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A Source for the Special Catalytic Power of Enzymes: Orbital Steering*

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Abstract. The velocities of acid catalyzed esterification and γ-lactonizations were studied to test the sensitivity of a chemical reaction to the orientation of the reacting atoms. Variation in orientation of the attacking oxygen atom relative to the carbon atom of the carboxylic acid was achieved by using bicyclic ring systems to limit the conformational mobility of the γ-hydroxy acids. Factors as high as 2×10^4 were observed for the acceleration of a reaction due to this "orientation factor" even after corrections for proximity and torsional strain have been made. The orientation factor is related to the shape of the electron orbitals and must have an angular preference far greater than previously estimated. Such sensitivity to orientation would provide factors large enough to explain the gap in our understanding of enzyme catalysis. It is suggested that the catalytic efficiency of enzymes depends on their ability not only to juxtapose the reacting atoms but also to "steer" their orbitals along a path which takes advantage of this strong directional preference.

Nature's catalyst, the enzyme, is capable of accelerating a chemical reaction faster than any man-made catalyst under the mild conditions of aqueous solution, room temperature, and neutral pH. The source of this catalytic power has puzzled chemists and biochemists for many years. The increasing information from protein modification studies and X-ray crystallography has served on the one hand to increase our information about this process[1–5] and at the same time to heighten the dilemma. It has, for example, become apparent that excellent nonenzymatic chemical analogs can be found for essentially every enzymatic reaction. Yet a quantitative comparison of the velocity of these nonenzymatic analogs with the velocity of the enzymatic reaction reveals differences as high as 10^{12} even after corrections for all the understood catalytic features have been made.[5] A summary of some illustrative ratios of this sort is given in Table 1.

Various suggestions have been made for the source of the special catalytic power of enzymes, such as strain,[6, 7] push-pull mechanisms,[8] proximity,[9–11] and orientation,[9] but quantitative evaluation of these concepts has been elusive. Such quantitative evaluations are essential, however, if one is to determine whether a proposed mechanism contributes significantly to enzyme catalysis. An experimental and theoretical approach to the quantitative contribution of orientation is described in this paper. The results suggest that it may be a major contributor to enzyme catalysis and possibly the source of the remaining gap in our understanding of the catalytic power of enzymes.

TABLE 1. *Comparison of enzymatic rates with nonenzymatic analogs.*

Enzyme	Nonenzymatic analog	Enzymatic velocity V_e (sec^{-1})*	Nonenzymatic velocity V_o, corrected for proximity (sec^{-1})†	V_e/V_o
Lysozyme	Acetal hydrolysis general base catalyzed	5×10^{-1}	3×10^{-9}	2×10^{8}
Chymotrypsin	Amide hydrolysis general base catalyzed	4×10^{-2}	1×10^{-5}	4×10^{3}
Beta amylase	Acetal hydrolysis general base catalyzed	1×10^{3}	3×10^{-9}	3×10^{11}
Fumarase	Alkene hydration general acid and general base catalysis	5×10^{2}	3×10^{-9}	2×10^{11}

* Rates obtained from turnover number of enzyme at saturation.

† Rates obtained from best analog based on knowledge of amino acid residues at active site and known model reaction velocities. Proximity factors of 55 used for each substrate and catalytic group to make V_e and V_o comparable in terms of units and proximity effects.

In order to clarify the calculations and experiments, a schematic illustration of orientation and proximity in an enzymatic reaction involving two substrates and two catalytic groups is shown in Figure 1. For illustrative purposes the generalized picture of Figure 1*A* is made specific in Figure 1*B* for the atoms involved when ATP and glucose are the substrates and histidine and aspartic acid are the catalytic groups. The pie-shaped wedges of Figure 1 are used to illustrate the possibility that reaction might occur over only a fraction of the total solid angle at the atom in question.[9] For example, the observation that Walden inversion occurs in many attacks on saturated alkyl carbon atoms suggested that the pie-shaped wedge in an S_N2 reaction should represent no more than 25% of the solid angle around the alkyl carbon atom.[12] The reciprocal of this fraction, designated θ, represents the additional rate enhancement an enzyme might achieve by optimizing orientation.

