, it is not yet apparent why some aldol condensations do not utilize the structural advantages afforded by opposed groups in a cleft so that the reaction takes place with inversion rather than by the one-sided arrangement implied by the retention mode (Figure 3).

3. Minimal Motion Rule. Mechanistic deductions from the observed stereochemistry of enzymic reactions usually include an unstated, and perhaps unconscious, assumption that the proposed intermediates remain oriented through consecutive steps in a minimal motion relationship to the interaction groups (*e.g.*, Figures 3 and 5). A further example is provided by mannosephosphate isomerase.[47a] When C-2 of the *cis*-enediol derived from D-fructose 6-phosphate (Figure 6) becomes tetrahedral by addition of H from above the reference plane to the 2*si* face, the internal coordinates of the chain alter in such a way as to bring the 5-OH (from below the plane) and C-1 into closer proximity. As long as this aldose intermediate has limited torsional freedom about the C-1–C-2 bond (perhaps restricted by chelation through the vicinal oxygens to the Zn^{2+} of the enzyme), ring closing can only lead to the β anomer. The product is most likely to be in its most stable conformation, as shown ($^4C_1$: bulky $-CH_2OPO_3^{2-}$, equatorial; 1-OH, equatorial; 2-OH, axial). Any sequence generating an α anomer would involve extensive conformational changes during the reaction. In the aldose to ketose reaction there is at least a 100-fold preference for the β anomer.[47b] Related arguments for the (2*R*)-aldose isomerases predict that α-D- and β-L-aldopyranoses should be better substrates, and this has been confirmed for D-xylose and L-arabinose isomerases, respectively.[47b] The minimal motion rule with respect to torsion about the C-1–C-2 bond may be relaxed for glucose-6-phosphate isomerase to allow the enzyme to catalyze a mutarotation.[47c]

Minimal motion appears to be an integral part of catalytic efficiency. It seems likely that, for reactions in which a reorientation of the substrate at the active site is a necessary part of the overall mechanism, the reaction sequence can be divided into minimal-motion steps and reorientation steps. In the conversion of citrate to (2*R*,3*S*)-isocitrate catalyzed by "aconitase" (aconitate hydratase), the reorientaton step is believed to be a change in position of *cis*-aconitate on the inner coordination sphere of an enzyme-bound ferrous ion.[48] A reorientation step not associated with metal coordination probably occurs in the biosynthetic conversion of the ergot alkaloid chanochavine-I to agroclavine.[49]

The constraints upon motion imposed by the active site may not prevent motion of small groups, particularly those that are torsiosymmetric[4b] such as $-CH_3$ and $-NH_3^+$. The fact that these, even when bound, frequently spin at rates which are greater than their rate of reaction allows deductions about the occurrence of such groups in the intermediate steps of enzymic reactions.[3a,b]

Selection for Minimal Motion. One may rephrase much of the extensive literature on the role of substrate binding in enzyme catalysis to explain selection for minimal motion. (a) Reversible enzymes must bind reactants, products, and intermediates, and binding implies restriction of motion.[50] The result of selection for a balance between this restriction and the freedom necessary for chemical transformations is likely to approximate minimal motion. (b) Selection must occur for enzymes that effectively juxtapose reacting groups.[51] Too much flexibility reduces the chance of collision. (c) Selection occurs for processes in which the free energy of the transition state is minimized by such stereoelectronic factors as maximal overlap between adjacent p orbitals and π systems.[52] (d) Selection occurs for active sites in

(45) Tightly coupled: a process with one or two transition states and with a minimal redistribution of attacking and/or leaving groups during the process; contrast the situation in which one group must leave before a second group attacks (Figure 3).

(46) An aci-thioester ion intermediate not formed in a tightly coupled process might require stabilization by a counterion if it is not to produce a ketene which could inactivate the enzyme by acylation [ketenes are formed from certain *p*-nitrophenyl esters: B. Holmquist and T. C. Bruice, *J. Amer. Chem. Soc.*, 91, 2993, 3003 (1969)]. The syn elimination could thus be catalyzed by a single base and an opposed counterion.

(47) (a) I. A. Rose, E. L. O'Connell, and K. J. Schray, *J. Biol. Chem.* 248, 2232 (1972); (b) K. J. Schray and I. A. Rose, *Biochemistry*, 10, 1058 (1971); (c) K. J. Schray, S. J. Benkovic, P. A. Benkovic, and I. A. Rose, *J. Biol. Chem.*, 248, 2219 (1973).

(48) J. P. Glusker, *J. Mol. Biol.*, 38, 149 (1968).

(49) H. G. Floss, M. Tcheng-lin, C-j. Chang, B. Naidoo, G. E. Blair, C. I. Abou-Chaar, and J. M. Cassady, *J. Amer. Chem. Soc.*, 96, 1898 (1974).

(50) The use of cyclic sugars by the aldose–ketose isomerases requires that the acyclic intermediates must be very tightly bound. This, in turn, imposes constraints on motion which result in absolute anomeric specificity.