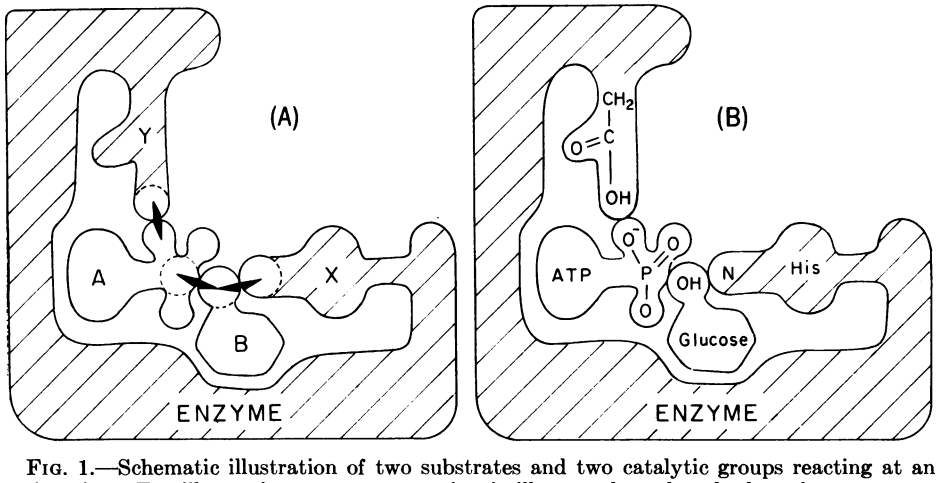


FIG. 1.—Schematic illustration of two substrates and two catalytic groups reacting at an active site. For illustrative purposes, reaction is illustrated as though the substrates were ATP and glucose and the catalytic groups were histidine acting as a base and aspartic acid acting as an acid (reacting atoms are circled). Pie-shaped wedges illustrate in two dimensions the fraction of solid angle of the atom designated over which reaction can occur.

Measured values for these orientation factors are not available and quantum mechanics gives no reliable method for quantitative estimations in polyatomic molecules. A factor of 4 would be suggested from the Walden inversion observation and factors as high as 10 might be guessed from preferences for other cases, e.g., the preferred *trans* addition to double bonds.[13] Factors of this magnitude could not bridge the gap between the enzymatic and nonenzymatic analogs shown in Table 1. However, if each θ factor were 10^3–10^5, a combination of two substrates and two catalytic groups could produce a factor of 10^9–10^{15}, just what is needed to bridge the gap in the enzymatic to nonenzymatic velocity rates. That factors of this order of magnitude might exist was suggested by studies previously reported on thiol subtilisin[14] and led to these experiments in which the angle of approach between attacking atoms is necessarily different whereas the atoms themselves are unchanged. Thus, in Figure 2 the reactive portions of two atoms can be made to coincide completely, partially, or not at all, depending on the angles in the remaining part of the cyclic structure. By designing the proper compounds, the sensitivity of a reaction to the orientation factor might be elucidated.

In the actual experiments esterification was studied both in bimolecular reactions such as acetic acid and ethyl alcohol and intramolecular lactonizations of γ-hydroxy butyric acid (I), 2-hydroxymethyl benzoic acid (II), 2-endo-hy-

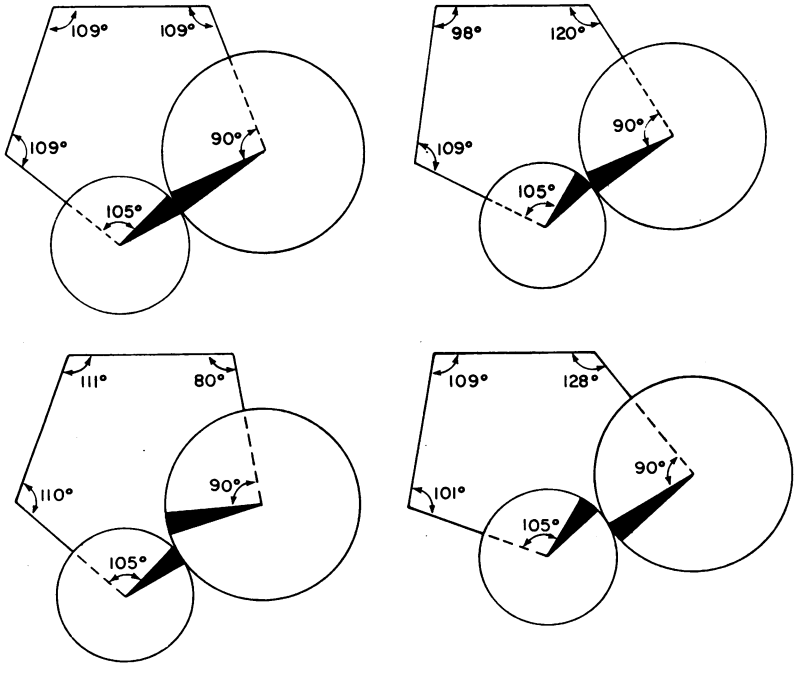


FIG. 2.—Effect of varying the angles of a cyclic ring system on direction of approach of the two reacting atoms. Bond lengths and fraction of surface of the reacting atom over which reaction can occur (*pie-shaped wedge areas*) are kept constant in each case. Angles in bonds of nonreacting atoms that are altered as indicated limit the possibilities in the angle of approach of the attacking atoms.

droxymethylnorborane-3-endo-carboxylic acid (III), and 6-endo-hydroxy-norborane-3-endo carboxylic acid (IV). Dreiding and space filling models showed that the cyclic organic structures would steer the hydroxylic oxygen so that its angle of approach to the carbon of the carboxylic acid groups would be limited relative to the random collisions in a bimolecular esterification. The experiments were designed to eliminate factors other than orientation, e.g., strain or solvation, as a source of acceleration. It could not be predicted *a priori* that any one compound would necessarily show a rate far greater than expected on the basis of proximity alone since the optimal angles are not known. If θ factors are very large, however, it follows that some compounds in which the approach angle is limited should show very great accelerations and there should be a reasonable probability of finding such a greatly enhanced rate.

Materials and Methods. Butyrolactone and phthalide were purchased from Aldrich Chemical Co. The lactones of the hydroxy acids III and IV and the corresponding thiolactones of I, II, and III were synthesized by published procedures.[15-18] The thio analog of IV was prepared by heating endo-5-norbornene-2-thiocarboxylic acid. The sodium salts of the γ-hydroxy and γ-mercapto acids were prepared from the lactones and thiolactones by saponification.

Rates of acid-catalyzed lactonizations were followed by monitoring the change in optical density at the following wavelengths: I, III, and IV (233 mμ); thioanalogs of I, III, and IV (240 mμ); II (254 mμ); and thioanalog of II (265 mμ). These rates were also followed whenever possible with a pH stat or by the hydroxamate assay for lactones and thiolactones.[19] The observed first-order rate constants for lactonizations and thiolactonizations were corrected for the extent of lactone or thiolactone formation at equilibrium. The second-order specific-acid rate constants were determined from the acid catalyzed reactions studied over a variety of hydrogen ion concentrations.

Results. In Table 2 are shown the rates of esterification of these compounds compared to the esterification of acetic acid by ethyl alcohol. It can be seen that compound IV shows an enormous acceleration (10^6) over the bimolecular reaction and an extremely high acceleration (10^4) when compared with the other lactonizations. It should be emphasized that factors such as strain in the lactone ring and steric hindrance to lactone formation, if they are important, should decrease these ratios.

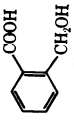
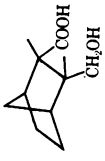
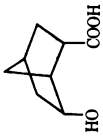
Some correction factors are desirable. The bimolecular reaction must be multiplied by 55 to correct for proximity to give a rate that is directly comparable to the unimolecular reaction in units and magnitude.[9] In some cases the ring closure results in unfavorable nonbonded interactions between vicinal hydrogens which are not present in the starting hydroxy acid and not present in the bimolecular reaction. The decrease in reactivity resulting from this torsional strain can be calculated by estimating the dihedral angles from the nuclear magnetic resonance coupling constants[20] and calculating the interaction energies from the dihedral angles.[21] Such corrections appear to be important only in compounds I and III and lead to factors of 64 and 4, respectively.