(51) For extensive discussions of proximity effects see ref 26b and T. C. Bruce, *Enzymes, 3rd Ed.*, 2, 217 (1970); also, S. Milstien and L. A. Cohen, *Proc. Nat. Acad. Sci. U.S.*, 67, 1143 (1970).

which the motions are partially frozen, so that the relative loss of translational, rotational, and internal entropy in going to a transition state is minimized.[53]

Conclusion

Enzymes as we know them are the result of a long process of natural selection. Rapid advances in our understanding of enzyme evolution are taking place, and it seems pertinent to reexamine enzyme catalysis from this perspective. The reaction stereochemistry of an enzymic process is an important index of the organization of the active site and of details of mechanism. We have, therefore, tried to interpret part of the extensive literature on enzyme stereochemistry in terms of the selective pressures encountered in evolution.

Two extreme situations have been noted above: A single ancestral protein may give rise to a whole class of related enzymes that share a common stereochemistry because the complexity of the protein structure associated with substrate binding has survival value. Alternatively, the stereochemistry of the reaction catalyzed may be independent of any contribution by the enzyme so that enzymes with the same reaction stereospecificity may have evolved separately. We believe, however, that the main power and novelty of our approach lie in its ability to identify mechanistic factors in enzyme catalysis whose survival value depends upon the subtle ways in which the catalytic groups at the active site are deployed and reused.

We have identified two general determinants of stereochemical uniformity in reaction classes: (a) the use of the minimal number of catalytic groups (often with the multiple use of a single base to permit proton recycling within the reaction sequence) and (b) the use of a maximal separation of catalytic groups (often upon opposite walls of a cleft in the enzyme). Undoubtedly these broad categories require further analysis or modification. It remains for the future to provide adequate structural, thermodynamic, or kinetic explanations for the preferential selection in a given reaction class according to one or the other determinant.

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(52) The extent to which the energy barrier to bond formation depends upon orbital alignment has been debated; see, *e.g.*, A. Dafforn and D. E. Koshland, Jr., *Biochem. Biophys. Res. Commun.*, **52**, 779 (1973), and references therein.

(53) W. P. Jencks and M. I. Page (in "Enzymes Structure and Function"), *FEBS (Fed. Eur. Biochem. Soc.) Symp.*, **29**, 45 (1972).

The Return of Sulfenes

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This Account, though mainly an orthodox review of the chemistry of a class of useful reactive intermediates, is also a tale of the return of a simple chemical idea.

Sulfenes[1] are molecules of the formula $RR'C{=}SO_2$ and may be regarded either as the sulfonyl analogs of ketenes or as derivatives of sulfur trioxide formally obtained by replacement of one oxygen atom by a CRR' group. They are most often formed by the action of amines on alkanesulfonyl chlorides, as, for example, in the ordinary preparation of methanesulfonate esters and other "mesylate" derivatives by the reaction of methanesulfonyl chloride and pyridine with alcohols and the like. Paradoxically, sulfenes may very well be more often used but less well known than either ketenes or sulfur trioxide. One purpose of this review is to provide the information which may rectify this anomaly.

J. F. King was born in Moncton, New Brunswick, Canada, in 1934. He obtained both his B.Sc. and Ph.D. degrees at the University of New Brunswick, the latter in 1957 under K. Wiesner. After 2 years of postdoctoral work, the first at Imperial College with D. H. R. (now Sir Derek) Barton and the second at Harvard with R. B. Woodward, he joined the faculty of the University of Western Ontario where is now Professor of Chemistry. His research interests include organic sulfur reactions, stereochemistry, and reaction mechanisms—either straight or mixed.

Historical Background

Wedekind and Schenk[2] proposed the name "sulfene" in 1911, and made the first planned attempt to synthesize one. Staudinger's discovery of the relative stability of diphenylketene,[3] taken with Wedekind's previous work on the formation of ketenes from acid chlorides, led them to hope that the reaction of a tertiary amine and diphenylmethanesulfonyl chloride might yield diphenylsulfene as a stable compound. After failing to obtain the starting chloride, however, they settled for the reaction of phenylmethanesulfonyl chloride with triethylamine, which led to the isolation of *trans*-stilbene and triethylammonium chloride. They suggested that these products arose as follows:

(1) G. Opitz' review (*Angew. Chem., Int. Ed. Engl.*, 6, 107 (1967)) gives authoritative coverage of the literature to the latter part of 1966; this is extended to early 1970 by (a) a supplementary review by W. E. Truce and L. K. Liu (*Mech. React. Sulfur Compounds*, 4, 145 (1969)) and (b) brief summaries in The Chemical Society Specialist Periodical Reports, "Organic Compounds of Sulphur, Selenium and Tellurium," D. H. Reid, Senior Reporter, Vol. 1, pp 199–201 and 290–296 (1970), Vol. 2, pp 85–86, 116–118, 225–228, and 317–320 (1973).

(2) E. Wedekind and D. Schenk, *Ber.*, 44, 198 (1911).

(3) H. Staudinger, *Ber.*, 38, 1735 (1905).