Compounds I, II, and III can exist in three conformational minima by rotation about the C_β—C_γ bond, and compound I would have additional rotational minima resulting from rotation about the C_α—C_β bond. The observed rates are multiplied by factors 4.5, 3, and 3 to make the appropriate "proximity" correction. All compounds can in principle undergo the same rotations about

the C_α—C=O and the C_γ—OH bonds and, therefore, no correction is made for these isomers. Although these corrections are approximations, they could not affect the qualitative conclusions unless they were in error by several orders of magnitude, a highly unlikely possibility. The results of the corrected rates are shown in Table 2. Compound IV is seen to undergo esterification at 10^4 times the bimolecular rate even after corrections for proximity have been made. Similarly, its velocity of esterification is 40 times that of compound I, 10 times that of compound III, and 1000 times that of compound II—analogous lactonizations corrected for proximity and strain effects.

It might be suggested that the great rate accelerations observed in the intramolecular reactions may be due to solvation effects. These, however, can be shown to be minor by the following evidence: (*a*) the esterification rate of acetic acid by ethanol is not sensitive to large changes in the solvent composition. Rates of esterification in pure water, 10% ethanol, and 60% acetone were measured and found to vary less than fourfold. (*b*) The acid-catalyzed lactonization of γ-hydroxy butyric acid and γ-hydroxy valeric acids are 0.086 and 0.13 M^{-1} min^{-1}, respectively. The added hydrophobic groups around the hydroxyl carbon and the secondary carbon versus primary carbon effects in these two compounds are, therefore, seen to be minor influences on the rate. (*c*) The pK differences between the hydroxy acids and their deoxy analogs in all cases are less than 0.2 pK units indicating that

TABLE 2. *Relative rates of esterification.*

	CH_3CH_2OH + CH_3COOH	 I	II	 III	 IV
A. *Uncorrected rates:*					
Observed rates*	1.09×10^{-3}†	8.60×10^{-2}	3.32×10^{-2}	7.23	1.12×10^3
Uncorrected relative rates	1	79	305	6630	1,027,000
B. *Corrected rates:*					
		Torsional strain: 64X Conformational		Torsional strain: 4.4X Conformational	
Correction factors:	Proximity: 55X	isomers: 4.5X‡	Conformational isomers: 3X	isomers: 3X	
Corrected rates	6.0×10^{-2}	2.48×10^1	9.96×10^{-1}	9.95×10^1	1.12×10^3
Corrected relative rates	1	413	17	1660	18,700

* All reactions performed at 25°C, 10% ethyl alcohol, μ = 1.0.
† Second-order rate constants, M^{-1} min^{-1}.
‡ Factor of 4.5 arises because two of the given conformations juxtapose OH and C=O.

the γ substituents do not affect the ionization of the acid any more than would be expected on the basis of minor variations between compounds. (*d*) The esterification rate of acetic acid is very similar for ethanol, propanol, and butanol.

Thus, it appears that a large rate enhancement is found with intramolecular reactions as compared to a bimolecular reaction after correction for proximity, nonbonded interactions, and conformational isomers. Solvation has been eliminated as a factor, and strain would operate to decrease the differences observed. The remaining reasonable explanation for these enhanced rates is that the angle of approach for the interacting bonding orbitals of the attacking hydroxyl and attacked carboxyl carbon is 2×10^4 times more favorable for reaction in one case and about 10^3 better in others. Examination of space-filling models shows that the angles of approach are indeed restricted and that they vary somewhat from compound I to compound IV. Since some rotation is allowed about both the C_γ-hydroxyl bond and the C_α—COOH bond and since the angles of approach in the compound synthesized may not be optimal, the observed accelerations are minimal. Thus, the orientation factor in acid-catalyzed esterification is at least 2×10^4 and may well be much greater.

Another test of this hypothesis could be made. If the velocity of a reaction is very sensitive to orientation, replacement of oxygen by sulfur might in a favorable case show a dramatic change in rate. Again one cannot predict *a priori* which rate would be affected the most since the optimal orientation of sulfur versus oxygen is not known. However, the relative rates of lactonization to thiolactonization for compounds I, II, III, and IV are 70, 115, 2.5×10^4, and 426, respectively. Thus, compound III shows an abnormally large deceleration in rate of lactonization when sulfur is substituted for oxygen. Apparently in this compound the angles of approach are such that the thiol structure is far less favorably oriented than the oxygen compound. In Figure 3 a schematic illustration with actual bond angles and bond lengths is shown to indicate the steric consequences resulting from the substitution of S for O in this γ-lactonization.

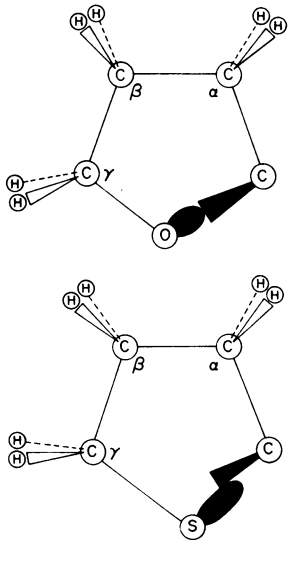


FIG. 3.—Effect of substituting sulfur for oxygen on the angle of approach of reacting atoms in lactonization of a five-membered ring system. Bond angles and distances are based on the reported values for the structure of compound III. Electron orbitals indicated as diffuse clouds but results here suggest a very high angular preference which requires considerable fine structure within the cloud.

An effect similar to this was observed in the conversion of the OH group of the active serine of subtilisin to the sulfhydryl group.[14] From chemical modification studies and structural studies in solution, it was postulated that the only change was caused by the change in the size of the attacking atom and the orbital electron structure associated with

it.[14] Solution studies, however, could not establish details of structure as precisely as crystallography. However, the complete crystallographic detail is now available from Kraut and co-workers and it appears clear that the amino acid residues in thiol subtilisin occupy positions identical to those of subtilisin except for the change from OH to SH.[22] Thus, the analogy between the model studies and the enzymatic study is further supported.

Discussion. It should be emphasized that the "orientation" discussed here is not the gross orientation of substrates and catalytic groups on the enzyme surface or the juxtapositioning of the reacting atoms. All these factors including nonproductive binding are corrected for in the "proximity effect."[9] The factor being discussed here, therefore, is the orientation of the orbitals in the reacting atoms, either in the catalyst or the substrates. Although steric occupation of some orbitals on an atom might account for θ factors of 2 to 4, factors of 10^4 or more after juxtapositioning of the atoms indicate that we are dealing with the precise orientation of orbitals.

The accumulated information on enzymatic reactions strongly supports the hypothesis that they follow the principles of physical organic chemistry.[1, 5, 23] Groups which can play the role of general acids and general bases appear at the active sites of enzymes whose known nonenzymatic analogs are catalyzed by general acids and general bases. Simple extrapolation of these velocities, however, cannot explain the high catalytic power of enzymes. If the hypothesis stated here is correct, the major qualitative difference between the enzymatic and nonenzymatic reactions might be explained by the "orbital steering" properties of the enzyme. Because of its binding and specificity properties, it places the reactive atoms in juxtaposition. The proximity effect resulting from this juxtaposition is important and contributes significantly to the enzyme velocity but simple calculations show that it is insufficient by itself to explain enzyme catalytic power.[5] However, the juxtaposition allows the enzyme to capitalize on a second feature of these reactions which has been hidden in the over-all kinetics, the sensitivity of the reaction to a precise orientation of the electron orbitals of the reacting atoms.

Practically all the enzymes which have been extensively characterized by modification studies and X-ray crystallography involve two or more catalytic groups and two or more substrates. If each orbital steering effect gives a θ factor of the order of 10^4, precise orientation of the four groups could lead to rate enhancements up to 10^{12} times greater than that accounted for by extrapolation from known nonenzymatic mechanisms.

The ramifications of this hypothesis are widespread. Four may be worthy of mention at this time. First, the high orbital orientation factors can explain the enormous accelerations by neighboring groups even when considerable ring strain might be expected. These factors cannot be explained by proximity alone. Secondly, specificity of enzymes reflected in V_m is readily understandable. A modification in the substrate that slightly disorients the approach of a catalytic group could cause order of magnitude changes in V_m with little or no concomitant change in Michaelis constant. Thirdly, "orbital overlap" has been a useful qualitative concept for the quantum mechanical explanation of chemical

properties as in directed valencies, spectral properties, and reactivity.[24-28] Unfortunately, the complexities in the mathematics for polyatomic molecules prevents reliable quantitative calculations of most properties of the ground state molecules and certainly precludes accurate calculations of the transition state properties such as the θ factors discussed here. Perhaps, experiments of the type indicated here can reveal quantitative features of orbital overlap and lead to empirical relationships in this area similar to the limited but highly useful Hammett sigma-rho relationships in reaction kinetics. Fourthly, the sensitivity of reaction to orbital orientation explains how allosteric effectors need produce only minor disorientations to cause large changes in velocity. These and other ramifications will be discussed in detail elsewhere.

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[1] Hollaway, M. R., *Annual Reports*, **65**, 601 (1969).
[2] Perutz, M. F., *European J. Biochem.*, **8**, 455 (1969).
[3] Vallee, B. L., and J. F. Riordan, *Ann. Rev. Biochem.*, **38**, 733 (1969).
[4] Singer, S. J., *Advan. Protein Chem.*, **22**, 1 (1967).
[5] Koshland, D. E., Jr., and K. E. Neet, *Ann. Rev. Biochem.*, **37**, 359 (1968).
[6] Lumry, R., *Enzymes*, **1**, 157 (1959).
[7] Jencks, W. P., in *Current Aspects of Biochemical Energetics*, ed. N. O. Kaplan and E. P. Kennedy (New York: Academic Press, 1966), p. 273.
[8] Swain, C. G., and J. F. Brown, Jr., *J. Amer. Chem. Soc.*, **74**, 2534, 2538 (1952).
[9] (*a*) Koshland, D. E., Jr., *J. Theoret. Biol.*, **2**, 85 (1962); (*b*) Koshland, D. E., Jr., *Advan. Enzymol.*, **22**, 45 (1960).
[10] Bruice, T. C., *J. Polymer Sci.*, **49**, 98 (1961).
[11] Bruice, T. C., and S. T. Benkovic, *Bioorganic Mechanisms* (New York: W. A. Benjamin & Co., 1966), p. 119.
[12] (*a*) Hughes, E. D., and C. K. Ingold, *J. Chem. Soc.*, 1196 (1937); (*b*) Brewster, P., E. D. Hughes, C. K. Ingold, and P. A. D. S. Rao, *Nature*, **166**, 178 (1950).
[13] Young, W. G., R. T. Dillon, and H. J. Lucas, *J. Amer. Chem. Soc.*, **51**, 2528 (1929).
[14] Neet, K. E., A. Nanci, and D. E. Koshland, Jr., *J. Biol. Chem.*, **242**, 6392 (1968).
[15] Vaughan, W. R., C. T. Goetschel, M. H. Goodrow, and C. L. Warren, *J. Amer. Chem. Soc.*, **85**, 2282 (1963).
[16] Beckmann, S., and H. Geiger, *Chem. Ben.*, **94**, 48 (1961).
[17] Truce, W. E., and D. J. Abraham, *J. Org. Chem.*, **28**, 964 (1966).
[18] Protiva, M., M. Rajsner, E. Alderova, V. Seidlova, and Z. J. Vejckelek, *Collection Czech. Chem. Commun.*, **29**, 2161 (1964).
[19] Hestrin, S., *J. Biol. Chem.*, **180**, 249 (1949).
[20] Karplus, M., *J. Chem. Phys.*, **30**, 11 (1959).
[21] Wilson, E. B., *Advan. Chem. Phys.*, **2**, 367 (1959).
[22] Kraut, J., private communication.
[23] Jencks, W. P., *Catalysis in Chemistry and Enzymology* (New York, McGraw Hill Book Co., 1969).
[24] Pauling, L., *J. Amer. Chem. Soc.*, **53**, 1367, 3225 (1931).
[25] Mulliken, R. S., *Phys. Rev.*, **40**, 55 (1932); **41**, 751 (1932).
[26] Roberts, J. D., *Molecular Orbital Calculations* (New York: W. A. Benjamin, Inc., 1961).
[27] Ballhausen, C. J., and H. B. Gray, *Molecular Orbital Theory* (New York: W. A. Benjamin, Inc., 1964).
[28] Woodward, R. B., and R. Hofmann, *Ang. Chem.* **8**, 781 (1969).